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PREFACE

At the end of the last International Symposium on avian corona- and pneumovirus infections in the year 2004 it became clear, that new insights into the pathogenesis, host range and molecular aspects are about to become known. This were the reason for an additional symposium within a time frame of three years. Indeed, in recent years additional and valuable information was obtained in various laboratories that show - among others - an expansion of data on pathogenesis on the molecular level, deeper insights into immunogenesis, epidemiology and host rage of avian coronaviruses. In particular, the emergence of the so-called Italian and Chinese strains of infectious bronchtis virus needed more detailed evaluation.

Also, the avian pneumoviruses – now re-grouped as metapneumoviruses – tend to spread, cause disease in various hosts and are obviously difficult to control. The world-wide presence of diseases due to these viruses demands more and better measures for control. The proceedings of this symposium contain the papers presented and the summaries of each of the eight sessions that were provided by the chairpersons.

It is a special obligation of the organizers to express their sinsere gratitude to all sponsors who contributed significantly to the success of this symposium. Also, the University of Giessen made it again possible to accommodate this symposium in this excellent meeting site. Thanks go also to all speakers for their presetations and fruitfull discussions of all participants. Last but not least the assistance of the staff of the Clinic for Birds, Reptiles, Amphibians and Fish is greatly acknowledged.

Erhard F. Kaleta
Ursula Heffels-Redmann

CHARACTERISATION OF ITALIAN AVIAN PNEUMOVIRUS STRAINS

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SUMMARY

Avian Pneumovirus infection in Italy is reported from its first appearance in 1987 to present day. The B type virus has been found in different geographical regions but more recently, A type virus has also been detected. To establish the identity and heterogeneity among the APV strains that circulated in Italy from 1987 to 2004, the nucleotide sequences of whole Fusion protein gene were determined and compared with European APV field strains and commonly available vaccine strains.

INTRODUCTION

The first Avian Pneumovirus (APV) virus isolation in Italy were made in the late 1980s (Fabris & D'Aprile, 1990). Serological evidence of Avian Pneumovirus infections was reported in turkeys, chickens (Massi, 1997; Fabris et al., 1998) and reared and free living pheasants (Catelli et al., 2001). Since 2001 B type viruses were frequently found in the most poultry populated areas in the North of Italy using virological and molecular testing applied during epidemiological studies of turkeys and chickens (Catelli et al., 2004). Recently A type virus has also been detected (Cecchinato et al., 2003). Little information is available on the molecular epidemiology of APV Italian strains, except for one of the first Italian isolates which was sequenced in the Fusion protein (F) gene by Naylor et al. (GenBank accession number Y14292).
To establish the identity and heterogeneity among the APV strains that circulated in Italy from 1987 to date, the nucleotide sequence of the whole F gene of some of them was determined and compared with international field strains and commonly available vaccine strains.

MATERIAL and METHODS

Seven APV strains were examined (Table 1). Between 1987 and 2004, 6 B type viruses were isolated on farms belonging to a large Italian poultry company which had reported frequent TRT outbreaks since the late 1980s. The flocks were located in a highly populated area of Northern Italy (Veneto Region). Four of the six isolates were recovered from turkeys showing clinical signs typical of TRT. The others derived from broiler flocks with mild respiratory disease. Only one APV strain subtype A was isolated which was isolated in 2004 from turkeys in Central Italy. APV isolates were grown in chicken embryo tracheal organ cultures and RNA was extracted using a commercial Kit (QIAamp viral miny Kit, Quiagen).
All sequences were determined from 3 overlapping independent RT-PCR products and covering the entire F gene. Different primers, specific for A and B APV types, were used. Purified PCR products were sequenced in both directions and the nucleotide sequences were edited using Bioedit software. These were aligned with previously published sequences using the Clustal X programme. The B type European APV F sequences available on the web were all from strains isolated before 1997.
Phylogenetic analysis was carried out under distance criterion, with neighbor-joining as algorithm, using MEGA software. Bootstrap values were obtained with 1,000 replicates.

RESULTS and DISCUSSION

As expected, the phylogenetic analysis of F gene nucleotide sequences produced three different groups corresponding to APV types A, B and C (Figure 1).
The 6 B type Italian isolates were clustered with the previously published European APV B sequences. The APV B types exhibited more than 98% identity over a period of about twenty years, indicating that all strains had a common origin and that very little changes had occurred.
Moreover, within the group, significant sub clustering was apparent, as the more recent isolates (from 2001 to 2004) formed one cluster clearly separated from viruses isolated in the former decade supported by a high bootstrap value. If we look at the B Italian strains it is also possible to see a clear trend with years (Table 2). During the last two decades, the degree of identity between the strains slowly decreased from 99.5 % (between APV Italy/turkey/88 and APV Italy/turkey/87) to 98.0 % (between APV Italy/turkey/88 and the last isolates Italy/turkey/04a and Italy/turkey/04b). A question arises as to whether this trend had been driven in response to vaccination. Since 2002 the poultry company has started vaccination of meat turkeys with a B type vaccine. Slightly increased differences were observed in 2 years after vaccination was introduced but data are needed to confirm this trend.
Three strains isolated over a period of a few months from chickens and turkeys, were compared. The turkey isolate showed 99.6 % and 99.8 % identity with two chicken's strains respectively and none of the mutations coded for amino acid changes. These findings in the fusion gene do not support genetic differences correlated to the host tropism.
Finally, analyzing the F gene nucleotide sequence of the A type 309/04/Italy/turkey/04 strain, we observed strong similarity, 99.8%, with one of the

vaccine sequences included in the philogenetic tree. 309/04 was isolated in 2004 from an Italian flock of young poults (18 days old) showing mild respiratory signs which had been vaccinated with an APV type A in the hatchery. Full sequencing the 309/04 confirmed its vaccinal origin (Catelli et al., 2006). However other A type field viruses from the database also gave similar identities in F.

CONCLUSION

Phylogenetic analysis of the F gene of B type APV Italian and European strains shows differences between the strains that seems to be related with time. In fact it is possible to identify two different clusters, one including strains isolated before 1997 and another after this year. Viruses were all similar irrespective of the area of Europe isolated or the host species.
Only one A type APV strain has so far been analyzed. Its F gene identity was very close to that of a known vaccine strain and further work (Catelli et al., 2006) has proved this to be vaccine derived. More sequence data are required to understand if genuine A type APV field strains are circulating in Italy.

REFERENCES

Catelli E., De Marco M.A., Delogu M., Terregino C., Guberti V., 2001. Serological evidence of avian pneumovirus infection in reared and free-living pheasants. Veterinary Record, 149: 56-58.

Catelli E., Cecchinato M., Delogu M., De Matteo P., Ortali G., Franciosi C., De Marco M.A., Naylor C.J., 2004. Avian Pneumovirus infection in turkey and broiler farms in Italy: a virological, molecular and serological field survey. Italian Journal of Animal Science, 3(3): 287-292.

Catelli E., Cecchinato M., Savage C.E., Jones R.C., Naylor C.J., 2006. Demonstration of loss of attenuation and extended field persistence of a live avian metapneumovirus vaccine. Vaccine. Accepted for pubblication.

Cecchinato M., Catelli E., Savage C.E., De Matteo P., Faenzi M., Naylor C.J., 2003. Evidenza di pneumovirus aviare sottotipo A in corso di un focolaio di TRT in tacchini da carne in Italia. XLII Convegno Società Italiana Patologia Aviare, Forlì 2-3 ottobre 2003. Large Animal Review, 9 (6): 121-122.

Fabris G., D'Aprile P.N., 1990. Rinotracheite infettiva del tacchino: osservazioni sul campo ed indagini di laboratorio. Zootecnica International, I (6): 36-40.

Fabris G., Della Valentina M., Gavazzi L., Gozzini P., 1998. TRT nei tacchini. Indagine sierologica in animali vaccinati e non vaccinati. Selezione Veterinaria 39: 645-654.

Massi P., 1997. Riepilogo delle principali patologie aviarie osservate nel corso del 1996 nelle diverse regioni italiane. Selezione Veterinaria 38:537-541.

Table 1 - APV strains examined.

Strain	Year of isolation	Species	APV type
Italy/turkey/87	1987	Turkey	B
Italy/turkey/01	2001	Turkey	B
Italy/chicken/02a	2002	Chicken	B
Italy/chicken/02b	2002	Chicken	B
Italy/turkey/04a	2004	Turkey	B
Italy/turkey/04b	2004	Turkey	B
309/04/Italy/turkey/04	2004	Turkey	A

Figure 1 - Phylogenetic tree of Italian APV and International strains based on F gene sequence.

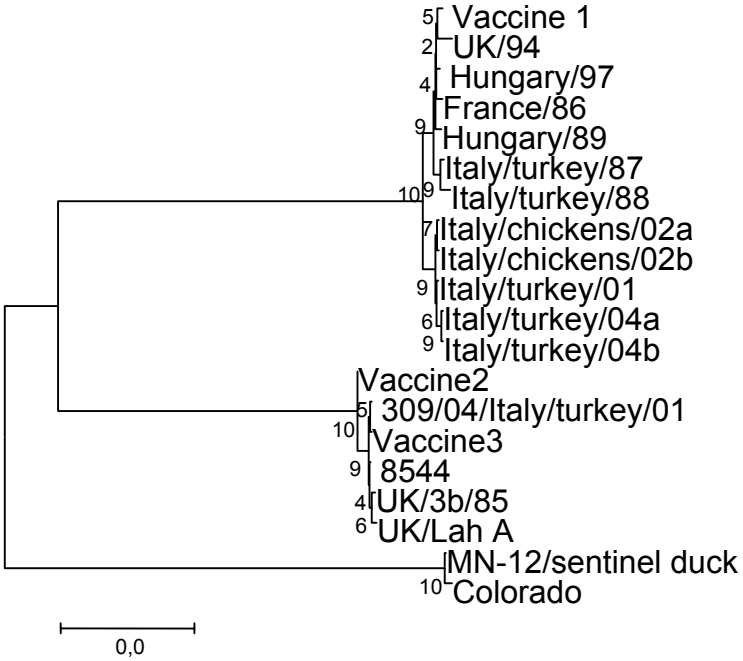

Table 2 - Nucleotide identities (%) comparing Italian APV B types.

	Italy/turkey/87	Italy/turkey/88*
Italy/turkey/87	-	99.5
Italy/turkey/01	98.5	98.2
Italy/chicken/02a	98.5	98.2
Italy/chicken/02b	98.5	98.2
Italy/turkey/04a	98.4	98.0
Italy/turkey/04b	98.4	98.0

*accession number Y14292

SEQUENCE ANALYSIS OF THE COMPLETE GENOME OF AVIAN METAPNEUMOVIRUS SUBGROUP C COLORADO STRAIN: DEVELOPMENT OF A REVERSE GENETICS SYSTEM FOR THIS VIRUS

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SUMMARY

The availability of the complete genome information is essential for development of a reverse genetics system to study the molecular biology and rescue infectious avian metapneumovirus (aMPV) from cloned cDNA. Therefore, we determined the nucleotide (nt) sequence of the complete genome of aMPV-C Colorado strain (aMPV-C-CO) propagated in Vero cells in our laboratory (here designated as SEPRL variant). The full-length genome is comprised of 13,136 nt encoding eight genes, a 40 nt leader at its 3' end and a 45 nt trailer at its 5' end. It is two nt longer than the aMPV-C-CO strain propagated in the University of Minnesota (UMN variant, Lwamba et al., 2005), and 1,014 nt shorter than the same strain of virus propagated in the University of Maryland (UMD variant, Govindarajan and Samal, 2005). The significant difference in length between these variants was found in the coding region of the G gene, where the SEPRL and UMN variants were 1,015 nt or 333 amino acids (aa) shorter when compared with the UMD variant. In addition, there were 23 nt differences scattered along the genome of the variants. Nine of them resulted in eight aa coding changes in five genes, three of which were located in the L gene. Based on the genomic sequence of the SEPRL variant, we developed a reverse genetics minireplicon system using a green fluorescence protein (GFP) gene as a reporter, which allowed us to assess the effects of coding differences in the L gene on viral gene expression. It was found that one of the coding differences (position 1371 Leu vs Phe) in the RNA-dependent polymerase L gene was critical for the polymerase functionality.

INTRODUCTION

Avian Metapneumovirus (aMPV), known as turkey rhinotracheitis virus, causes turkey rhinotracheitis (TRT) and is also associated with swollen head syndrome (SHS) of chickens (Cook, 2000). aMPV is a non-segmented, single-stranded negative sense RNA virus, and belongs to the genus *Metapneumovirus* within the subfamily

Pneumovirinae of the family *Paramxoviridae* (Pringle, 1998). aMPV genome contains eight genes with a gene order of 3'-Leader-N-P-M-F-M2-SH-G-L-Trailer-5' (Ling et al., 1992, Yu et al., 1992). Based on the level of genetic variation in the attachment G protein and their antigenic differences, aMPVs have been classified into four subgroups: aMPV-A, aMPV-B, aMPV-C and aMPV-D. The US isolates of aMPV belong to subgroup C, and exhibit genetic and antigenic differences from isolates of other subgroups (Seal, 2000).
To date, two complete genome sequences for the aMPV-C Colorado strain isolated from turkeys have been reported (Govindarajan & Samal, 2005, Lwamba et al., 2005). Surprisingly, these two published sequences generated from the supposedly same strain of aMPV-C are significantly different in length of the genome. The complete genome sequences generated from the virus stock prepared in the University of Maryland (here so called UMD variant) was 1,016 nt longer than that generated from the virus stock prepared in the University of Minnesota (UMN variant). This length difference was mainly located in the coding region of the G gene, resulting in the deduced G protein of the UMD variant 333 aa larger than that of the UMN variant. Recently, Bennett et al. (Bennett et al., 2005) have reported that an aMPV-C strain isolated from Canadian goose possesses the same size of the G gene as the UMD variant. To clarify this situation and achieve a reliable consensus of the aMPV-C-CO strain for development of a reverse genetics system for this virus, we determined the nucleotide sequences of the complete genome of aMPV-C-CO virus stock prepared in our lab (SEPRL variant). Three complete genome sequences of aMPV-C-CO were compared and a MVA-T7 based aMPV-GFP minigenome expression system was developed for analysis of *cis*- and *trans*-acting requirements for viral gene expression.

MATERIALS and METHODS

Virus and RNA preparation

The aMPV-C Colorado strain (aMPV/CO/96/C) was obtained from Dr. Bruce Seal (USDA-ARS, Athens, GA, USA) and propagated in Vero cells grown at 37^0C in 5 % CO_2 in D-MEM medium supplemented with 10 % fetal bovine serum (FBS) and antibiotics (Invitrogen, Baltimore, MD). The virus stock was maintained at passage level five. Total cellular RNA from infected Vero cells was extracted using Trizol-LS reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA).

cDNA synthesis and nucleotide sequencing

Five overlapped cDNA fragments spanning the entire genome of the virus except its genomic termini were generated by RT-PCR amplification of the viral RNA with five pairs of specific primers using a Superscript™ III One-Step RT-PCR system with Platinum T*aq* Hi-Fi Polymerase (Invitrogen, Carlsbad, CA). cDNAs of the viral genomic termini were obtained by RT-PCR amplification of termini-ligated RNA as described previously (Lwamba et al., 2005). RT-PCR products were purified and sequenced directly with M13 universal primers and gene-specific primers using the Applied Biosystems-PRISM fluorescent big dye sequencing kit and the ABI 3730 DNA sequencer (ABI, Foster City, CA). Nucleotide sequence editing, assembling and comparison were accomplished using the DNASTAR program (Madison, WI).

Construction of an aMPV-GFP minireplicon and plasmids expressing the N, P, M2-1, and L proteins

cDNAs containing the 3' leader and the N gene start signal, and the 5' trailer and the L gene end signal region were generated by RT-PCR amplification of genomic RNA using a Superscript™ III One-Step RT-PCR system kit (Invitrogen, Carlsbad, CA). A cDNA copy of the GFP gene was generated by PCR amplification from pAAV-hrGFP plasmid (Stratagene, La Jolla, CA) using AccuPrime pfx superMix kit (Invitrogen, Carlsbad, CA). These cDNA fragments were then cloned and assembled into a modified pBluescript plasmid (Stratagene, La Jolla, CA) to generate an aMPV-GFP minireplicon (Figure 1). cDNAs coding for the aMPV N, P, M2-1, and L proteins were generated by RT-PCR from viral RNA, and then cloned into a modified expression vector, pTM1 (Moss et al., 1990), under the control of the T7 promoter and followed by a poly A tail (Figure 2). Plasmids containing nt differences presented in the L gene of three variants were also constructed by using a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

Transfection and examination of GFP expression

A mixture of 1.0 ug of paMPV-GFP, 1.0 ug of pN, 0.5 ug of pP, 0.1 ug of pL and pM2-1 plasmids was transfected into HEp-2 cell monolayers, previously infected with MVA/T7 recombinant virus (Wyatt et al., 1995), using Lipofectamine™ 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). A negative control monolayer was transfected as described above, but omitting the pL plasmid, thus not supplying the essential viral polymerase gene. At 6 h post transfection, the cells were washed 1x with PBS and maintained in D-MEM medium supplemented with 5% FBS and antibiotics (Invitrogen, Carlsbad, CA). At 72 h post transfection, the medium was removed and the cells were examined for GFP expression under an UV light microscope and digitally photographed (Olympus).

RESULTS

Nucleotide sequence analysis revealed that the full-length genome of the SEPRL variant was comprised of 13,136 nt encoding eight genes, a 40 nt leader at its 3' end and a 45 nt trailer at its 5' end. As listed in Table 1, it was two nt longer than the UMN variant by adding a G residue between the 3rd and 4th nt upstream of the M2-1 translation start codon and an extra U at the end of the M2 gene poly A signal. The SEPRL variant genome was 1,014 nt shorter than the UMD variant. The striking difference in length between the variants was found in the coding region of the G gene, where the SEPRL and UMN variants were lacking 1,015 nt between 18th and 19th nt upstream of the G gene-end signal, when compared with the UMD variant. Thus, the deduced G protein of the SEPRL and UMN variants (252aa) was 333 aa shorter than that of the UMD variant (585 aa). In addition to the genome length differences, there were 23 nt differences scattered along the genome of the variants. Nine of them resulted in eight aa coding changes in five genes (Table 1).

Based on the genomic sequence of the SEPRL variant, an aMPV minreplicon was constructed and designated as paMPV-GFP (Figure 1). The aMPV-GFP minireplicon encoded a negative-sense copy of the GFP gene flanked by aMPV leader and the N gene start signal at 3' end, and the trailer and the L gene end signal region at 5' end. RNA transcription of the aMPV-GFP minireplicon was driven by the T7 RNA

polymerase promoter sequence and terminated at the T7 terminator signal region. Plasmids expressing the aMPV N, P, M2-1, and L proteins were constructed and named as pN, pP, pM2-1, and pL, respectively (Figure 2). Three additional L gene plasmids containing single nt substitutions that represented the differences found in the UMN variant were also constructed and designated as $pL_{1002Tyr}$, $pL_{1331Gly}$, or $pL_{1371Leu}$, respectively. Expression of the viral proteins was driven by T7 promoter sequence and enhanced by insertion of a ribosome binding sequence (RBS) between the T7 promoter and the aMPV genes.

To analyze the requirements of *cis*-acting elements and the viral protein components for viral gene expression, the aMPV-GFP minireplicon and a mixture of pN, pP, pM2-1 and pL plasmids were co-transfected into MVA/T7 infected-HEp-2 cells. The T7 transcripts from the aMPV-GFP minireplicon in the transfected cells would be cleaved between the trailer and HDV sequences because of the self cleavage activity of the HDV ribozyme, and produce an aMPV genomic analog with an exact 3' terminus as in the virus genome. Simultaneously, the nascent aMPV genomic analog would be encapsided by the N protein and associated with the P and L proteins that were expressed from the supporting plasmids to form a ribonucleoprotein complex, which is the active template for RNA replication and transcription. As shown in Figure 3, co-transfection of all four support-gene plasmids directed the synthesis of GFP (Figure 3. C), whereas in the absence of the L gene plasmid, no expression of GFP was detected (Figure 3. B). This result demonstrated that the aMPV genomic analog contained the essential *cis*-acting elements for RNA synthesis and all four viral proteins expressed from the plasmids were functional and required for viral gene expression.

To examine if the aa coding differences found in the L gene of the UMN variant affect the polymerase function, three L gene mutant plasmids were included in the co-transfection to replace the pL plasmid. As shown in Figure 4, the aa differences at position 1002 (Tyr vs Asn) and 1331 (Gly vs Ser) of the L protein did not apparently affect the level of GFP expression (Figure 4. A, B, and C). However, the aa change at 1371 (Phe to Leu) remarkably decreased the GFP expression (Figure 4. D).

DISCUSSION

Sequence comparisons of three variants of aMPV-C-CO isolate revealed a striking difference in length for the G gene, such that the deduced G protein of the SEPRL and UMN variants was even smaller than half of the UMD variant G. The G protein of members of the *pneumovirinae* subfamily is thought to mediate attachment of virus to a host cell receptor. In order to escape from the immune-pressure or adapt to a new host, these viruses may have to undergo evolutionary changes, resulting in genetic and antigenic variations of the G protein (Sullender, 2000). It has been reported that pneumoviruses with a partial or complete deletion of the G gene can still grow in cell cultures (Elliott et al., 2004, Karron et al., 1997, Naylor et al., 2004). Thus, it is possible that the length difference in the G gene between the aMPV-C variants was due to a deletion mutation of the G gene during adaptation of virus to the non-avian origin cell line, Vero cells.

Minireplicon systems are helpful tools for analyses of *cis*- and *trans*-acting elements required for gene expression of negative stranded RNA viruses. In the case of

pneumoviruses, most minireplicon systems have been developed using the Chloramphenicol Acetyl Transferase (CAT) as a reporter (Grosfeld et al., 1995, Naylor et al., 2004, Randhawa et al., 1997). The detection of the CAT expression requires lysis of transfected cell monolayers, detection of activity by chemical substrates and determination of the intensity of the reactions by means of spectrophotometry or thin layer chromatography. It is laborious and time consuming. In the present study, we developed an aMPV-C minireplicon system using the GFP gene as a reporter. The advantage of this aMPV-GFP minireplicon system is that the GFP expression can be detected directly under a UV light microscope without a need of any sample preparations or chemical reactions. The GFP expression in this minireplicon system is highly specific without any background and statistical analyses are possible by counting fluorescent cells in monolayer or by flow cytometry.
Sequence analysis of the polymerase proteins of various non-segmented negative strand RNA viruses denoted that there are six conserved domains (I-VI) along the length of the protein, and the function of the L protein probably depends on these distinct domains (Poch et al., 1990). Sequence alignment of the L protein of three aMPV-C CO strain variants, aMPV-A and human metapnumovirus revealed that the coding differences at aa position 1002, 1331 and 1371 in the L protein of the UMN variant were not located in any one of those domains. However, the amino acids at these three positions were conserved among members of metapneumovirus except the UMN variant, suggesting they may play a role in the polymerase function. By using this aMPV-GFP minireplicon system, we were able to identify that two codon differences resulting in aa changes at position 1002 (Tyr vs Asn) and 1331 (Gly vs Ser) had no obvious effect on polymerase function in supporting viral gene expression. Thus, these two codon differences may represent either quasi species of the virus populations or cloning errors. In contrast, the aa change at 1371 of the L gene (Phe to Leu) resulted in a significant decrease of the GFP expression, demonstrating that the Phe residue at this position was critical for maintaining the polymerase functionality. Most likely, this coding change found in the UMN variant was a cloning error, otherwise, this virus would be considerably attenuated or might not be viable for next generation.

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REFERENCES

Bennett, R. S., LaRue, R., Shaw, D., Yu, Q., Nagaraja, K. V., Halvorson, D. A. & Njenga, M. K. (2005). A wild goose metapneumovirus containing a large attachment glycoprotein is avirulent but immunoprotective in domestic turkeys. *J Virol* 79, 14834-42.

Cook, J. K. (2000). Avian pneumovirus infections of turkeys and chickens. *Vet J* 160, 118-25.

Elliott, M. B., Pryharski, K. S., Yu, Q., Parks, C. L., Laughlin, T. S., Gupta, C. K., Lerch, R. A., Randolph, V. B., LaPierre, N. A., Dack, K. M. & Hancock, G. E. (2004). Recombinant respiratory syncytial viruses lacking the C-terminal third of the attachment (G) protein are immunogenic and attenuated in vivo and in vitro. *J Virol* 78, 5773-83.

Govindarajan, D. & Samal, S. K. (2005). Analysis of the complete genome sequence of avian metapneumovirus subgroup C indicates that it possesses the longest genome among metapneumoviruses. *Virus Genes* 30, 331-3.

Grosfeld, H., Hill, M. G. & Collins, P. L. (1995). RNA replication by respiratory syncytial virus (RSV) is directed by the N, P, and L proteins; transcription also occurs under these conditions but requires RSV superinfection for efficient synthesis of full-length mRNA. *Journal of Virology* 69, 5677-5686.

Karron, R. A., Buonagurio, D. A., Georgiu, A. F., Whitehead, S. S., Adamus, J. E., Clements-Mann, M. L., Harris, D. O., Randolph, V. B., Udem, S. A., Murphy, B. R. & Sidhu, M. S. (1997). Respiratory syncytial virus (RSV) SH and G proteins are not essential for viral replication in vitro: clinical evaluation and molecular characterization of a cold-passaged, attenuated RSV subgroup B mutant. *Proceedings of the National Academy of Sciences of the United States of America* 94, 13961-13966.

Ling, R., Easton, A. J. & Pringle, C. R. (1992). Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. *J Gen Virol* 73 (Pt 7), 1709-15.

Lwamba, H. C., Alvarez, R., Wise, M. G., Yu, Q., Halvorson, D., Njenga, M. K. & Seal, B. S. (2005). Comparison of the full-length genome sequence of avian metapneumovirus subtype C with other paramyxoviruses. *Virus Res* 107, 83-92.

Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. & Fuerst, T. R. (1990). Product review. New mammalian expression vectors. *Nature* 348, 91-2.

Naylor, C. J., Brown, P. A., Edworthy, N., Ling, R., Jones, R. C., Savage, C. E. & Easton, A. J. (2004). Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. *J Gen Virol* 85, 3219-27.

Poch, O., Blumberg, B. M., Bougueleret, L. & Tordo, N. (1990). Sequence comparison of five polymerases (L proteins) of unsegmented negative-strand RNA viruses: theoretical assignment of functional domains. *J. gen. Virol.* 71, 1153-1162.

Pringle, C. R. (1998). Virus taxonomy--San Diego 1998. *Arch Virol* 143, 1449-59.

Randhawa, J. S., Marriott, A. C., Pringle, C. R. & Easton, A. J. (1997). Rescue of synthetic minireplicons establishes the absence of the NS1 and NS2 genes from avian pneumovirus. *J Virol* 71, 9849-54.

Seal, B. S. (2000). Avian pneumoviruses and emergence of a new type in the United States of America. *Anim Health Res Rev* 1, 67-72.

Sullender, W. M. (2000). Respiratory syncytial virus genetic and antigenic diversity. *Clin Microbiol Rev* 13, 1-15, table of contents.

Wyatt, L. S., Moss, B. & Rozenblatt, S. (1995). Replication-deficient vaccinia virus encoding bacteriophage T7 RNA polymerase for transient gene expression in mammalian cells. *Virology* 210, 202-5.

Yu, Q., Davis, P. J., Li, J. & Cavanagh, D. (1992). Cloning and sequencing of the matrix protein (M) gene of turkey rhinotracheitis virus reveal a gene order different from that of respiratory syncytial virus. *Virology* 186, 426-34.

Table 1. Sequence comparison between variants of the AMPV-C-CO strain

	Nucleotide position [a]	Nucleotide changes [b]			
Gene/ region	3' end of vRNA	UMN variant	SEPRL variant	UMD variant	Amino acid differences [c]
N	164	U	U	**C**	no change
	973	U	**C**	U	**Asn-Ser (307)**
M	2276	C	**A**	C	**Phe-Val (36)**
F	3123	**U**	C	C	**Tyr-Cys (28)**
	3166	**C**	U	U	no change
M2-1	4683	C	**G**	C	non-coding
	4684	-	**extra G**	-	non-coding
	4768	A	**G**	A	no change
	4772-4774	AGG	**UCA**	AGG	no change
	4780	A	**U**	A	no change
M2-2	5381	A	**U**	A	no change
	5416-5422	**-**	extra U	extra U	non-coding polyA
SH	6004	C	C	**U**	non-coding
	6178	G	G	**A**	no change
G	6620	**C**	U	U	**Ala-Thr (157)**
	6891-6892	-	-	**extra 1,015 nt**	**extra 333 aa**
	6899-6900	UG	UG	**CU**	**Arg-Glu (250)**
L	9942	**A**	U	U	**Tyr-Asn (1002)**
	10929	**C**	U	U	**Gly-Ser (1331)**
	11049	**G**	A	A	**Leu-Phe (1371)**
	11189	**U**	C	C	no change

a. Nucleotide position number is based on the number of the SEPRL variant sequence.
b. Nucleotide sequences are shown in the genomic sense.
c. Number in the bracket denotes the position of amino acid changes.

The nucleotide and amino acid differences between the variants are highlighted by bold. Nucleotide sequence of the UMN variant of the AMPV-C-CO strain was reported by Lwamba et al. (2005); the UMD variant published by Govindarajan and Samal (2005); and the SEPRL variant determined by us.

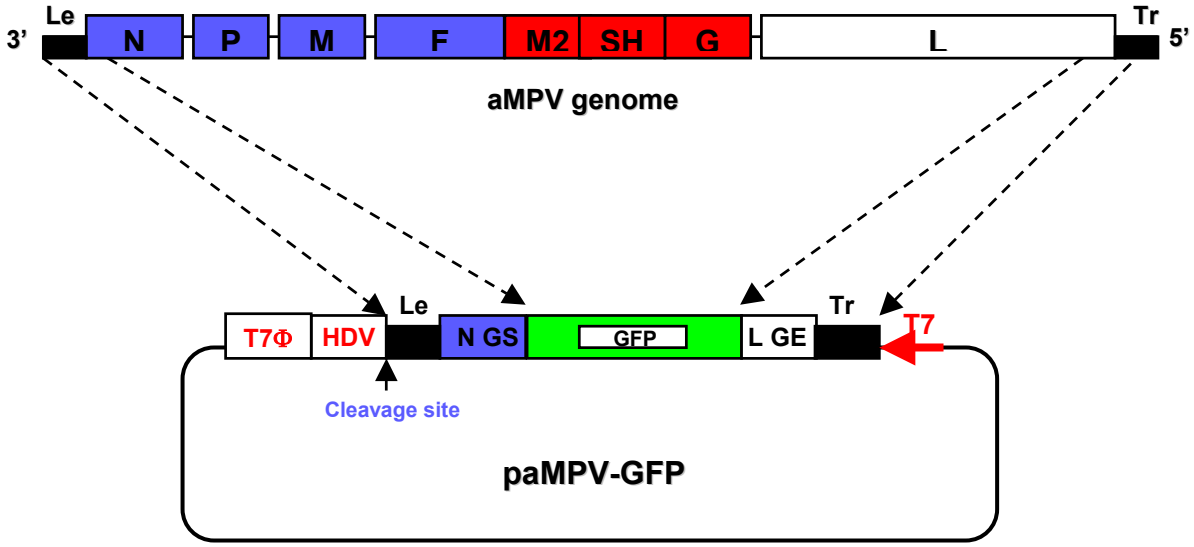

Figure 1. Schematic presentation of a paMPV-GFP minireplicon. T7 promoter direction is indicated by a red arrow and hepatitis delta virus (HDV) ribozyme cleavage site is marked by a black arrow. T7Φ, N GS, GFP and L GE denote the T7 terminator, the N gene start, green fluorescence protein and the L gene end sequences, respectively.

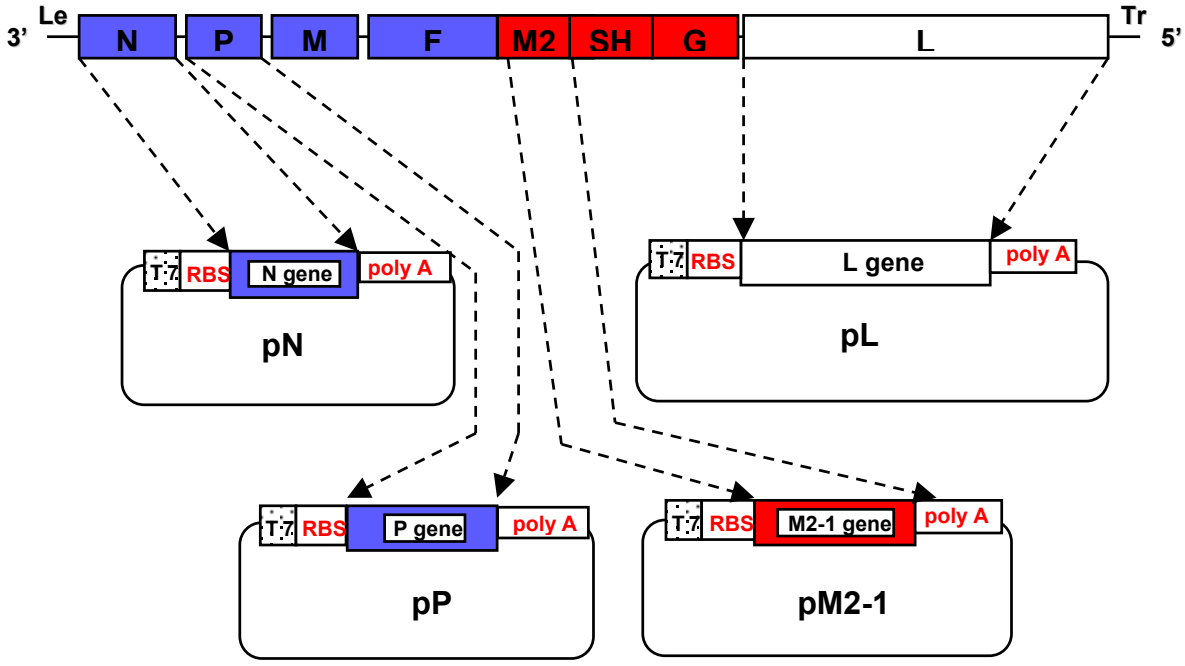

Figure 2. Schematic presentation of viral gene expression plasmids. T7, RBS and poly A represent T7 RNA polymerase promoter, ribosomal binding sequence and poly A tail sequences, respectively.

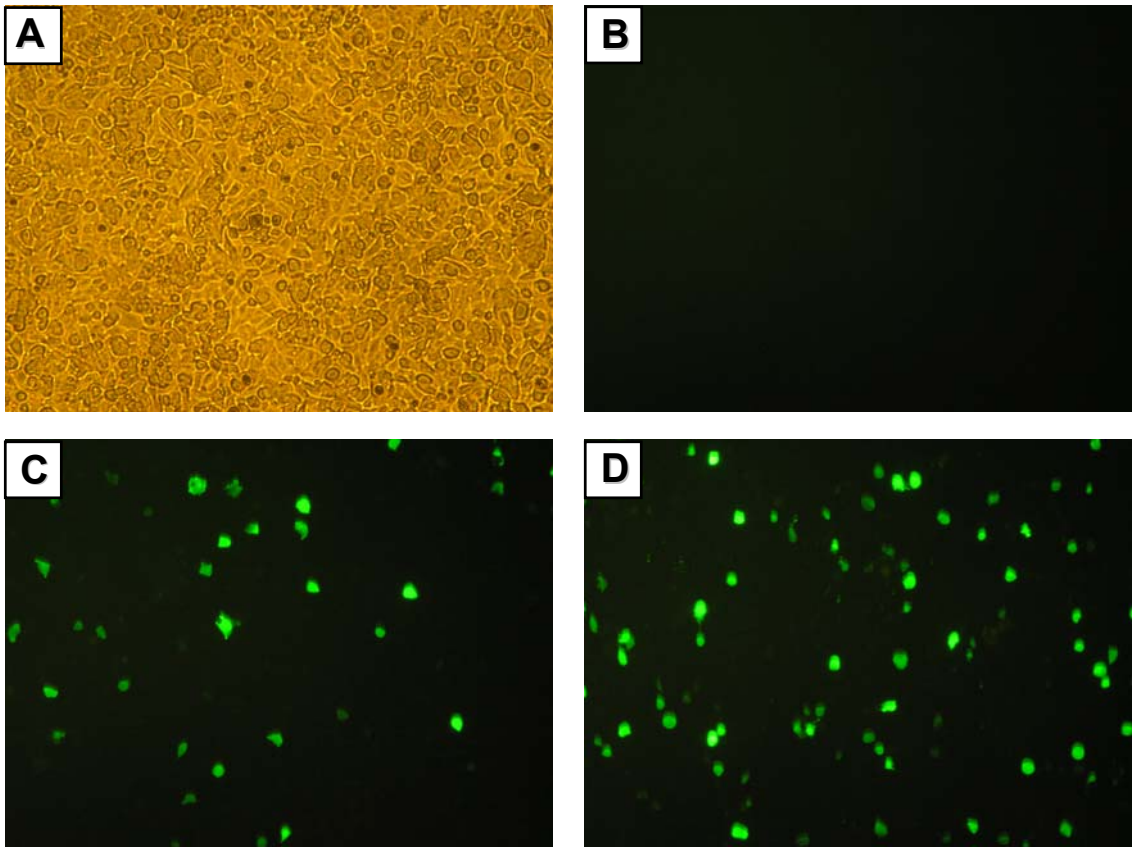

Figure 3. Examination of GFP expression from an aMPV-GFP minigenome. MVA-T7-infected HEp-2 cell monolayers were transfected with paMPV-GFP minireplicon, and a combination of either all four (N, P, M2-1, and L) gene-expression plasmids (C), or three gene-expression plasmids without pL (B). After 72 h post transfection, GFP expression was detected under an UV light microscope and digitally photographed. (A) is a cell control without any treatment, and (D) is the MVA-T7 infected cells that were transfected with pTM1-GFP plasmid as a positive transfection control.

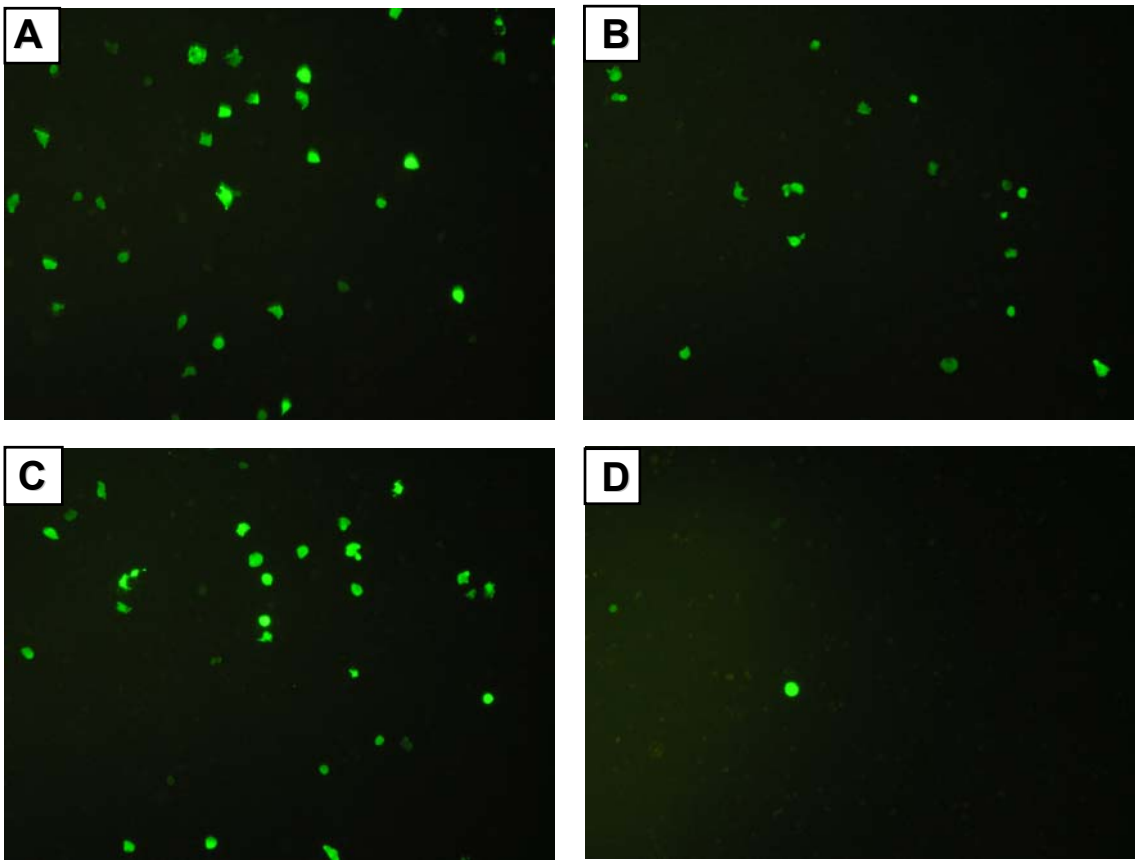

Figure 4. Effects of L gene mutations on GFP expression. MVA-T7-infected HEp-2 cell monolayers were transfected with paMPV-GFP minireplicon, and a combination of three gene-expression plasmids (pN, pP, and pM2-1), plus pL (A), or one of three L gene mutant plasmids $pL_{1002Tyr}$ (B), $pL_{1331Gly}$ (C), or $pL_{1371Leu}$ (D). At 72 h post transfection, GFP expression was detected under an UV light microscope and digitally photographed.

REVERSE GENETIC STUDIES OF AVIAN METAPNEUMOVIRUS SUBGROUP C

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SUMMARY

With a long-term goal of developing a better vaccine for controlling avian metapneumovirus (AMPV) in the US, a reverse genetics system to produce infectious AMPV of subgroup C entirely from cDNA was established. A cDNA clone encoding the entire genome of AMPV strain Colorado (AMPV/CO) was generated by assembling five cDNA fragments between the T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme of a transcription plasmid pBR 322. Transfection of this plasmid, along with the expression plasmids encoding the N, P, M2-1 and L proteins of AMPV/CO, into cells stably expressing T7 RNA polymerase resulted in the recovery of infectious AMPV/CO. Characterization of the recombinant AMPV/CO showed that its growth properties in tissue culture were similar to that of the parental virus. These results demonstrate that infectious AMPV can be generated entirely from cloned DNA using reverse genetics techniques. The potential of AMPV/CO to serve as a viral-vector was examined using green fluorescent protein (GFP) as a reporter. The recovered rAMPV/CO-GFP stably expressed GFP for at least five serial passages and showed characteristics similar to that of the parental virus, except that there was a one-log reduction in the virus titer. These results demonstrated that the established reverse genetics system can be utilized effectively for various studies involving AMPV molecular biology, pathogenesis and vaccine development.

INTRODUCTION

Avian metapneumovirus (AMPV) causes an acute respiratory disease in turkeys and is associated with “swollen head syndrome” in chickens. The virus also affects many other species of birds. AMPV is a member of the genus *Metapneumovirus* in the subfamily *Pneumovirinae* of the family *Paramyxoviridae* (Pringle, 1998). The genus *Metapneumovirus* contains the AMPV and its mammalian counterpart, the human metapneumovirus (HMPV). HMPV causes respiratory tract disease, especially in young children (van den Hoogen et al., 2001). Based on nucleotide (nt) sequence divergence in the attachment glycoprotein genes and their antigenic differences,

AMPVs have been classified into four subgroups – AMPV-A, AMPV-B, AMPV-C and AMPV-D (Juhasz & Easton, 1994; Eterradossi et al., 1995 and Bäyon-Auboyer et al., 1999). The US isolates of AMPV belong to subgroup C, and exhibit genetic and antigenic differences from isolates of other subgroups (Seal, 1998; Cook et al., 1999 and Toquin et al., 2000). Interestingly, several reports have shown that AMPV-C shows a closer resemblance to HMPV than to other AMPV subgroups (van den Hoogen et al., 2002; Yunus et al., 2003; Toquin et al., 2003; Govindarajan & Samal, 2004, Govindarajan et al., 2004 and Govindarajan & Samal, 2005).
AMPV subgroup C strain Colorado (AMPV/CO) contains a non-segmented negative-stranded RNA genome which is 14,150 nt long (Govindarajan & Samal, 2005). The genome contains eight genes, which encode the nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix proteins (M2-1 and M2-2), small hydrophobic protein (SH), attachment glycoprotein (G) and the large polymerase protein (L). As encountered in other non-segmented negative-sense RNA viruses, the genomic RNA of AMPV is not infectious by itself. Other components of the ribonucleoprotein complex (N, P and L proteins) are essential to initiate virus-specific mRNA synthesis and thus the production of infectious virus. Due to technological advances, by co-transfecting plasmids expressing the full-length antigenomic viral RNA and the support plasmids (N, P, and L proteins), all under the control of T7 promoter, it is possible to recover infectious virus entirely from cloned cDNA (Schnell et al., 1994). Using this approach, we have recently generated infectious recombinant AMPV/CO (Govindarajan et al., 2006). This newly established virus rescue system will provide important applications for the development of safe and efficacious attenuated vaccine strains. In addition, the manipulation of the AMPV genome at the DNA level will be highly useful for further studies on AMPV virulence, pathogenesis and molecular biology.
An important application of reverse genetics system is to engineer paramyxoviruses as vaccine vectors by expressing protective antigens of other pathogens. Several studies have shown that paramyxoviruses can stably express foreign proteins for many passages (Conzelmann, 2004). Therefore, the reverse genetics system developed by us was used to study the potential of AMPV/CO as a virus vector by generating a recombinant AMPV/CO that stably expresses GFP as a foreign protein. The biological characteristics of recombinant AMPV/CO-GFP was similar to that of the wild type virus except that there was a slight reduction in virus titer. These results indicate that it is possible to engineer AMPV/CO to stably express foreign proteins through reverse genetic techniques.

MATERIALS and METHODS

Cells and virus

Vero cells were grown in MEM (Invitrogen GIBCO) supplemented with 10% fetal calf serum. Baby hamster kidney cells that constitutively express T7 RNA polymerase (BSR T7/5, Buchholz et al., 1999) were a generous gift from Dr. Karl-Klaus Conzelmann (Ludwig-Maximilians-University Munich, Munich, Germany). These cells were maintained in Glasgow MEM (Invitrogen GIBCO) supplemented with 10% fetal calf serum, glutamine and amino acids, under geneticin (1mg/ml) selection every second passage. AMPV/CO was obtained from the National Veterinary Services Laboratory (Ames, IA, USA).

Viral RNA extraction

AMPV/CO and the recombinant AMPVs described below were grown in confluent monolayers of Vero cells supplemented with 2% fetal calf serum. The virus propagation, purification and RNA extractions were performed in a manner similar to that described elsewhere (Govindarajan & Samal, 2004). Briefly, Vero cells grown to confluency were infected with the parental AMPV/CO or the recovered recombinant viruses. Virus was harvested when maximum cytopathic effect (CPE) was evident as extensive syncytia. The infected cells were scraped into the medium and lysed by three cycles of freezing and thawing to release the cell-associated virus. The cell lysate was clarified at 3000 g for 15 min, and the supernatant was made 10% with respect to PEG 8000 (Sigma) and incubated for 3 h at 4 °C. Subsequently, the virus was pelleted at 4000 g for 30 min at 4 °C. Viral genomic RNA was extracted from the viral pellet using TRIzol reagent (Invitrogen) according to the manufacturer's directions, except that an additional extraction with phenol plus chloroform was performed.

Construction of expression plasmids

The open reading frames (ORFs) of N, P, M2-1 and L genes of AMPV/CO were generated by RT-PCR using gene-specific primers and cloned into plasmid pTM-1, that possesses the encephalomyocarditis virus internal ribosome entry site (IRES) downstream of the T7 RNA polymerase promoter and uses the translation start codon contained in the Nco I site of the IRES. The ORFs of N, P and M2-1 were cloned individually in pTM-1 between Nco I and BamH I sites. The ORF of L gene was cloned between the Nco I and Xho I sites by a two-step cloning procedure using the Xma I site as the third restriction site. The Xma I site was introduced in the L gene ORF without any alteration of amino acid sequence by changing two nt (nt 9656 A→C and nt 9659 A→G). The ORFs of N, P, M2-1 and L in pTM-1, designated as pN, pP, pM2-1 and pL respectively, were sequenced to their entirety using an ABI 3100 DNA sequencer (Applied Biosystems).

Construction of full-length plasmid

The complete genome of AMPV/CO was cloned into the transcription plasmid pBR322/dr. Plasmid pBR322/dr was a modified form of plasmid pBR322 which contained a 72-nt oligo linker between the EcoR I and Pst I sites and hepatitis delta viral 84-nt antigenome ribozyme sequence and T7 RNA polymerase transcription termination signal between the Rsr II and Fse I sites (Krishnamurthy et al., 2000). The complete genome of AMPV/CO was cloned into pBR322/dr as 5 cDNA fragments (Fig. 1). All cDNA synthesis reactions were carried out using Superscript II reverse transcriptase (Invitrogen) and gene-specific primers.

Three restriction sites (Xho I, Pvu I and Mlu I) were created during the cloning procedure so as to help in cloning as well as to serve as markers to confirm the identity of the recovered recombinant virus. The Xho I site was created between the M and F genes, in the non-coding region after the M gene termination codon, by altering three nt (nt 2988 T→G, nt 2989 C→A and nt 2990 A→G). The Pvu I site was created in the SH-G intergenic region by altering one nt (nt 6124 A→G). The Mlu I site was created between the G and L genes, in the non-coding region after the G gene termination codon, by altering three nt (nt 7916 G→A, nt 7918 A→G and nt 7919 A→C). None of the artificially created marker sites involved amino acid-coding

sequences. The Age I restriction site (nt 12960) was an unique site already present in the genome, which was utilized for the cloning process. The five fragments were cloned in the order given in Fig.1. After ligation into the plasmid, each fragment was sequenced completely using an ABI 3100 DNA sequencer (Applied Biosystems). The resulting AMPV/CO full length expression plasmid was termed pAMPV/CO. This plasmid contained three non-viral G residues adjacent to the T7 promoter, at the 5' end of the antigenome, to enhance promoter efficiency (Biacchesi et al., 2004).

Construction of full-length plasmid encoding GFP

In order to study the vector potential of AMPV/CO, we generated a full-length plasmid encoding GFP as an additional gene (Fig. 2). The antigenomic pAMPV/CO plasmid mentioned above was modified by the insertion of a transcription cassette containing the ORF for enhanced GFP (Clontech, Inc.). The cassette was introduced as a single fragment at the Mlu I site created between the G and L genes, in the non-coding region after the G gene termination codon, by altering 3 nt (nt 7916, 7918 and 7919). The transcription cassette contained the Mlu I recognition sequence followed by the 14-nt putative G gene-end sequence (TAGTTAATTAAAAA, positive sense, AMPV/CO nt 7922-7935), followed by a 2-nt intergenic sequence (CC, positive sense), followed by the 16-nt putative N gene-start sequence (GGGACAAGTGAAA*ATG*, positive sense, AMPV/CO nt 41-56, N ORF initiation codon italicized and underlined), followed by the GFP ORF, followed by the Mlu I recognition sequence. The fragment was digested with Mlu I and cloned into pAMPV/CO resulting in the final construct pAMPV/CO-GFP (Fig. 2). The length of the encoded rAMPV/CO-GFP antigenome, excluding the non-viral sequences, would be 14,905 nt.

Transfection and recovery of infectious recombinant viruses

Transfection experiments were performed in BSR T7/5 cells grown to 90-95% confluency in 6-well plates. The cells were transfected with 5 µg each of the full length plasmids (pAMPV/CO and pAMPV/CO-GFP), 2 µg each of pN and pP, 1 µg each of pM2-1 and pL plasmids in a volume of 100 µl of Opti MEM per well. Transfection was carried out with Lipofectamine 2000 (Invitrogen), according to the manufacturer's directions. After 6 h of incubation at 37^0 C, the transfection mixture was removed and the cells were washed and maintained with Glasgow MEM containing 3% fetal calf serum. Cells transfected with pAMPV/CO-GFP were monitored by fluorescent microscopy (Zeiss) for the expression of GFP. Three to four days after transfection, all the media was used for infecting a fresh batch of Vero cells in 6-well plates or T-25 flasks and observed for the development of virus-induced CPE and also expression of GFP (cells transfected with pAMPV/CO-GFP plasmid). After one additional passage, the supernatant was harvested and clarified for further purification of the recombinant viruses.

Virus growth

Multiple-step growth characteristics of the recombinant viruses were compared with those of the parental virus. Briefly, Vero cell monolayers grown in 6-well plates were infected with 0.01 MOI of the parental and the rAMPV/CO-GFP. Supernatants (0.2 ml) were collected on indicated days post-infection and replaced by an equivalent volume of fresh medium. The collected samples were flash frozen, stored at -70^0 C,

and titrated later in parallel by plaque assay. Each growth curve is based on the average of the virus titers from two infected monolayers. For the GFP-expressing rAMPV/CO-GFP, the number of viral foci was counted by fluorescent microscopy and was also confirmed with the number obtained following an immunostaining reaction.

Antibody staining of plaques

The recovered recombinant viruses along with the parental wild-type virus were grown in Vero cells maintained in MEM supplemented with 2% fetal calf serum and 0.8% methyl cellulose (Sigma). After incubation for 4 days at 37^0 C, the overlay was removed and the monolayer was fixed with 4% paraformaldehyde solution. The cells were then permeabilized with 1:1 acetone in phosphate-buffered saline at -20^0 C for 2 min. The plaques were then incubated with rabbit polyclonal antisera (1:1000 dilution) raised against a polypeptide of the AMPV/CO N protein (KDNSGPIPQNQRPSS, aa 140 to 154 on N ORF), followed by incubation with goat anti-rabbit IgG tagged with horseradish peroxidase (KPL, MD, USA). The viral plaques were visualized using light microscopy after staining with DAB substrate (Sigma).

RESULTS

Construction of a plasmid encoding the full-length AMPV/CO genome

A cDNA clone encoding the complete 14,150-nt antigenome of AMPV/CO was constructed by sequential cloning of five individual cDNA fragments into the low copy number plasmid pBR 322, as detailed in Fig. 1. Five overlapping cDNA fragments were generated: fragment 1 contained the putative N, P, and M genes, flanked by T7 RNA polymerase promoter at the upstream end and an Xho I site at the downstream end. The Xho I site was created by three nt substitutions in the putative non-coding region following the termination codon of the M gene and served as a genetic marker to distinguish between the recombinant and the wild-type parental viruses. Fragment 2 contained the putative F, M2 and SH genes and was flanked by the Xho I site at the upstream end and a Pvu I site at the downstream end. The Pvu I site was created by one nt substitution in the putative SH-G intergenic region and also served as a genetic marker to distinguish between the recombinant and the wild-type parental viruses. Fragment 3 contained the putative G gene and was bordered on the upstream end by the Pvu I site and on the downstream end by an Mlu I site that was created by three nt substitutions in the putative non-coding region following the termination codon of the G gene. Fragment 4 contained the upstream sequences of the putative L gene and was bordered on the upstream end by the Mlu I site and on the downstream end by a naturally occurring Age I site. Fragment 5 contained the remaining sequences of the putative L gene and the trailer sequence, flanked by the Age I site at the upstream end and part of the hepatitis delta virus ribozyme sequence ending in an Rsr II site at the downstream end. The Rsr II site is a naturally occurring restriction site in the hepatitis delta virus ribozyme. The transcription plasmid vector pBR322 supplied the remaining part of the hepatitis delta virus ribozyme followed by a T7 RNA polymerase terminator sequence. The full-length plasmid was named as pAMPV/CO and was sequenced to its entirety. Sequence analysis revealed that pAMPV/CO was a faithful copy of the 14,150-nt AMPV/CO

genome except for the artificially introduced genetic markers at the Xho I, Pvu I and Mlu I sites.

Construction of full-length plasmid encoding GFP

In order to assist in the recovery and identification of the cDNA-derived virus, the full-length plasmid pAMPV/CO was modified such that it contained a transcription cassette encoding the GFP gene at the Mlu I site created between the G and L genes in the putative 5' untranslated region. In addition to providing a means to monitor the recovery of rAMPV, this also helped in studying the potential of AMPV to serve as a viral vector. The procedure of cloning the GFP transcription cassette into the full-length plasmid pAMPV/CO is shown in Fig. 2. The foreign gene cassette consisted of appropriate viral gene-start and gene-end signals to enable proper transcription. The resulting plasmid, pAMPV/CO-GFP, was sequenced at the flanking sites of the Mlu I cloning site to confirm the insertion of the foreign cassette.

Recovery and characterization of recombinant viruses

The antigenome plasmids pAMPV/CO and pAMPV/CO-GFP, along with the support plasmids encoding the N, P, M2-1 and L proteins, were transfected into BSR T7/5 cells that stably express the T7 RNA polymerase. In a parallel transfection, the plasmid encoding the L protein was excluded to serve as a negative control. In the case of pAMPV/CO-GFP, the transfected cells were examined by fluorescent microscopy on successive days after transfection. Green fluorescent cells were visualized by the day after transfection. Initially, they appeared as scattered isolated cells and subsequently they formed foci of more cells that later on developed into well-formed syncytia, similar to the CPE induced by AMPV/CO. When the transfection mixture was transferred to fresh Vero cells, green fluorescent cells were visualized after about 24 h post-infection denoting that the virus was derived entirely from the cDNA and not a laboratory contamination of AMPV/CO. Recovery of infectious recombinant rAMPV/CO-GFP was also possible without the inclusion of M2-1 protein in the transfection mixture (data not shown). DNA sequencing of the RT-PCR products derived from the recovered viruses denoted the presence of the artificially-introduced restriction markers, while being absent in the biologically derived AMPV/CO (data not shown). The recovered recombinant viruses will henceforth be known as rAMPV/CO and rAMPV/CO-GFP.

Multiple-step growth cycle replications of rAMPV/CO, rAMPV/CO-GFP and wild-type AMPV/CO were compared following inoculation of Vero cells at an MOI of 0.01 (Fig. 3). Both the kinetics and magnitude of replication of rAMPV/CO were similar to those of the wild-type virus indicating that rAMPV/CO was fully competent for multicycle growth in vitro. However, the kinetics and magnitude of replication of rAMPV/CO-GFP were slightly lower than those of the wild-type AMPV/CO as well as rAMPV/CO. Virus titers of rAMPV/CO-GFP in the supernatants collected at 72 h post-inoculation were around $10^{5.3}$ pfu/ml while the titers of the other two viruses tested were around $10^{6.0}$ (Fig. 3). Monitoring Vero cells by fluorescence microscopy following infection with rAMPV/CO-GFP also showed that GFP was stably expressed for at least five serial passages.

The wild-type AMPV/CO, rAMPV/CO and rAMPV/CO-GFP viruses induced CPE, consisting of large syncytia, in cultured Vero cells (Fig. 4A). The syncytia induced by the three viruses were similar and were indistinguishable. Furthermore, the recovered

rAMPV/CO and rAMPV/CO-GFP were compared with the wild-type AMPV/CO for their antigenic characteristics in an immunoperoxidase plaque-staining reaction (Fig. 4B). We observed that the plaques induced by all three viruses were similar in size and shape. These results indicated that the recovered recombinant viruses exhibited similar growth properties as those of the wild-type virus in tissue culture and that the insertion of a foreign gene does not drastically affect the in vitro replication characteristics of AMPV/CO.

DISCUSSION

In this study, we report for the first time the recovery of infectious AMPV-C strain Colorado (rAMPV/CO) and infectious AMPV-C strain Colorado expressing GFP (rAMPV/CO-GFP) entirely from cloned cDNAs. The complete genome sequence data available for AMPV/CO (Govindarajan & Samal, 2005) was used to generate a reverse genetics system for this emerging turkey pathogen. The recombinant virus representing the full-length AMPV/CO (rAMPV/CO), developed in this study, showed growth characteristics similar to those of the biologically derived parental virus in tissue culture. Hence, the rescue of rAMPV/CO entirely from cloned cDNA confirmed that the currently available nucleotide sequences of AMPV/CO (Govindarajan & Samal, 2005) are accurate and functional.

Among the AMPVs, the complete genome sequence is currently known only for strains CVL14/L and Colorado of AMPV subgroups A and C, respectively (Randhawa et al., 1997; Govindarajan & Samal, 2005). The entire genome of AMPV/CO was 14,150 nucleotides long while that of CVL14/1 was 13,373 nucleotides long. Since both these genome lengths are not a multiple of six, avian metapneumoviruses do not seem to obey the “rule of six”, similar to the HMPVs (Biacchesi et al., 2003; Herfst et al., 2004).

Ever since its identification in the US, AMPV-C has become a major problem for the turkey farmers. Currently, no effective vaccine is available to control AMPV infections in affected birds, thus leading to major economic losses to the US turkey industry. Hence, engineering a safe and effective live-recombinant vaccine for the US subgroup of AMPV becomes imperative. Unfortunately, the recently developed reverse genetic system for AMPV-A (Naylor et al., 2004) cannot be used to generate live-recombinant vaccines to control AMPV infections in the US for two main reasons: (i) AMPV-A is very different both genetically and antigenically from subgroup C and hence would not be effective against the latter and, (ii) an AMPV-A based vaccine in the US would mean introduction of a new subgroup of AMPV into the US, which is prohibited in the US. Hence, our newly developed reverse genetics system for AMPV subgroup C will be a very effective tool towards development of a live attenuated vaccine against AMPV infections. In addition, this reverse genetic system will also be helpful in understanding the role of individual metapneumovirus genes in pathogenesis in its natural host, turkey.

An important application of reverse genetics system is the ability to engineer paramyxoviruses expressing additional foreign genes (Conzelmann, 1998). Studies over the last many years have indicated the genome of paramyxoviruses to be very elastic and that they can be manipulated to stably express foreign proteins to very high levels. Recently, the recovery of recombinant HMPVs expressing GFP and multiple extra genes indicated that the HMPV genome can accommodate an

additional 30 % of its original genome size (Biacchesi et al., 2004). In the present study, the reverse genetics system developed by us was used to generate a recombinant AMPV/CO that expresses GFP. GFP coding sequence was inserted as a transcription cassette in G/L intergenic region of full length AMPV/CO cDNA. The recovered rAMPV/CO-GFP virus showed growth properties in tissue culture similar to those of the parental virus but yielded viral titers one-log lower than the parental virus. We also observed that rAMPV/CO stably expressed GFP for at least five serial passages in Vero cells. These results indicated that the AMPV/CO can be genetically manipulated through reverse genetics to stably express foreign proteins to relatively high levels and thus has a great potential to serve as a vaccine vector for other pathogens. Furthermore, the ability to express a foreign protein from an artificially inserted transcription cassette also confirmed the identification of the transcription signal sequences of AMPV/CO. This rAMPV/CO-GFP will also be helpful in future studies involving virus tropism and pathogenesis.
We believe that this newly developed virus-recovery system will be very helpful in studying basic molecular biology of metapneumoviruses in general and in developing attenuated live-recombinant vaccines to control this emerging poultry pathogen. Several live-attenuated and chimeric recombinant viruses can be engineered through this technology. Gene-deleted recombinant AMPV/COs can also be generated and used as vaccine candidates. Recombinant AMPV/CO lacking the G glycoprotein can be expected to be a promising vaccine virus because it will replicate efficiently but not spread to multiple tissues and hence be attenuated. Using this system, AMPV/CO can also be engineered to carry foreign proteins of viruses causing diseases in other avian, non-avian or human species and their potential as a vaccine vector remains to be investigated.

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REFERENCES

1. Bäyon-Auboyer, M.H., Jestin, V., Toquin, D., Cherbonnel, M., and Eterradossi, N., 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Arch. Virol. 144, 1091–1109.

2. Biacchesi S., Skiadopoulos M.H., Boivin G., Hanson C.T., Murphy B.R., Collins P.L., and Buchholz U.J. 2003. Genetic diversity between human metapneumovirus subgroups. Virology 315, 1–9.
3. Biacchesi, S., Skiadopoulos, M.H., Tran, K.C., Murphy, B.R., Collins, P.L., and Buchholz, U.J., 2004. Recovery of human metapneumovirus from cDNA: optimization of growth in vitro and expression of additional genes. Virology 321, 247-259.
4. Buchholz, U.J., Finke, S., and Conzelmann, K., 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. J. Virol. 73, 251-259.

5. Conzelmann, K.K. 1998. Nonsegmented negative-strand RNA viruses: Genetics and manipulation of viral genomes. Annu. Rev. Genet. 32, 123-162.
6. Conzelmann, K.K. 2004. Reverse genetics of mononegavirales. Curr. Top. Microbiol. Immunol. 283, 1-41.
7. Cook, J.K.A., Huggins, M.B., Orbell, S.J., and Senne, D.A., 1999. Preliminary characterization of an avian pneumovirus isolated from commercial turkeys in Colorado USA. Avian Pathol. 28, 607–617.
8. Eterradossi, N., Toquin, D., Guittet, M., and Bennejean, G., 1995. Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. Zentbl Veterinarmed B 42, 175–186.
9. Govindarajan, D., and Samal, S.K., 2004. Sequence analysis of the large polymerase (L) protein of the US strain of avian metapneumovirus indicates a close resemblance to that of the human metapneumovirus. Virus Res. 105, 59–66.
10. Govindarajan, D., and Samal, S.K., 2005. Analysis of the complete genome sequence of avian metapneumovirus subgroup C indicates that it possesses the longest genome among metapneumoviruses. Virus Genes 30, 331-333.
11. Govindarajan, D., Yunus, A.S., and Samal, S.K., 2004. Complete sequence of the G glycoprotein gene of avian metapneumovirus subgroup C and identification of a divergent domain in the predicted protein. J. Gen. Virol. 85, 3671-3675.
12. Govindarajan, D., Buchholz, U.J., and Samal, S.K., 2006. Recovery of avian metapneumovirus subgroup C from cDNA: Cross-recognition of avian and human metapneumovirus support proteins. J. Virol. 80(12): 5790-5797.
13. Herfst, S., de Graaf, M., Schickli, J.H., Tang, R.S., Kaur, J., Yang, C.F., Spaete, R.R., Haller, A.A., van den Hoogen, B.G., Osterhaus, A.D., and Fouchier, R.A. 2004. Recovery of human metapneumovirus genetic lineages A and B from cloned cDNA. J. Virol. 78, 8264-8270.
14. Juhasz, K., and Easton, A.J., 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J. Gen. Virol. 75, 2873–2880.
15. Krishnamurthy, S., Huang, Z., and Samal, S.K., 2000. Recovery of a virulent strain of Newcastle disease virus from cloned cDNA: expression of a foreign gene results in growth retardation and attenuation. Virology 278, 168-182.
16. Naylor, C.J., Brown, P.A., Edworthy, N., Ling, R., Jones, R.C., Savage, C.E., and Easton A.J., 2004. Development of a reverse-genetics system for avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. J. Gen. Virol. 85, 3219-3227.
17. Pringle, C.R., 1998. Virus taxonomy—San Diego. Arch. Virol. 143, 1449–1459.
18. Randhawa, J.S., Marriott, A.C., Pringle, C.R., and Easton, A.J. 1997. Rescue of synthetic minireplicons establishes the absence of the NS1 and NS2 genes from avian pneumovirus. J. Virol. 71, 9849-9854.
19. Schnell, M.J., Mebatsion, T., and Conzelmann, K.K. 1994. Infectious rabies viruses from cloned cDNA. EMBO J. 13, 4195-4203.
20. Seal, B.S., 1998. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first U.S. avian pneumovirus isolate is distinct from European strains. Virus Res. 58, 45–52.

21. Toquin, D., Bayon-Auboyer, M.H., Senne, D.A., and Eterradossi, N., 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis. 44, 977–982.
22. Toquin, D., de Boissesson, C., Beven, V., Senne, D.A., and Eterradossi, N., 2003. Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. J. Gen. Virol. 84, 2169–2178.
23. van den Hoogen, B.G., de Jong, J.C., Groen, J., Kuiken, T., de Groot, R., Fouchier, R.A., and Osterhaus, A.D., 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat. Med. 7, 719–724.
24. van den Hoogen, B.G., Bestebroer, T.M., Osterhaus, A.D., and Fouchier, R.A., 2002. Analysis of the genomic sequence of a human metapneumovirus. Virology 295, 119–132.
25. Yunus, A.S., Govindarajan, D., Huang, Z., and Samal, S.K., 2003. Deduced amino acid sequence of the small hydrophobic protein of US avian pneumovirus has greater identity with that of human metapneumovirus than those of non-US avian pneumoviruses. Virus Res. 93, 91–97.

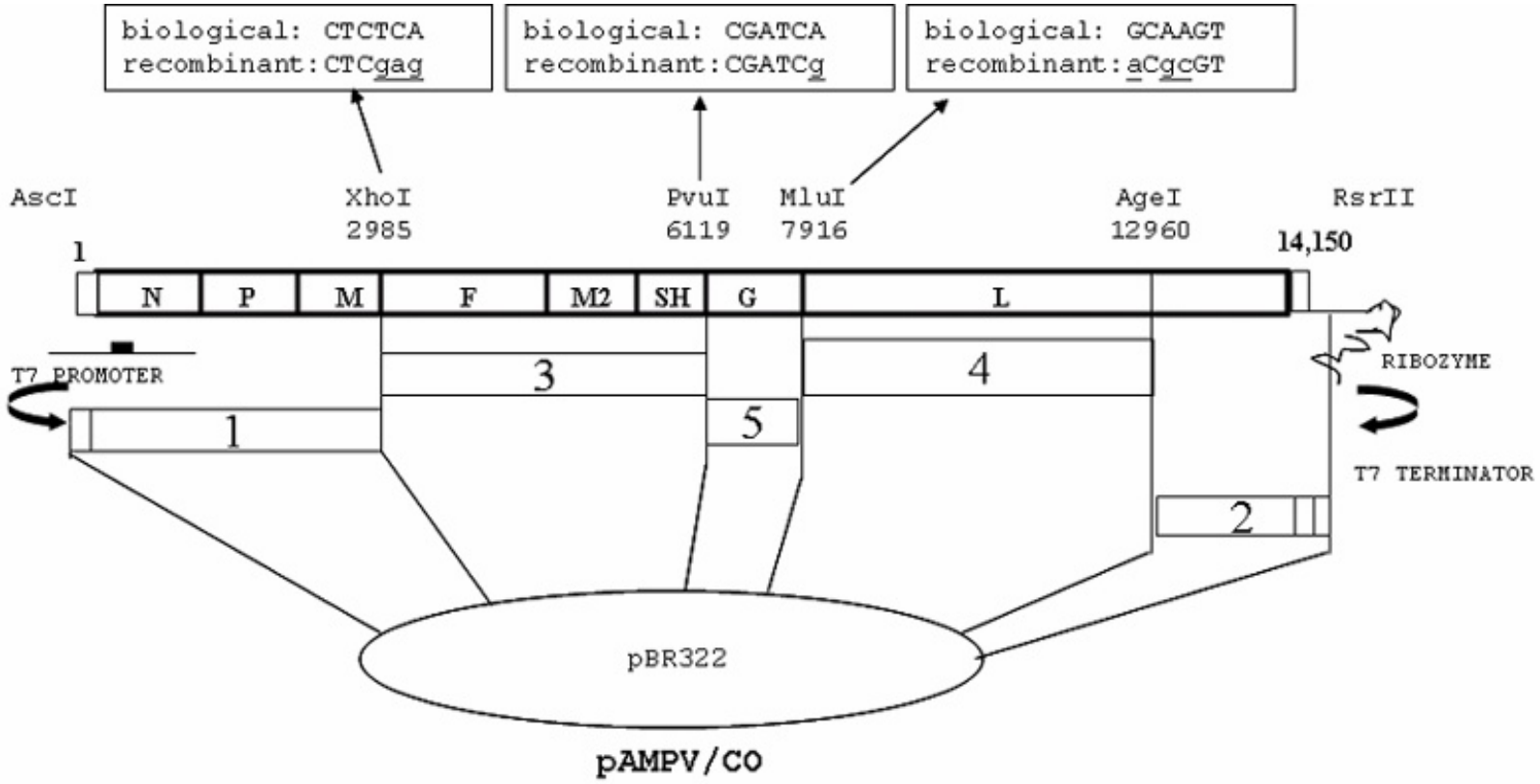

Figure 1. Generation of full-length AMPV/CO plasmid. Full-length AMPV/CO cDNA was assembled in pBR 322 from five subgenomic cDNA fragments that were generated by high-fidelity RT-PCR. The fragments were inserted in between the T7 RNA polymerase promoter sequence and the hepatitis delta ribozyme autocatalytic sequence which was followed by the T7 terminator sequence. The names of the restriction enzymes used for the assembly are shown at the top and the order in which the fragments were assembled is shown on each of them. The Xho I, Pvu I and Mlu I sites were introduced to facilitate construction and serve as markers (sequence changes shown in lower case and underlined).

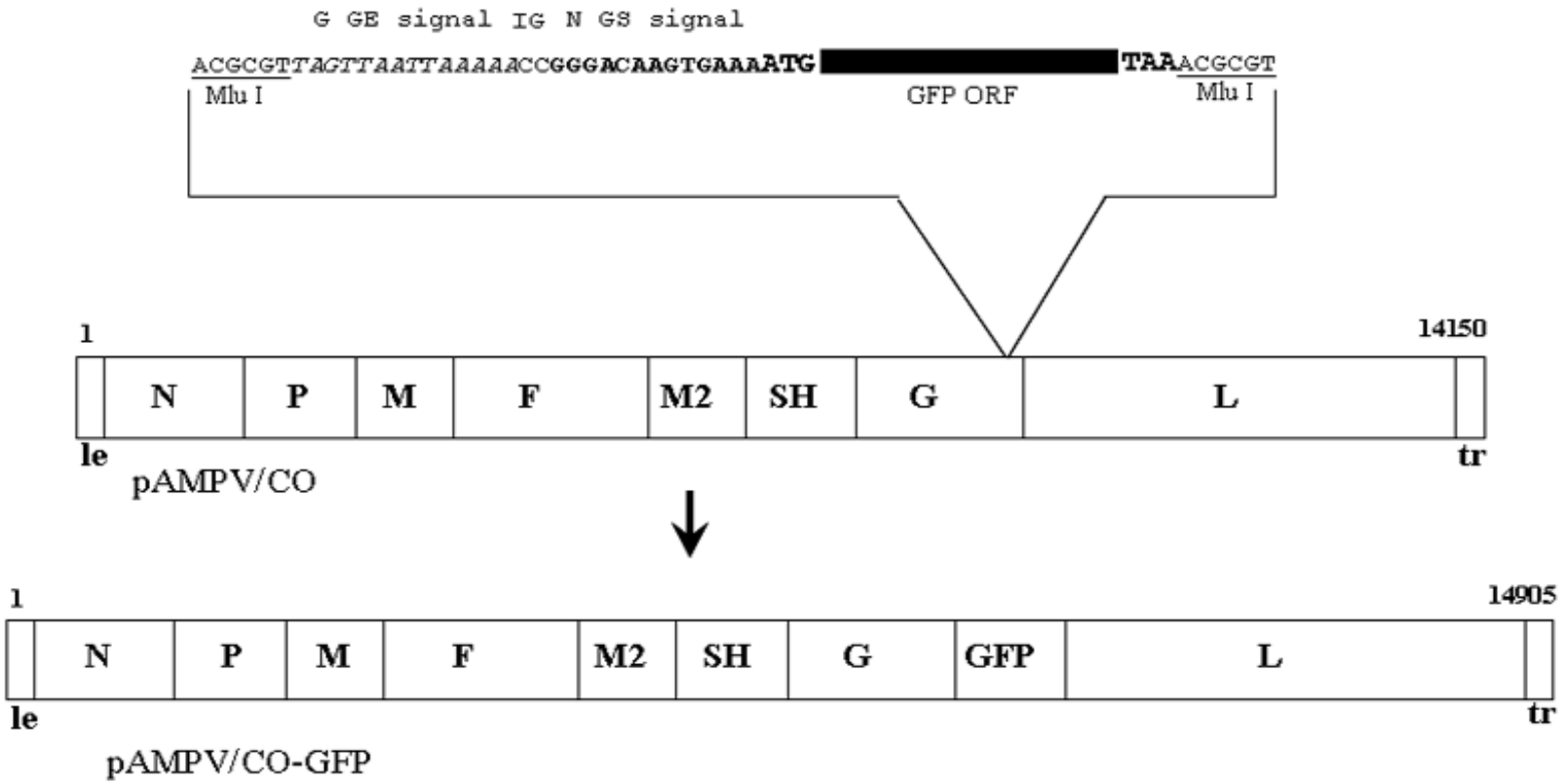

Figure 2. Construction of plasmid pAMPV/CO-GFP expressing the complete antigenomic RNA of AMPV/CO and GFP as a foreign gene. GFP was inserted in the putative 5' non-coding region of the G gene as a separate transcription cassette. The cassette contained the GFP ORF (black rectangle) that was flanked on the upstream side by a Mlu I site (underlined), the G GE motif (italicized), an IG region comprised of two C residues, and the N GS motif (bold). The GFP ORF was flanked on the downstream side by a Mlu I site (underlined). GE – gene end, GS – gene start, IG – intergenic, ORF – open reading frame.

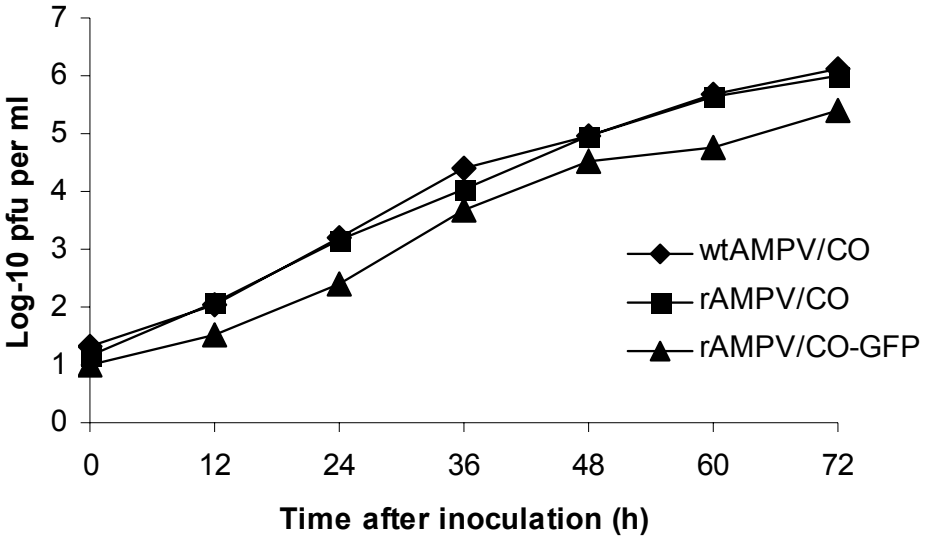

Figure 3**.** Replication kinetics of wild-type AMPV/CO, rAMPV/CO and rAMPV/CO-GFP. Vero cells were infected at a multiplicity of infection of 0.01 with wtAMPV/CO (♦), rAMPV/CO (■), or rAMPV/CO-GFP (▲). Supernatants (0.2 ml out of a total volume of 3 ml per well) were taken at the indicated time points post-inoculation and replaced by an equivalent amount of fresh medium. The samples were flash frozen and analyzed later for virus titers by plaque assay and immunostaining. Each time point was represented by two wells, and each titration was performed is duplicate. The mean virus titers are shown.

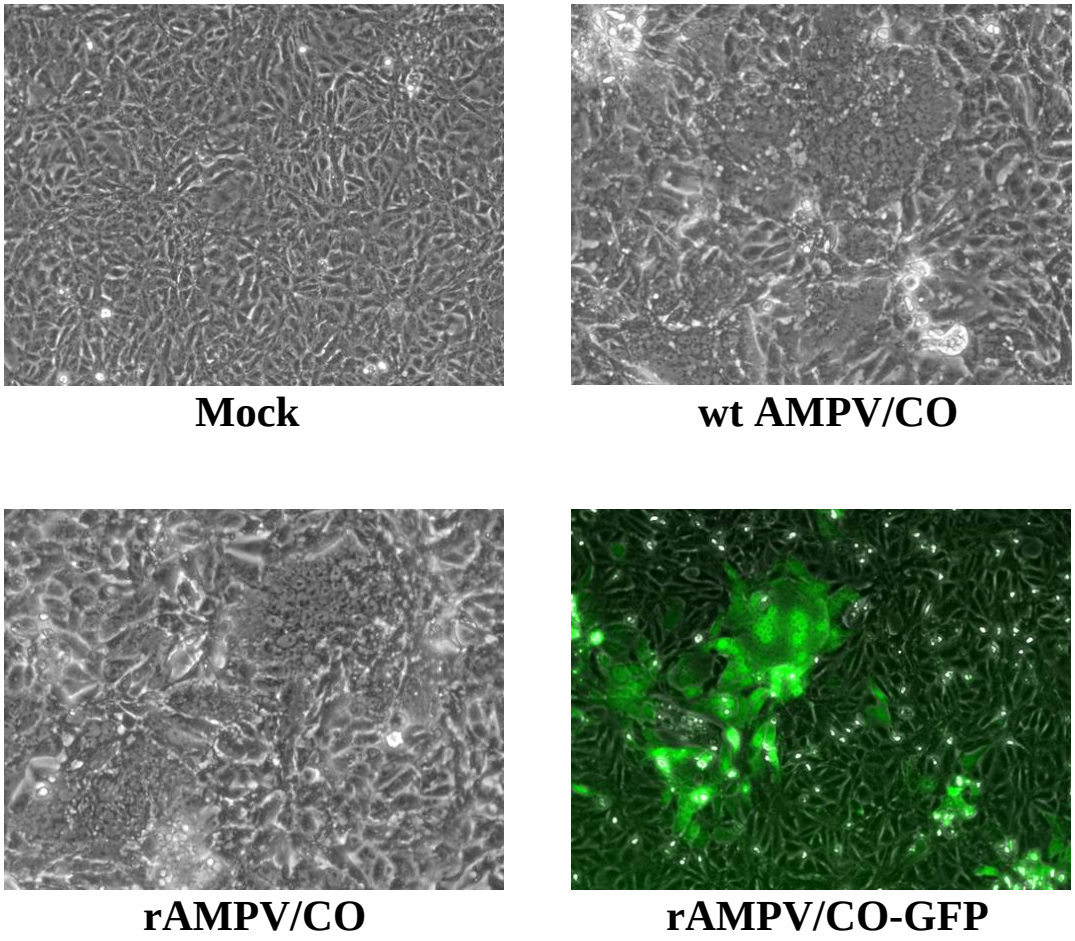

Figure 4A**.** Cytopathic effect of recombinant AMPV/CO in Vero cells. Vero cells were either infected with wtAMPV/CO, rAMPV/CO-GFP or mock-infected with PBS and observed for appearance of CPE characteristic of AMPV. The recovered recombinant viruses induced syncytia indistinguishable from those of wild type AMPV/CO, at 72 h post infection, at an MOI of 0.1.

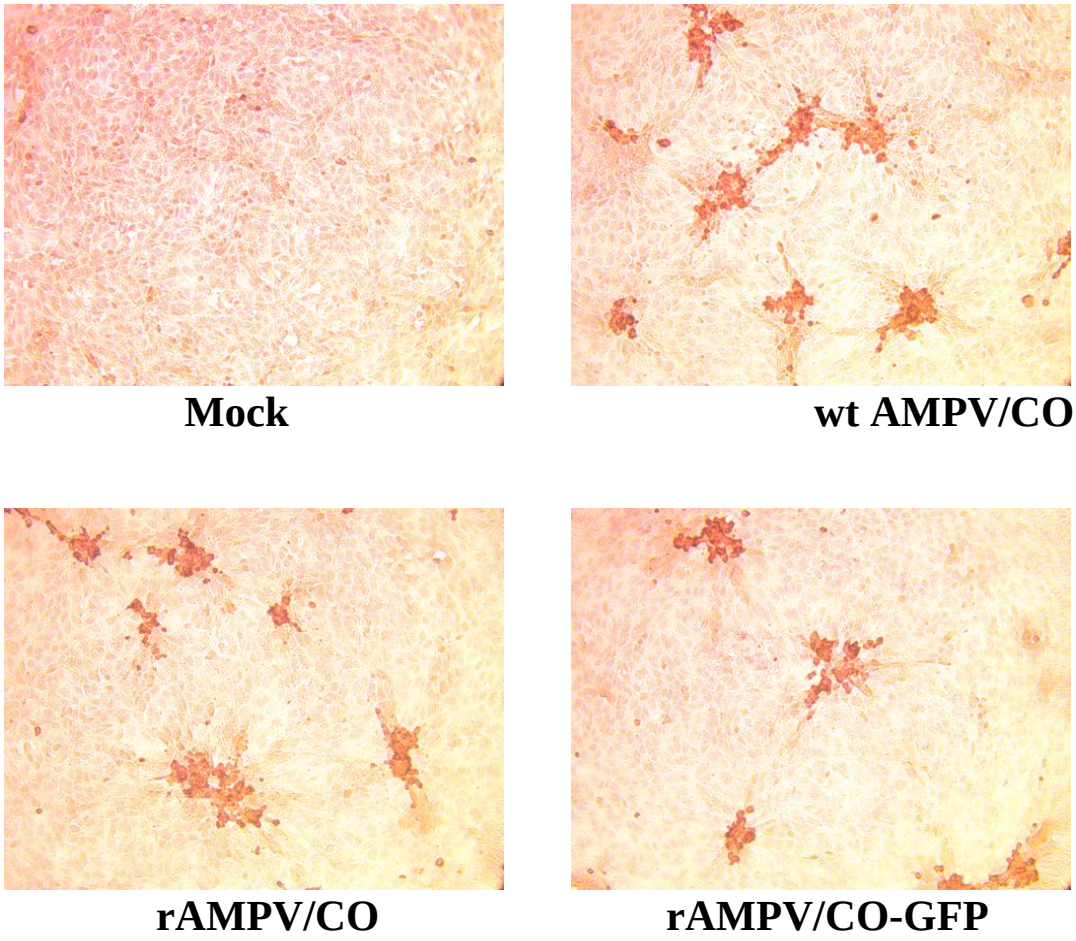

Figure 4B. Immunostaining of AMPV/CO-infected Vero cells. Vero cells were either infected with 0.1 MOI of wtAMPV/CO, rAMPV/CO-GFP or mock-infected with PBS and the infected monolayer was grown in 2 % EMEM and 0.8 % methyl cellulose overlay. After 72 h of infection, the overlay was removed and the monolayers were subjected to immunoperoxidase staining using an anti-peptide antibody raised against the N protein of AMPV/CO.

RNA INTERFERENCE OF AVIAN METAPNEUMOVIRUS (APV) PRODUCTION BY TARGETING RNA NUCLEOPROTEIN GENE (N) IN CULTURED CELLS *

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SUMMARY

Avian pneumovirus (APV) is an enveloped, non-segmented, negative-stranded RNA virus that is the primary causative agent of severe rhinotracheitis in turkeys and has been associated with swollen head syndrome of chickens. A potential strategy is the RNA interference technology (RNAi), based on the introduction of small interfering RNA molecules (siRNAs), which can promote cleavage of a complementary and specific mRNA sequence. The RNAi can be specifically used to silence important protein expression for the viral replication. In this study, we designed specific siRNAs targeting the nucleoprotein (N) and fusion protein (F) genes in an AMPV strain. The siRNA transfections were performed in chicken embryo related cells (CER) in 24 well plates and after 24 h of growth (90% confluent), using Lipofectamine 2000 (Invitrogen), according to manufacturer's recommendations. Transfected cell monolayer was infected with AMPV after 6 h post transfection. Seventy-two hours post virus infection, a semi-quantitative RT-PCR and virus titration assay were performed, to verify the N and F genes inhibition by siRNAs and validate the siRNA effect on replication of aMPV. The association of siRNA N + F reduced both N and F RNAs production, while the siRNA F used alone showed no reduction of F RNA production, as verified by PCR reactions. In addition, a decrease in virus titers of siRNA N and N+F transfected CER cells was evident. Probably, in the presence of siRNA N, the newly synthesized N, further viral transcription and replication are blocked, as is new virion production. Further analyses using quantitative techniques, such as Real-time PCR, have to be performed to confirm these findings; however, the targeted mRNA degradation and the resulting another viral RNA transcription make the siRNA N especially potent inhibitors as an antiviral therapy or a tool to study AMPV infections.

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INTRODUCTION

RNA interference (RNAi) is a process of sequence specific post-transcriptional gene silencing initiated by double-stranded RNA (dsRNA). This phenomenon was first observed in the nematode Caenorhabditis elegans (1) and is conserved in mammalian cells. RNAi dependent silencing in C. elegans can be initiated through dsRNA injection, soaking of worms in dsRNA, or feeding the worms with dsRNA-producing bacteria. After delivery of long dsRNA to C. elegans, it is subsequently processed into 21–25 bp functional small interfering RNA (siRNA) by an enzyme called Dicer that belongs to the RNase III family (2). siRNAs are incorporated into an enzyme complex RISC (RNA-induced silencing complex), which upon activation unwinds the siRNA. This siRNA is used by RISC for selecting the target RNA, which is later degraded in the region of homology directed by the original siRNA (3). Recently, RNAi has been successfully used against several viruses in mammalian cells (4) used siRNA to silence the mRNAs produced by the respiratory syncytial virus (RSV), a negative strand virus that causes a form of respiratory disease. However, authors of this work could not inhibit the full-length viral genomic strand that might be due to the fact that the RNA was associated with structural proteins.
Avian metapneumovirus (AMPV) or Turkey rhinotracheitis virus (TRTV) causes acute rhinotracheitis characterized by coughing, nasal discharge and conjunctivitis in turkeys. In chickens the respiratory tract infection playing a role, in association with bacteria, on the development of swollen head syndrome (5-9). Pneumoviruses are members of the family *Paramyxoviridae*, subfamily *Pneumovirinae*, within the genus *Metapneumovirus* (5-7, 10) that contain a nonsegmented, negative-sense RNA genome of approximately 15 kb in length. Viruses related to AMPV include human (hRSV), bovine, ovine and caprine respiratory syncytial viruses and pneumonia virus of mice (10), as well as the recently identified human metapneumovirus (hMPV). The metapneumovirus genome encodes eight distinct transcription units in a linear array, with each unit separated by a short segment of untranscribed sequence.
These include the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), second matrix protein (M2), small hydrophobic protein (SH), surface glycoprotein (G), and a viral RNA-dependent RNA polymerase (L), encoded by genes with the same designation. The RNA genome in association with the virus nucleoprotein (N) and large (L) proteins and the phosphoprotein (P) and the L protein is often referred to as the RNA polymerase since it isthought to contain all of the catalytic activities necessary for virus RNA synthesis, although it does not function as such without the N and P proteins. The M2-1 transcriptional enhancer protein is also thought to be associated with the nucleocapsid, although this has not yet been determined directly (10).
The N protein forms an integral part of the nucleocapsid complex of the virion and is an essential component of the polymerase complex. The N protein is thought to be responsible for giving the RNA genome its helical structure through a tight association with the virus genome RNA (10).
The virion has two main surface glycoproteins in the viral membrane: the attachment (G) protein, which mediates virus binding to the cell receptor (11), and the fusion (F) protein, which is responsible for fusion of the viral and cell membranes (12). A third small hydrophobic (SH) surface glycoprotein of unknown function is expressed abundantly at the surface of infected cells, but is incorporated only in small amounts

in the virus particle (13). The F protein of hRSV was first was identified by immunoprecipitation using monoclonal antibodies which inhibit the formation of multicell syncytia in cell culture. The pneumoviruses have an F protein that promotes cell fusion, but these viruses do not hemagglutinate, nor do they have neuraminidase activity in their G attachment protein. This is an important distinguishing characteristic from the other paramyxoviruses (14).
The F protein is synthesized as an inactive precursor that is cleaved by furin-like proteases during transport to the cell surface to yield two disulfide-linked olypeptides, F2 from the N terminus and F1 from the C terminus (15). There are three hydrophobic sequences in the F polypeptide. The first is the signal peptide, located at the N terminus of the F2 chain. The second is the fusion peptide at the N terminus of the F1 chain, and the third is the transmembrane region, located near the C terminus of F1.(16).
The aim of study is demonstrate that RNAi mediated silencing of AMPV N gene (portion N2) could effectively inhibit expression of AMPV antigen but not F gene (portion F2), hence RNAi based strategy should be further explored as a more efficacious antiviral therapy of AMPV infection.

MATERIALS AND METHODS

siRNAs

A set of four 21-nt siRNAs corresponding to aMPV N gene (GenBank accession number U39295.1) and aMPV F gene (GenBank accession number D00850.1) were obtained and subjected to BLAST analysis to ensure lack of homology to sequences other than the target gene.
siRNAs included siRNA_ N [sense, r(CCGGCGUGCCUCAAGGGUAUUU)dTdT], siRNA_F [sense, r(UCUCCUAUUGUUCCUUAUAUC)dTdT]. A double stranded siRNA GFP gene [sense, r(GACGGGAACUACAAGACACGU)dTdT] that does not share identity with any known sequence was also obtained and used as non-silencing control. All RNA oligonucleotides were synthesized by IDT (Coralville, IA, USA). The oligonucleotides were deprotected according to the manufacturer's instructions. Equimolar amounts of complementary oligonucleotides were mixed and annealed by heating to 95°C for 5 min, then reducing the temperature slowly to 25°C for 20 min and stored in -20°C. The resulting siRNA duplexes were analyzed for completion of duplex formation by gel electrophoresis.

Cell culture and transfection

The CER (Chicken related embryo) cell line was cultured cultivated in Eagle's minimal essential medium (E-MEM) with 10% bovine foetal serum without antibiotics. The CER cells were plated onto 24 well plates at a density of 2 x 10e5 cells per well and cultured at 37^{o}C with 5% CO_2 overnight for transfection. The transfection was performed at 90% cell confluence with a total amount of 75nM siRNA (siRNA_N, siRNA F, siRNA N+F, and as irrelevant gene the siRNA_GFP) per well using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol.

AMPV culture and Virus Infection

AMPV (strain SHSBR/662/03, 13th passage, GenBank accession number AAU87838), provided by the Laboratory of Animal Virology, Brazil, was propagated

on CER cells. For the test of inhibition of the virus by siRNA, the transfected CER cells (with siRNA) were infected with 0.01 multiplicity of infection (MOI) of AMPV per well 6 h post transfection in a final volume of 0.2 ml of E-MEM with 2% FBS for 1 h at 37OC. After it, E-MEM was added 1 ml to allow growth for 72 h at 37oC with 5% CO2. Then, the cells were lysed and assayed for the virus titration and RNA extraction to investigate the inhibition of AMPV.

Virus titration
CER cells were infected with cells treated by siRNA (siRNA_N (portion N2), siRNA_F (portion F1, an association of siRNA_N + F and siRNA_GFP) The virus was allowed to infect the cells for 1 h and then the cells were completely with E-MEM. After incubation for 72 h, the cells were observed and the titre calculated as.

RNA extraction and reverse transcription (RT) - PCR
Total RNA was extracted from the infected CER cultures using Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's recommendations. Viral RNA was reverse transcribed using 60 ng of random primer (Invitrogen, Carlsbad, CA, USA) with the kit Superscript III Rnase H- reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For the N gene amplification, the primers designed were: Nf and Nr. And for the F gene amplification the primers designed were: Ff and Fr and we detected also beta actin (house keeping gene). The PCR products (N gene– 698 bp and F gene-698 bp; beta-actin– 360 bp) were fractionated on 1% agarose gel electrophoresis and stained with ethidium bromide.

Statistical analysis: Pairs of groups were compared by Student´s t-test. Differences between groups were considered significant at $p<0.05$. Data for all measurements are expressed as means +- s.d.

RESULTS

Two different siRNA oligonucleotide sequence siRNA_N and siRNA_F for AMPV were designed. One siRNA for GFP was used as irrelevant gene and none effect on AMPV was observed. The controls plates (Figure 1B; GFP infected with AMPV) had high titers of virus (Figure $2{,}10^{6{,}85}$/mL), suggesting the virus replicated in the cells during the 72 h culture period. In contrast, the virus titer was substantially decreased in the plate where the cells were transfected with siRNA_N and siRNA_N + F (Figure 1C, 1E; Figure 2, $10^{4{,}05}$/mL), demonstrating that the siRNA inhibited virus replication and a significant ($p<0{,}05$) decrease for both virus titres. Moreover, no significant collateral off-target silencing effects were observed. In addition, The siRNA N reduced the N RNA production (Figure 3C) and the association of siRNA N + F reduced both N and F RNAs production (Figure 3E and 3F, respectively). In marked contrast, the siRNA_F showed no reduction of virus titre as compared to controls (Figure 2) and also did not decrease F RNA production (Figure 3D) as verified by semi-quantitative RT-PCR reactions (positive controls: Figure 3B).

DISCUSSION

The vast majority of viral RNA genomes are transcribed exclusively in the cytoplasm, catalyzed by virally encoded RNA-dependent RNA polymerases (RdRP)(17). The viral genome is a single, linear, negative-sense (anti-mRNA sense) RNA molecule. The full-length genomic and antigenomic RNA (− and + sense, respectively) are neither capped nor polyadenylated, and are wrapped with N protein and hence, are not accessible to siRNA, in contrast to the mRNA. The RNA molecule is intimately wrapped with the nucleocapsid protein N. The resultant nucleoprotein complex, termed N-RNA template, associates itself with the RdRP subunits, primarily L and P, and is finally packaged inside the structural shell of the virion, mainly made up of viral glycoproteins (18).

It is not yet clear precisely how the polymerase complex alters its activity and switches from the transcription to the replication mode. (19) showed that increased expression of the N protein stimulated hRSV replication. The nucleocapsid inside the virion is, therefore, transcription-ready, and thus, primary viral transcription begins shortly after infection. A notable physical property of the N-RNA template is its extreme resistance to RNases, suggesting that the tightly bound N protein offers protection. Only this N-RNA complex serves as the biological template for the viral RdRP, which would not recognize pure deproteinized genomic RNA. This is consistent with the proposal made for other negative-sense RNA viruses, suggesting that replication is dependent on RNA encapsidation (18). However, there are some conflicting data suggesting that high levels of N protein may also stimulate transcription, thus probably, in the presence of siRNA N, the newly synthesized N, further viral transcription and replication are blocked, as is new virion production.

Broad inhibition of viral proteins as well as reduced viral titers may be attributed to the RNAi induced reduction, as reported recently in the intracellular amount of P protein and the subsequent lack of P protein function of AMPV subtype C (20). Along with L and N proteins, P protein is packaged in the assembled virions which together initiate transcription and replication during initial stages of infection. However, owing to the critical roles of P protein as RdRP complex subunit (Dupuy et al., 1999;, active synthesis of new P protein is crucial to efficient replication without which blockage of further transcription and genomic RNA replication is imminent. Furthermore, continuous production of new P protein is also required as it is packaged within new virions egressing from the cell (20).

The fusion protein, F, is generally essential for cell fusion (formation of syncytia), although the relative importance of the attachment proteins in fusion is under active study and Knockdown of F resulted in complete loss of syncytium as reported by (4) , however the present study can not reduce the production of F RNAs and no effect as observed.

Further analyses using quantitative techniques, such as Real-time PCR, have to be performed to confirm these findings; however, RNAi mediated viral gene silencing and replication block offers great potential for novel nucleic acid based antiviral approaches, provided efficient means for in vivo siRNA delivery could be developed.

REFERENCES

1. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) Nature 391, 806-11.
2. Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001) Nature 409, 363-6.
3. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) Nature 411, 494-8.
4. Bitko, V. & Barik, S. (2001) BMC Microbiol 1, 34.
5. Cook, J. K. (2000) Rev Sci Tech 19, 602-13.
6. Cook, J. K. (2000) Vet J 160, 118-25.
7. Njenga, M. K., Lwamba, H. M. & Seal, B. S. (2003) Virus Res 91, 163-9.
8. Turpin, E. A., Perkins, L. E. & Swayne, D. E. (2002) Avian Dis 46, 412-22.
9. Velayudhan, B. T., Lopes, V. C., Noll, S. L., Halvorson, D. A. & Nagaraja, K. V. (2003) Avian Dis 47, 764-8.
10. Easton, A. J., Domachowske, J. B. & Rosenberg, H. F. (2004) Clin Microbiol Rev 17, 390-412.
11. Levine, S., Klaiber-Franco, R. & Paradiso, P. R. (1987) J Gen Virol 68 (Pt 9), 2521-4.
12. Walsh, E. E. & Hruska, J. (1983) J Virol 47, 171-7.
13. Olmsted, R. A. & Collins, P. L. (1989) J Virol 63, 2019-29.
14. van den Hoogen, B. G., Herfst, S., Sprong, L., Cane, P. A., Forleo-Neto, E., de Swart, R. L., Osterhaus, A. D. & Fouchier, R. A. (2004) Emerg Infect Dis 10, 658-66.
15. Collins, P. L. & Mottet, G. (1991) J Gen Virol 72 (Pt 12), 3095-101.
16. Gonzalez-Reyes, L., Ruiz-Arguello, M. B., Garcia-Barreno, B., Calder, L., Lopez, J. A., Albar, J. P., Skehel, J. J., Wiley, D. C. & Melero, J. A. (2001) Proc Natl Acad Sci U S A 98, 9859-64.
17. Ahlquist, P. (2002) Science 296, 1270-3.
18. Barik, S. (2004) Virus Res 102, 27-35.
19. Fearns, R., Peeples, M. E. & Collins, P. L. (2002) J Virol 76, 1663-72.
20. Munir, S., Kaur, K. & Kapur, V. (2006) Antiviral Res 69, 46-51.

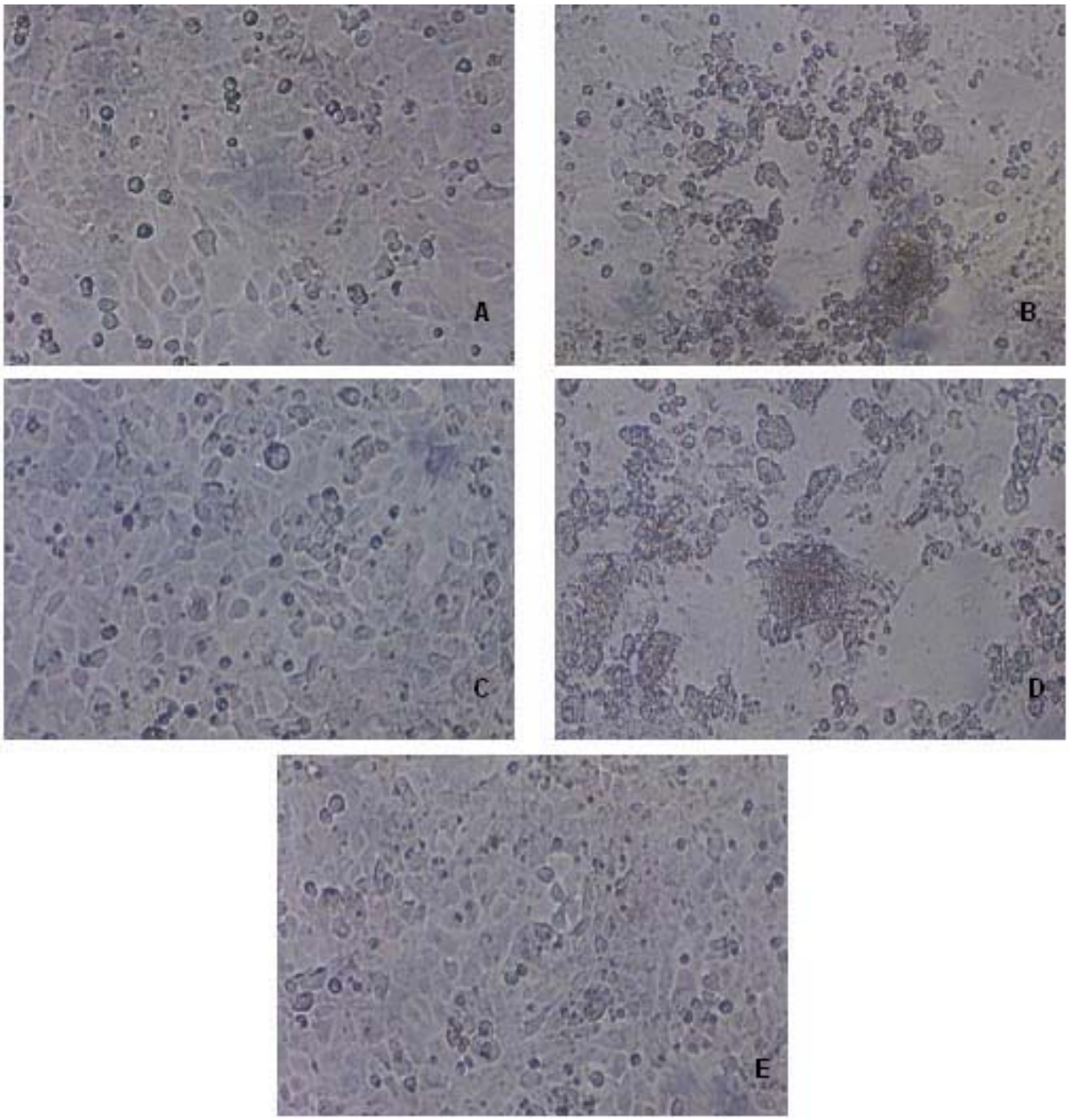

Figure 1: Cytopatict effect in transfected cells (after 72h). CER cells were infected with aMPVA/A (A662) after 6h in the presence of different siRNAs as indicated (200x): A- Negative control (without virus and siRNAs); B-siRNA_GFP Irrelevent gene (green fluorescent protein-GFP); C - siRNA N gene; D - siRNA F gene; E-siRNA N+F gene.

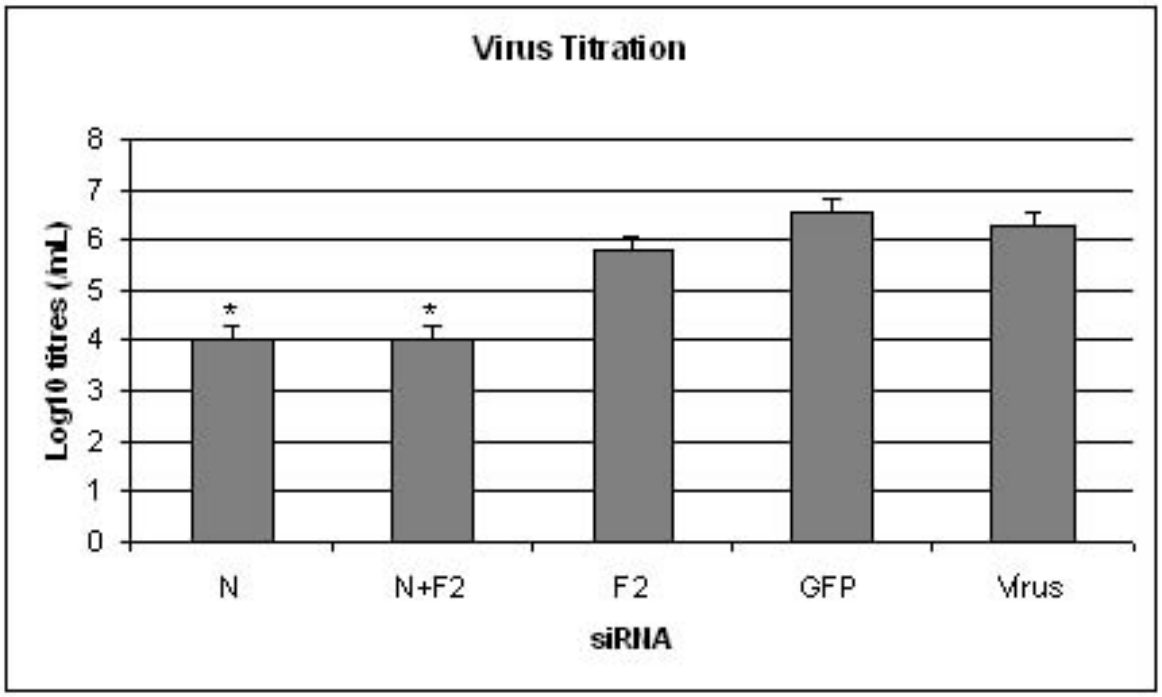

Figure 2: Transfected cell monolayer was infected with AMPV after 6 h post transfection and the virus titrations were determined for the supernatant of the cells cultured for 72 h after infection. * $p<0.05$ compared to control group.

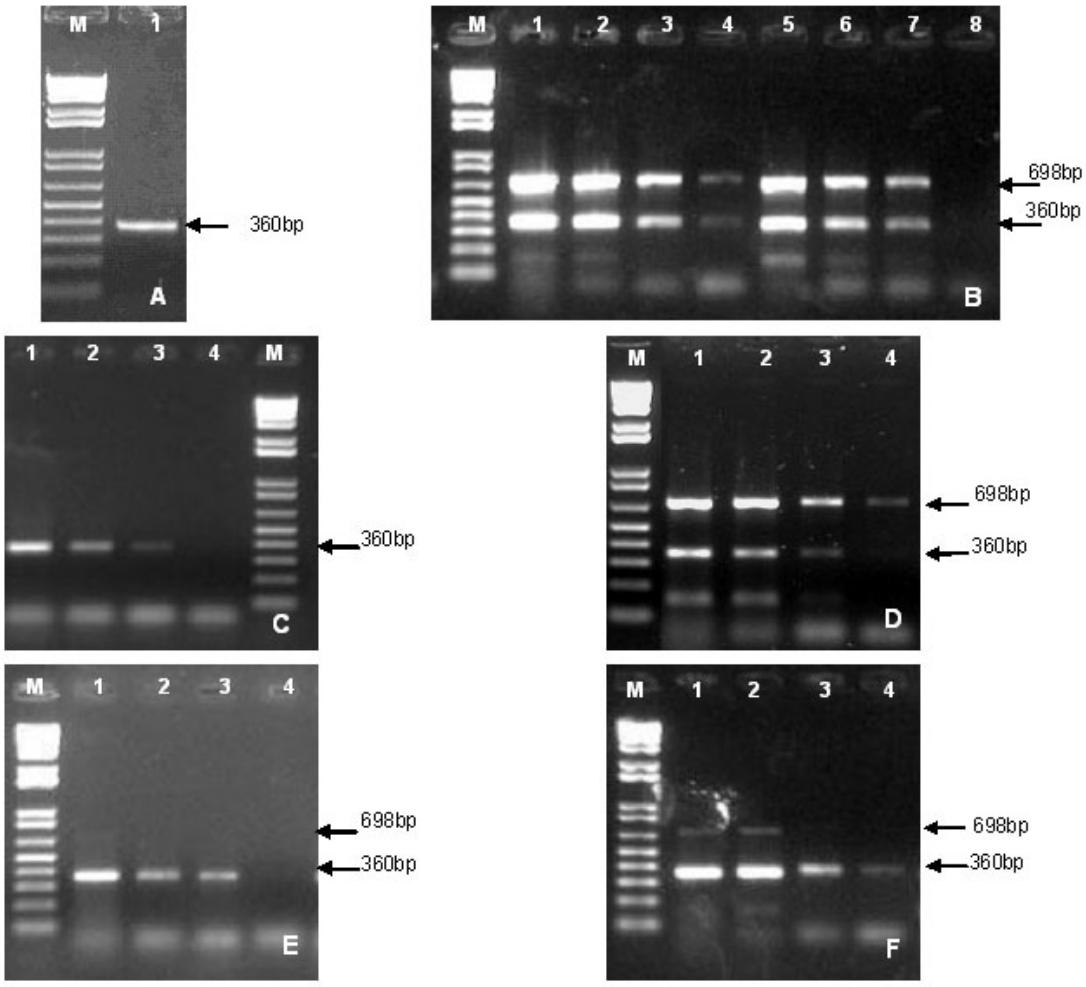

Figure 3: RT-PCR detection of N (698bp) and F gene (698bp) and the internal control, beta- actin (360bp), transcripts in CER cells infected with AMPV (strain A662) after 6h in the presence of different siRNAs as indicated: M: Leader 1kb plus; numbers 1-4 and 5-8:dilutions pure, 1/10, 1/100 and 1/1000, respectively. A- Negative control (without virus and siRNAs); B-siRNA Irrelevent gene (green fluorescent protein-GFP) (1-4: F gene; 5-8 : N gene); C- siRNA N gene- 25nM (N gene); D- siRNA F gene (F gene); E- siRNA N+F1+F2 gene (N gene); F- siRNA N+F1+F2 gene (F gene).

LABORATORY EVALUATION OF A QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTION PCR ASSAY FOR THE DETECTION AND IDENTIFICATION OF THE FOUR SUBGROUPS OF AVIAN METAPNEUMOVIRUS

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SUMMARY

Avian metapneumovirus (AMPV) is an important pathogen causing respiratory diseases and/or egg drops in various species of poultry including turkeys, chickens, ducks, guinea fowl or in wild birds as goose. According to their antigenic and molecular variations, AMPVs have been divided into four subgroups termed AMPV-A, -B, -C and -D . As the respiratory signs following infection are not AMPV-specific, many techniques are used for the laboratory diagnosis of AMPV infections. They rely on serological methods, on labour-intensive virus isolation procedures, or on recently developed subgroup-specific reverse transcription PCR (RT-PCR) protocols.
In the present study, the specificity and sensitivity of a commercial real-time reverse transcription PCR (RRT-PCR) for the detection and identification of the four AMPV subgroups were evaluated. It appeared that the RRT-PCR test allowed to detect only the AMPV viruses, the subgroup identification being in agreement with the previous molecular and antigenic subgroup assignment of each strain. Moreover, linear dose/ responses were observed using virus RNA and RNA runoff transcripts, thus allowing virus quantification. Finally, the results were reproducible under our experimental conditions.

INTRODUCTION

Since the early eighties, avian metapneumoviruses (AMPVs) have been recognized worldwide, Australia excepted, as a major cause of both respiratory and reproductive disorders in various species of poultry including turkeys, chickens, guinea fowl or in wild birds such as wild Canada goose (Bennett et al., 2002). First described in 1970

in South Africa, then in the 80ies in all Europe and more recently in 1996 in the United States, AMPVs have been, based on antigenic and molecular criteria, divided into four subgroups termed AMPV-A, -B (Juhasz & Easton, 1994), -C (Senne et al., 1997; Toquin et al., 1999) and -D (Bayon-Auboyer et al., 2000).
The diagnosis of AMPV infections cannot rely only on clinical observation, as signs are not AMPV-specific, and many laboratory techniques have been developed to support this diqgnosis. ELISA (Giraud et al., 1987; Wyeth et al., 1987) is the most commonly used serological method, however the antigenic variation between the four subgroups may negatively affect the assay sensitivity and serological results are delayed for the time needed for seroconversion. Virus isolation from tracheal swabs or respiratory organs of diseased animals relies on the labour-intensive culture of tracheal organs, cell lines or chicken cells. Conventional RT-PCR (Bayon-Auboyer et al., 1999; Cavanagh et al., 1999) provides an interesting alternative since this technique enables direct detection of the viral genome, hence allowing the early diagnosis of the infection from swabs. However, conventional RT-PCR is not convenient for large scale epidemiological studies and provides no indication on the amount of virus RNA present in samples, unless a semi quantitative preliminary step based on the dilution of the RNA or cDNA is implemented (Cavanagh et al., 1999).
In this study, we evaluated a further development of the molecular diagnosis of AMPV infection, the real-time RT-PCR (RRT-PCR). The TaqVet™ Avian Metapneumovirus (AMPV) A, B, C RRT-PCR kit designed by LSI, Laboratoire Service International, for the detection of AMPV subgroups A, B and C was evaluated with an additional combination of primers and probe developed for the detection of AMPV subgroup D.

MATERIAL and METHODS

Virus isolates

Three attenuated vaccines and twenty nine field strains or isolates of AMPV were used to validate RRT-PCR assay. The origin of most of these viruses was reported previously, and their subgroup assignment was determined both antigenically and molecularly (Bayon-Auboyer et al., 1999; Toquin et al., 2000; Toquin et al., 2003; Toquin et al., 2006). All viruses were used as crude supernatants of infected Vero cells. AMPV strain 85051 was selected as representative for AMPV-A, 86004 and 98103 for AMPV-B, 99350 for AMPV-C and 85035 for AMPV-D.
Fifteen non-AMPV avian viruses were also used : respiratory viruses (avian paramyxoviruses 1 to 4 and 6 to 9, orthomyxovirus H1swN1sw, fowl adenovirus type 1, reovirus, enteritic viruses (turkey haemorrhagic enteritis virus, infectious bursal disease virus serotypes I and II) and neurotropic virus (avian encephalomyelitis virus). These vieruses were propagated on specific pathogen free (SPF) chicken eggs, cell cultures, SPF embryos or SPF turkeys.

Determination of the virus titres

The infectious titres of the AMPV strains were determined in Vero cells by inoculating serial ten-fold dilutions. Presence or absence of cytopathogenic effect was observed directly and confirmed by an indirect immunofluoresence. The virus titres were determined by the Reed and Muench method (Reed & Muench, 1938) and

expressed as median Tissue Culture Infectious Doses ($TCID_{50}$) per ml of viral suspension.

RNA extraction
RNA extraction was performed using the QIAamp® Viral RNA Mini Kit (Qiagen), according to the manufacturer's instructions.
Standard curves to test the sensitivity of the RRT-PCR detection were prepared from the four viruses representing each subgroup, with samples of titrated virus suspensions that were serially diluted (ten fold dilutions) then were extracted as above.
To clone the AMPV genes in order to perform the *in vitro* transcription of the genes targeted by the RRT-PCR, viral RNA was extracted with the RNeasy® Mini kit (Qiagen) from the supernatant of Vero cells infected by the representative AMPV strains.

Design of oligonucleotides and Taqman ® probes for RRT-PCR
The primers and Taqman ® probes included in TaqVet™ Avian Metapneumovirus (AMPV) A, B, C RRT-PCR kit (LSI, Lyon, France) and the supplementary primers and probe designed for detecting subgroup D AMPV were defined by LSI using the Primer Express Version 2.0 software (Applied Biosystems). Based on the conserved regions of the nucleotide sequences available for the target genes, a forward primer, a reverse primer and a probe (labelled with FAM or VIC dye) were defined for each subgroup.

Taqman ® RRT-PCR assays
The Taqman ® RRT-PCR allows to monitor the progress of PCR as it occurs, in real-time, as the accumulation of fluorescence during successive PCR cycles parallels the accumulation of PCR products. A mix containing the D-specific primers and probe was added to the TaqVet® Avian Metapneumovirus (AMPV) A, B, C RRT-PCR kit to detect all subgroups, and was developed as a one step Taqman ® RRT-PCR assay designed under a 96-well format.
The RNA elution buffer, RNase-free water and RNA extracted from mock-infected Vero cells were included in each assay as negative controls. All samples were run in duplicates and the average of the two duplicates was considered as the final result. Data were analysed with the Sequence detection version 1.2.3 software (Applied Biosystems) using an automatically determined baseline and threshold values (Auto Ct option) for each detector (FAM and VIC).

Cloning and in vitro RNA synthesis from the target genes
The detectability of the RRT-PCR in terms of numbers of RNA copies was measured by testing serial dilutions of cRNAs of the targetted genes. To achieve this, the target genes of the 4 representative AMPV strains were cloned under the control of the T7 promoter into the pcDNA 3.1©Directional TOPO expression kit (Invitrogen). Stocks of the four plasmids were produced using the Endofree ® Plasmid Giga and/or Mega kits (Qiagen) according to the manufacturer's recommendations and stored at –20°C until used for the in vitro synthesis of synthetic RNA runoff transcripts.

In vitro synthesis of RNA runoff transcripts
RNA runoff transcripts were synthesized to generate a standard curve for the quantitation of RNA copy numbers. The pcDNA constructs were linearized at the *Eco RV* (New England Biolabs) restriction site located in the plasmid. Runoff transcripts were synthesized using the T7 RNA polymerase (Promega) according to standard protocols. Following the transcription reaction, the DNA templates were digested with the RQ1 RNase-free DNase (Promega,). The *in vitro* transcripts were extracted with phenol/chloroform, resuspended in nuclease-free water, aliquoted and stored at –70°C. Number of RNA molecules were calculated after spectrophotometer quantification of RNA using A_{260}, based on the expected length of the in vitro transcripts.

Assessment of detectability and linearity of response
Standard curves were constructed with RNA runoff transcripts for each AMPV subgroup. For each transcript, 10 serial ten-fold dilutions were analyzed and the curves were constructed from the resulting Ct values. The experiment was repeated three times, by different manipulators, on 3 different days, from the same RNA stocks.
Other standard curves were constructed using serial dilutions of the four titrated virus suspensions. Eight serial ten-fold dilutions were prepared from each virus suspension. Repeats were performed as with RNA runoff transcripts.
In order to know if the Ct values were linked with experimental parameters (subgroup, quantity and day of experiment), we performed two analyses of variance model (Anova test), one for the RNA runoff transcripts and the other for the RNA from virus. All statistical analyses were performed using the *GLM* procedure of the *SAS* software (SAS France). Predicted values of quantity, explained from Ct, were calculated from a linear fit, using the *REG* procedure of the *SAS* software. Confidence interval (95%) is given for the mean expected value of the variable.

RESULTS

Specificity of the RRT-PCR assays
Neither the mock infected Vero cells, nor the RNA templates isolated from the non-AMPV strains, yielded any Ct value lower than 35. Applied to 29 AMPV strains or isolates and to the three attenuated AMPV vaccines, the assay always allowed the detection of the virus under study and all subgroup assignments were in agreement with the antigenic and genetic typing performed earlier (Table 1).

Dose / effect curves of the RRT-PCR
Irrespective of the subgroup (A to D) and origin (runoff transcripts or virus) of the tested RNA, Ct values higher than 35 cycles were out of linearity. With RNA runoff transcripts, dose / effect curves exhibited a very significant linear correlation from 10^5-10^6 to 10^{12} copies / ml (R^2 between 0.972 and 0.997).
With RNA extracted from virus dilutions, very significant linear correlations were also observed. Assays proved linear from $10^{-1.50}$-$10^{0.50}$ to $10^{4.50}$-$10^{5.21}$ $TCID_{50}$/ml (R^2 between 0.979 and 0.997).
The standard curves as shown concerning subgroup B in Fig. 1 were used to determine the AMPV RNA copy numbers detected in the assay and the number of

copies per ml of virus. Knowing the virus titres for each virus stock, the ratio of RNA molecules per $TCID_{50}$ was found to be in the $10^{4.9}$ to $10^{6.2}$ range depending on the virus stock.

Reproducibility

We performed an Anova test in order to know whether or not, under our experimental conditions, the results were dependent of the day of assay.

Using RNA runoff transcripts, the statistical analysis revealed a trend to obtain slightly different results according to the day of assay (p = 7.66 %). However, the same analysis was done with RNA from virus stocks. Analysis of the variance showed clearly that Ct values were not significantly different depending on the day parameter (p = 75 %), meaning data were independent of the day.

The maximum range of the confidence intervals (95% mean confidence level) from the predicted values for the amounts of RNA molecules/ml or of $TCID_{50}$/ml were $10^{0.29}$ and $10^{0.36}$ for RNA copies and $TCID_{50}$ respectively (data not shown), thus illustrating the very good reproducibility of the quantitative approach based on the assay.

DISCUSSION

The Taqman chemistry used in the studied RRT-PCR assay relies on the definition of a combination of target-specific primers and probe. The high subgroup specificity of the sequence of the primers and probes forms the basis for the very good specificity of the assay : Indeed, none of the 15 non-AMPV viruses resulted in a false positive, and among 32 AMPV strains or isolates, all were identified in agreement with their previous subgroup assignment.

The late responses sometimes observed with the heterologous primers and probes (differences in Ct of at least 10 cycles) are unlikely to interfere with subgroup identification under field conditions when samples with a lower virus load than cell culture supernatants will be analysed. These heterologous responses might reveal well-to-well contamination via aerosols, a possibility that cannot be ruled out when RT-PCR assays are performed under the 96-well format and despite the efforts to prevent such contaminations. Such an hypothesis is indeed consistent with negative controls (mock infected Vero cells, RNase free water, AVE buffer) presenting a Ct value higher than 35, so that Ct values above 35 should be considered as non-specific.

The tested AMPV assays seem well-suited for a subsequent quantitative use, as all standard curves established with either RNA runoff transcripts or RNA extracted from serial dilutions of virus stocks revealed a very significant linear correlation between the RNA quantities and the Ct values (R^2 > 0.97) (Fig 1.), irrespective of the subgroup. For each assay, the limit of linearity in the dose response curves was around 35 cycles, a threshold that was consistent with the specificity limit previously defined with the negative controls and non-AMPV viruses. The ANOVA study of the assay repeatability showed that, under our experimental conditions, all data obtained with RNA from virus stocks were not significantly different, independent of the day of assay (p = 0.75), thus demonstrating the assay reproducibility. Although, reproducibility was not so good with RNA runoff transcripts (p = 0.07), the slopes of all the dose / effect curves were still closely related, which suggest that quantification

of the RNA load in samples treated on the three different days would have yielded consistent results (data not shown), a prerequisite for the robust use of RRT-PCR in quantitative assays.
Incidentally, it is interesting to note that different "number of RNA copies per $TCID_{50}$ were obtained with the different subgroups, a finding that could stem both from the viruses having different transcription / replication efficiencies, but also from the stock viruses being harvested at different stages of the virus replication cycle. In addition, the sensitivity with RRT-PCR appears similar or higher as compared with previous conventional RT-PCR methods for the detection of AMPV (Malik et al., 2004), (Toquin et al., unpublished data).
Altogether, the present study demonstrates that RRT-PCR assay can be considered as a powerful tool for the detection, the subgroup identification and the quantification of the four AMPV subgroups. AMPV-specific RRT-PCR could find further applications in the implementation of large scale on-farm epidemiological studies (with RRT-PCR performed as a screening test prior to virus isolation), in the quantification of virus excretion following challenge in birds vaccinated with vaccines from the homologous or an heterologous subgroup, etc ….

ACKNOWLEDGEMENTS

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REFERENCES

Bayon-Auboyer, M.H., Arnauld, C., Toquin, D. and Eterradossi, N., 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J Gen Virol 81, 2723-33.

Bayon-Auboyer, M.H., Jestin, V., Toquin, D., Cherbonnel, M. and Eterradossi, N., 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Arch Virol 144, 1091-109.

Bennett, R.S., McComb, B., Shin, H.J., Njenga, M.K., Nagaraja, K.V. and Halvorson, D.A., 2002. Detection of avian pneumovirus in wild Canada (Branta canadensis) and blue-winged teal (Anas discors) geese. Avian Dis 46, 1025-9.

Cavanagh, D., Mawditt, P., Britton, C. and Naylor, C.J., 1999. Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathol 28, 593-605.

Giraud, P., Toquin, D., Picault, J.P., Guittet, M., L'Hospitalier, R., Kles, V. and Bennejean, G., 1987. Utilisation de la méthode Elisa pour le sérodiagnostic de l'infection par le virus de la rhinotrachéite infectieuse chez la dinde, le poulet et la pintade. Bulletin d'information des laboratoires de Services Vétérinaires 27/28, 65-70.

Juhasz, K. and Easton, A.J., 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J Gen Virol 75 (Pt 11), 2873-80.

Malik, Y.S., Patnayak, D.P. and Goyal, S.M., 2004. Detection of three avian respiratory viruses by single-tube multiplex reverse transcription-polymerase chain reaction assay. J Vet Diagn Invest 16, 244-8.

Reed, L.J. and Muench, H., 1938. A simple method of estimating 50 per cent end points. Am J Hyg 27, 493-7.

Senne, D.A., Edson, R.K., Pedersen, J.C. and Panigraphy, B., 1997. Avian pneumovirus update. 134th Annual Congress of the American Veterinary Medical Association, 190.

Toquin, D., Bayon-Auboyer, M.H., Jestin, V., Eterradossi, N. and Morin, H., 1999. Isolation of a pneumovirus from a Musckovy duck. Vet. Rec. 145, 680.

Toquin, D., Bayon-Auboyer, M.H., Senne, D.A. and Eterradossi, N., 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses. Avian Dis 44, 977-82.

Toquin, D., de Boisseson, C., Beven, V., Senne, D.A. and Eterradossi, N., 2003. Subgroup C avian metapneumovirus (MPV) and the recently isolated human MPV exhibit a common organization but have extensive sequence divergence in their putative SH and G genes. J Gen Virol 84, 2169-78.

Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C. and Eterradossi, N., 2006. European and American Subgroup C Isolates of Avian Metapneumovirus belong to Different Genetic Lineages. Virus Genes 32, 97-103.

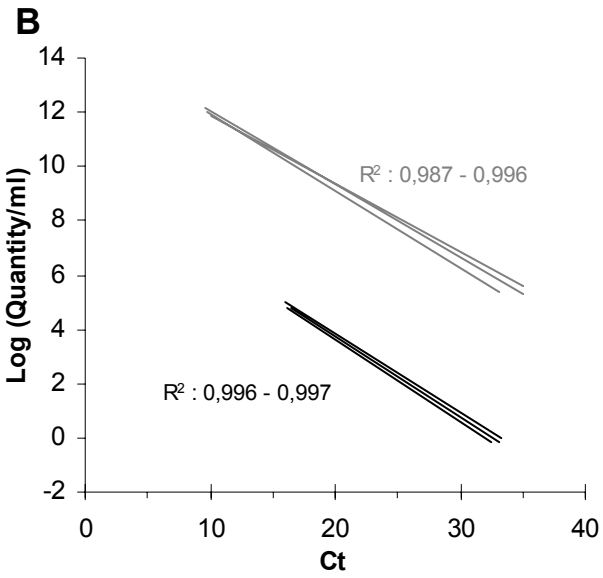
Wyeth, P.J., Chettle, N.J., Gough, R.E. and Collins, M.S., 1987. Antibodies to TRT in chickens with swollen head syndrome. Vet Rec 120, 286-7.

Table 1 : Subgroup identification using RRT-PCR in 29 AMPV strains or isolates and 3 attenuated AMPV vaccines
Subgroup assignment, history and source of the tested viruses as previously reported in (Bayon-Auboyer et al., 1999; Toquin et al., 2000; Toquin et al., 2003; Toquin et al., 2006). ND. : No Ct value could be determined at the end of the 45 PCR cycles.

Subgroup	Number of tested Strains	Origin	Range of Ct values obtained with probes			
			A	B	C	D
A	9	UK/France/ Israel/Germany	12.3-22.2	ND	36.9-ND	29.1-36.9
B	15	France/ Germany	30.9-ND	14.4-21.7	33.0-ND	31.3-36.7
C	6	France/USA	33.7-ND	33.7-ND	12.3-23.3	28.6-35.0
D	2	France	33.7-39.6	ND	32.5-ND	12.6-14.4

Fig. 1: Standard dose / response curves of RNA derived from runoff transcript or virus dilutions of representative virus strain belonging to the AMPV subgroup B.

Standard curves derived from runoff transcripts or virus dilutions are indicated in grey and black, respectively. Quantities for runoff transcripts or virus dilutions are indicated as "number of molecules" or "$TCID_{50}$" per ml, respectively. For each series of three curves, the range of the R^2 coefficient of determination is shown. This parameter, with a maximum value of 1, indicates the amount of variation in the studied data that can be accounted for by the linear regression model.



TOWARDS THE DEVELOPMENT OF NOVEL ELISAS FOR AVIAN PNEUMOVIRUS (APV) SEROLOGY

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SUMMARY

ELISAs are the method of choice for avian pneumovirus serology as they are rapid and can process many samples at the same time. Traditional ELISAs that use whole virus as antigen work well but have shown varied sensitivity with different subtypes. We address this here by expressing regions of the highly antigenic fusion (F) protein (subtype A) that may be equally sensitive for all subtypes or subtype- specific.
Six regions of the F gene were individually cloned into an expression vector specifically designed for use in an *E.coli* system. Expressed recombinant proteins were then purified using metal affinity chromatography. The presence of recombinant protein was detected using a monoclonal antibody against an engineered region of six amino acids placed at the amino terminal of each F section. Each F section gave higher absorbency readings with an APV-positive serum and lower readings with specific pathogen free (SPF) serum from 12 week old chickens when compared with a Liverpool-developed whole virus antigen ELISA. These initial test suggest that sections of the F protein are strong antigen candidates for APV ELISA serology. However, high background was observed when testing serum from 80week old SPF chickens. Serum absorption and agar gel precipitation techniques have confirmed that background absorbence is unlikely to be due to serum ab reactions with *E.coli*. The cause of these background reactions is under further study.

INTRODUCTION

The avian pneumovirus (APV) exists as different sub-types, A and B in Western Europe and most of the rest of the world and subtype C in the USA and to a limited extent in France. For diagnosis of APV infections, there is a wide range of tests available to detect viral antigens such as virus isolation, immunofluorescent staining and the polymerase chain reaction.
For detection of specific antibodies in serum, enzyme-linked immunosorbent assay (ELISA) is the method of choice, since the tests are rapid, specific, require small volumes of serum and allow the processing of many samples at the same time.

ELISAs that use antigen prepared from virus-infected cell cultures generally work well, but have the disadvantage that they contain many unwanted proteins in the preparation. These can lead to non-specific binding, which in turn reduces the specificity of the test.
In addition, it is known that ELISAs which use an empirical sub-type A antigen do not detect antibodies to sub-type B with the same sensitivity and vice versa [1]. Thus, to address this, novel ELISAs are being developed, and one approach is to use recombinant proteins. This should enable the expression of individual epitopes that are subtype-specific or alternatively, equally sensitive to all subtypes, depending on the protein used.
Our approach has been to express regions of the highly antigenic fusion protein (F). These were amplified using PCR, cloned, expressed in *E.coli* and purified using nickel columns. These recombinant proteins were tested for their suitability as antigens for ELISAs. Initial tests using pooled serum taken from APV infected SPF chickens showed improved performance with F section recombinants to that of Liverpool developed in-house ELISA.

MATERIALS and METHODS

Expression vector development
The full length F gene of APV, subtype A was amplified in six individual sections (see Figure 1) using PCR.

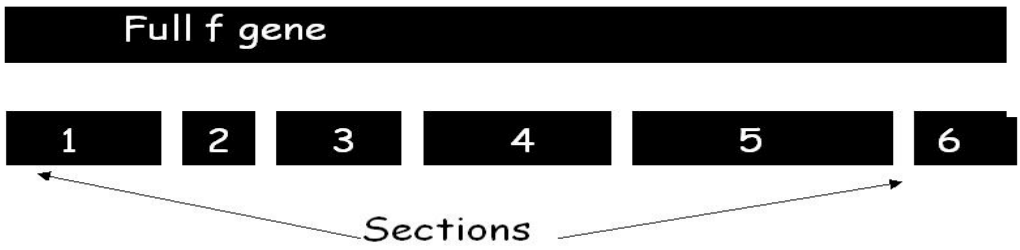


Fig 1. full F gene amplified in six sections

These sections were ligated into the EcoRV restriction enzyme site of plasmid vector pET 30 (Novagen) (See figure 2) and then used to transform *E.coli.*
Positive clones were identified using specific PCR primers.

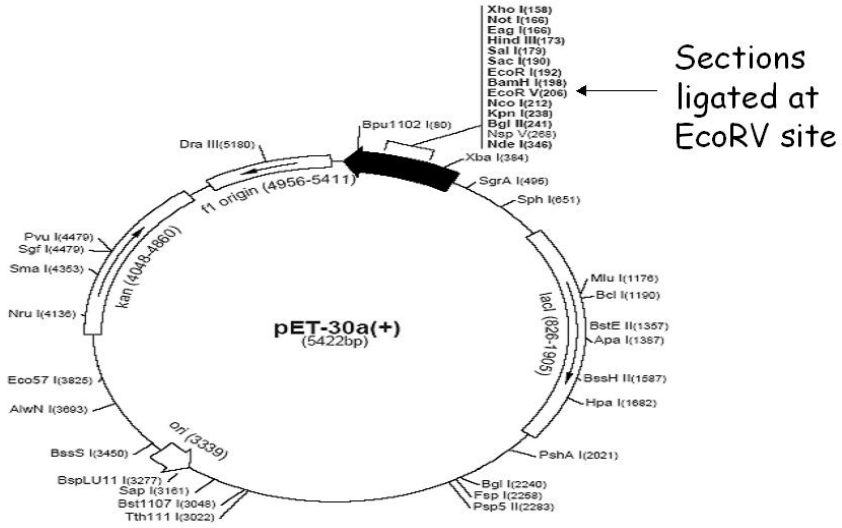


Fig 2. Ligation of F sections with *E.coli* expression vector

Expression and purification

E.coli cells (DE3 PlysS invitrogen) specialized for expressing recombinant proteins were transformed with the F section vectors.

Expression cultures were generated and expression induced for three hours.

Cell pellets from each culture were collected then lysed using detergents and sonication.

Recombinant proteins were captured using metal affinity chromatography that was specific for an engineered region of six Histidine (His) amino acids placed at the amino terminal of each F section (see Figure 3).

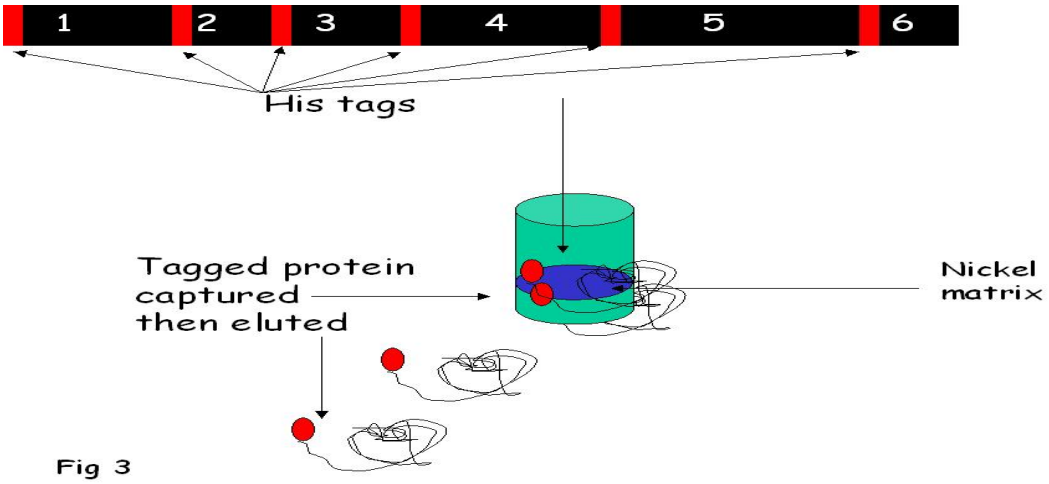


Fig 3

Metal affinity capture of His tagged recombinant protein

ELISA and Western blot identification
Initially Recombinant F section proteins were identified using a monoclonal antibody (ab) against the His tags (see Figure 4)
Protein sizes were examined using the for mentioned antibody in a Western blot

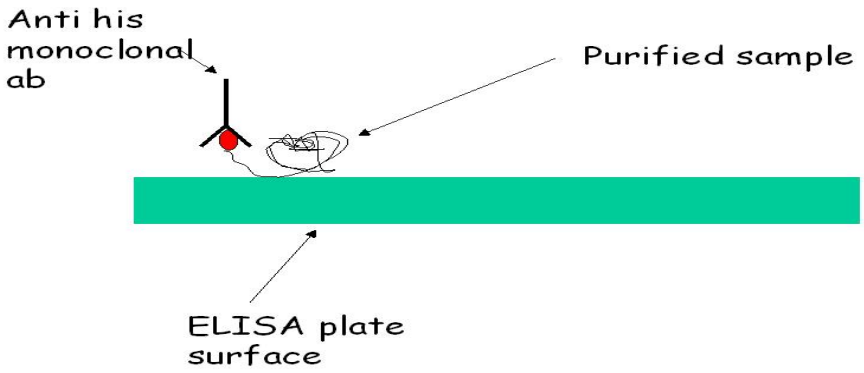


Fig 4

Detection F recombinants using an anti His monoclonal antibody

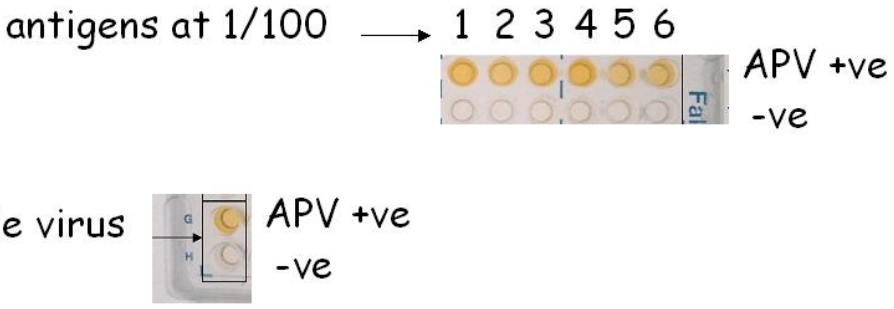
ELISA using APV positive and SPF serum
Plates were coated with recombinant F sections and a Liverpool subtype A whole virus antigen at 1/100 dilution. These were tested against APV positive serum and SPF serum from 12 week old chickens.
The above was repeated including SPF serum from 80 week old chickens and then again using *E.coli* adsorbed serum (see below).

Serum adsorption with E.coli
1ml of APV positive serum, SPF serums from 12 week and 80 week old chickens were each mixed with 1ml of *E.coli* culture. This culture had been washed 5x in pbs using centrifugation and re-suspension techniques. These mixtures were incubated for one hour at 37^0C. Adsorbed sera were collected after *E.coli* cells had been removed using centrifugation.
The removal of antibodies that react with *E.coli* proteins were checked using agar gel precipitation techniques.

Immunoperoxidase staining
Vero cells infected with APV in 48 well cell culture plates were used to check for the presence of APV antibodies in SPF serum. Cells were fixed in 70% ethanol for 10 mins before carrying out immunoperoxidase staining. The substrate used gave a brown colour in virus-infected cells.

RESULTS

Each F section was identified using the anti His monoclonal ab as described in Fig 4 and by Western blot conferred the correct sizes. Each of these recombinant F sections out performed the Liverpool whole virus antigen by giving greater absorbencies with APV positive serum and lower absorbencies with SPF serum from 12 week old chickens in Initial APV ELISAs (see Figure 5)



Absorbencies 492nm

F section	1	2	3	4	5	6	virus Ag
APV pos serum	1.6	1.7	1	1	1	1.3	0.6
spf serum	0.05	0.08	0.03	0.03	0.03	0.03	0.04

Fig 5
F sections tested against APV positive serum and SPF serum from 12 week old chickens

However when these antigens were tested against SPF serum from 80 week old chickens the absorbencies were even greater than those of the positive (see Figure 6).

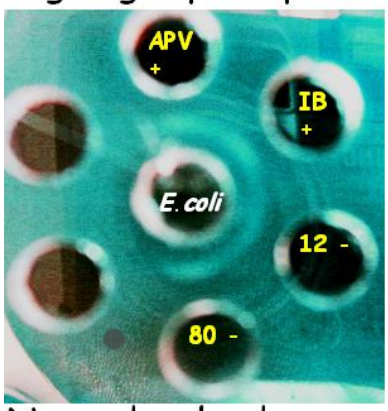
Absorbencies 492nm

F sections	1	2	3	4	5	6	virus Ag
APV pos serum	1.6	1.7	1	1	1	1.3	0.6
spf serum (12 wk)	0.05	0.08	0.03	0.03	0.03	0.03	0.04
spf serum (80wk)	1.9	1.8	0.8	0.7	1	1.5	0.2

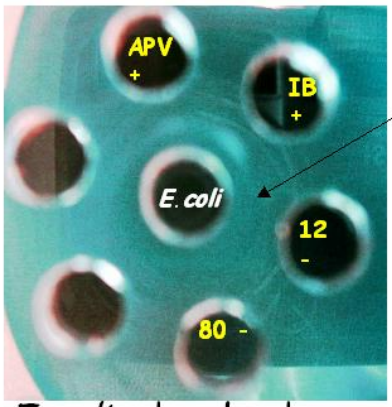
Fig 6

A repeat of the above ELISA but using serum that had been adsorbed with *E.coli* and checked using AGP (see Fig 7) gave the same high absorbencies with SPF serum from 80 week old chickens (see Fig 8).

Agar gel precipitation



Non adsorbed serum



Precipitation lines removed

E.coli adsorbed serum

Fig 7

Absorbencies 492nm (spf serum 80 week old chickens)

F section	1	2	3	4	5	6	virus Ag
non adsorbed	1.4	1.8	0.7	0.7	1.2	1.3	0.2
adsorbed	1.3	1.7	0.9	0.5	1	1.2	0.3

Fig 8

Figure 9 below, shows immunoperoxidase staining of APV infected Vero cells. A positive result was only observed when using APV positive serum (dark brown syncytia picture A) but not when using SPF serum from 12 or 80 week old chickens (picture B & C).

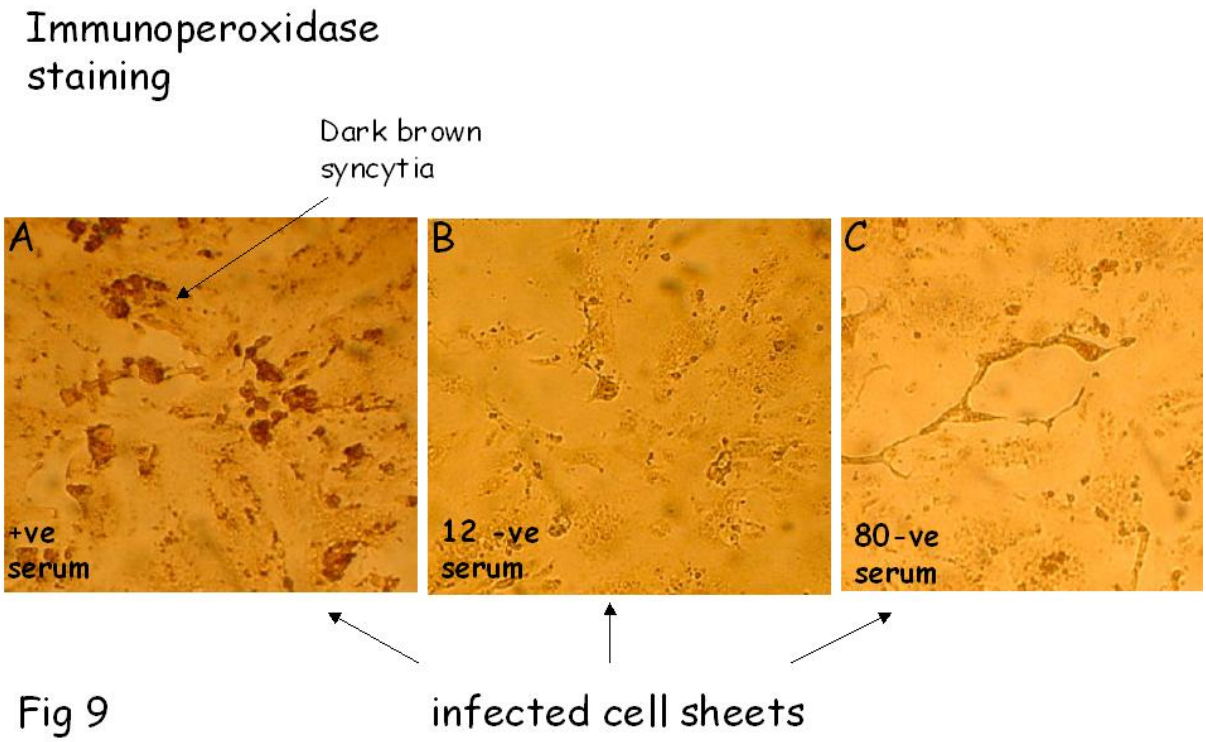


Fig 9

DISCUSSION

By generating vectors that contained regions of the fusion protein but not the full-length, expression and purification was achieved in an *E.coli* system. These recombinant proteins were quickly detected using a specific monoclonal antibody to the His tag. Recombinant proteins tested in an APV ELISA using APV positive serum and SPF serum from 12 week old chickens, out performed a Liverpool developed whole virus ELISA. However, when this ELISA was repeated using SPF serum from 80 week old chickens, high background was observed with the F recombinants.
High background absorbences are often seen when using *E.coli* expressed proteins as ELISA antigens [2-6]. It has been suggested that recombinant proteins expressed in *E.coli* often give high background due to the presence of homologous *E.coli* proteins, that have been co-purified with the recombinant heterologous proteins [7]. However in tHis study, the removal of antibodies (from test serum) that could have reacted with *E.coli* proteins did not improve background absorbance with SPF serum from 80 week old chickens. Therefore the background absorbencies shown in tHis study were likely due to other effects. Immunoperoxidase staining of APV infected

vero cells showed that no APV antibodies were present in SPF serum. Further work is required to determine the cause of these high backgrounds.
Future work will examine a variety of SPF serum from chickens of different ages in an attempt to identify a particular age at which these problems start to occur. Also reducing the high lipid content of serum from older chickens by absorbing with non-polar solvents may show if lipid content is an interfering factor.

REFERENCES

1. Eterradossi, N., Toquin, D., Guittet, M., Bennejean, G., Evaluation of different turkey rhinotracheitis viruses used as antigens for serological testing following live vaccination and challenge. Veterinary Medicine, 1995. 42(3): p. 175-86.
2. Crabb, B.S., MacPherson, C. M., Reubel, G. H., Browning, G. F., Studdert, M. J., Drummer, H. E., A type-specific serological test to distinguish antibodies to equine herpesviruses 4 and 1. Arch Virol, 1995. 140(2): p. 245-58.
3. De Diego, M., Brocchi, E., Mackay, D., De Simone, F., The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. Arch Virol, 1997. 142(10): p. 2021-33.
4. Ro, L.H., Lai, S. S., Hwang, W. L., Chou, H. H., Huang, J. N., Chang, E. L., Yang, H. L., Cloning and expression of an antigenic domain of glycoprotein gE of pseudorabies virus in Escherichia coli and its use as antigen in diagnostic assays. Am J Vet Res, 1995. 56(5): p. 555-61.
5. Warnes, A., A.R. Fooks, and J.R. Stephenson, Production of measles nucleoprotein in different expression systems and its use as a diagnostic reagent. J Virol Methods, 1994. 49(3): p. 257-68.
6. Wu, C.L., Leu, T. S., Chang, T. T., Shiau, A. L.,, Hepatitis C virus core protein fused to hepatitis B virus core antigen for serological diagnosis of both hepatitis C and hepatitis B infections by ELISA. J Med Virol, 1999. 57(2): p. 104-10.
7. Chang, P.C., Chen, K. T., Shien, J. H., Shieh, H. K., Expression of infectious laryngotracheitis virus glycoproteins in Escherichia coli and their application in enzyme-linked immunosorbent assay. Avian Dis, 2002. 46(3): p. 570-80.

MUCOSAL IMMUNITY IN AVIAN METAPNEUMOVIRUS

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SUMMARY

We examined the response of the upper respiratory tract (URT) of turkeys to two successive exposures to avian metapneumovirus (aMPV). The virus induced extensive cellular infiltrations in the mucosal surfaces of the respiratory sinuses following the first but not the second exposure. The infiltrating cells included IgG+ IgM+ and IgA+ cells. The number of IgA cells as well as secreted IgA appeared earlier following the second exposure to the virus than the first exposure.

INTRODUCTION

aMPV causes an acute, highly contagious URT infection in turkeys (1, 2). Clinical signs include depression, coughing, sinusitis, airsacculitis, and mortality (2). aMPV outbreaks in the United States were first reported in Colorado in 1996 (6). Since 1997, the virus has become endemic in Minnesota. The Minnesota virus belongs to subtype C, which has low sequence identity with subtypes A and B prevalent in Europe and other countries (5, 7). Lesions caused by aMPV are restricted to the URT (1). In the infected turkeys, viral antigen can be detected in the ciliated epithelial cells of nasal turbinates and infraobital sinuses (8). Our objective was to identify the phenotypes of B cells that infiltrate the mucosa following aMPV exposure. In addition we identified the presence of virus-specific IgA in the nasal secretion and the bile. .

MATERIALS and METHODS

The 41st passage of aMPV in Vero cells was used. Commercial turkeys were given two equal doses of the virus (10^4 $TCID_{50}$/bird) by the oculonasal route. The first dose was given at two weeks of age and the second dose 14 days after the first dose. At various days post inoculation (dpi), nasal turbinates were examined for histopathological lesions by Hematoxylin and Eosin (H&E) staining. IgG+, IgM+ and IgA+ cells were quantitated by immunohistochemistry (4). Nasal secretions and bile were examined by ELISA for anti-aMPV IgA..

RESULTS

Turkeys exposed to the first dose of aMPV developed extensive lymphoid cell infiltration in the mucosal surfaces of the turbinates. The intensity of the lymphoid cell infiltration was quantitated. Turbinate tissue section from each virus-exposed and control turkey was given a mean lesion score (MLS) of 1 to 5, based in increasing severity of infiltration. Following the first virus exposure, the MLS was 25 in the virus-exposed group compared to 7 in the virus-free control group ($P<0.05$). Following the second virus exposure, there was no statistical difference in the mean lesion scores of virus-exposed and control groups of turkeys ($P>0.05$). This result indicated that the first virus exposure protected the mucosa against reinfection.
At 7 dpi, the numbers of infiltrating IgG and IgM cells were similar in virus-exposed and control turkeys. However, the number of IgA+ cells was significantly higher in the virus-exposed group than the control group ($P<0.05$). After the second virus exposure, the number of IgM+ cells did not change; IgG+ cells were elevated at 3dpi but not at 7dpi. The numbers of IgA+ cells were higher in the virus-exposed group than in the control group at 3 and 7 dpi ($P<0.05$).
At 7 dpi following the first dose of the virus, nasal washes and the bile did not have detectable anti-aMPV IgA antibodies. Following the second virus dose, IgA antibodies were detected in the nasal washes at 3 and 7 dpi and in the bile at 7 dpi.

DISCUSSION

This study has shown that aMPV induces a strong lymphoid cell infiltration in the respiratory mucosa. Likely, these cells engage in a local immune response. Local mucosal immune responses often play a critical role in protection against most respiratory pathogens (3, 9). This immunity is mediated both by antibodies and immune cells. Among antibodies, IgA is considered most important for mucosal protection. IgA, secreted predominantly by B cells present in the mucosal surfaces, neutralizes viruses and prevents infection of susceptible host cells. Vaccines that stimulate strong IgA responses are considered most desirable for control of respiratory viruses. Our data indicate that aMPMV induces an IgA response in turkeys. The role of this antibody and other local immune responses in the immunopathogenesis of the virus needs further study.

REFERENCES

1. Chary, P., S. Rautenschlein, et al. (2002). Avian Dis 46(1): 153-61.
2. Goyal, S. M., S. J. Chiang, et al. (2000). J Vet Diagn Invest 12(2): 166-8.
3. Liew, F. Y., Russell, S. M. et al. (1984) Eur J Immunol, 14:350-6.1984.
4. Ohshima, K. and K. Hiramatsu (2002). Tissue Cell 34(2): 129-33.
5. Seal, B. S. (2000). Anim Health Res Rev 1(1): 67-72.
6. Senne, D. A., Edson, R. et al (1997). Proceedings of AVMA 134th Annual Congress, Reno, NV, USA.
7. Shin, H. J., K. T. Cameron, et al. (2002). J Clin Microbiol 40(5): 1687-93.
8. Jirjis, F. E., S. L. Noll, et al. (2001) J Vet Diagn Invest 13:13-6.
9. Weltzin, R., V. Traina-Dorge, K. et al. (1996) J Infect Dis 174:256-61.

PATHOGENESIS OF AVIAN METAPNEUMOVIRUS (AMPV) IN BROILER-TYPE CHICKEN: EXPERIMENTAL STUDIES AND FIELD OBSERVATIONS

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SUMMARY

Field observations indicate that avian Metapneumovirus (aMPV) may cause serious health problems in broilers and broiler parents. Although aMPV infections have been described in a variety of field cases in broilers not much is known about the pathogenesis of aMPV in these birds. We investigated the pathogenesis of turkey derived aMPV subtype A and subtype B in commercial broilers. The tissue distribution of the viruses and their effects on humoral and cell-mediated immunity were studied. Under experimental conditions, both aMPV subtypes induced only mild respiratory signs, which peaked at 6 days post inoculation (PI). aMPV genome was detected in the upper respiratory tract up to 6 days PI. Only subtype B was detected in the trachea at 11 days PI. This period of aMPV-detection in broilers by RT-PCR was shorter than in turkeys infected with the same virus strains. Correlations of field experiences and experimental pathogenesis data indicate that there is only a short time window to detect aMPV in the upper respiratory tract, which may be missed due the mild respiratory signs by single infections. The severe losses related to an aMPV infection in the field may not solely be due to aMPV, but to a combination of aMPV and other factors, exasperating the disease.

INTRODUCTION

Avian Metapneumovirus (aMPV) causes Turkey Rhinotracheitis (TRT) in turkeys. The virus is also associated with swollen head syndrome (SHS) in broilers and broiler breeders. Initially, the aetiological agent for SHS in broiler was hypothesized to be a combined infection of coronavirus and *Escherichia coli* (Morley and Thomson, 1984). Later TRT-like virus had been isolated from hens and guinea fowl in France showing SHS similar to that described in broilers (Buys et al., 1989; Morley and Thomson, 1984; Picault et al., 1987).
SHS due to aMPV has been reported in broiler and broiler breeder in many countries world wide (O' Brien, 1985; Wyeth et al., 1987; Perelman, 1988; Zellen, 1988;

Pattison et al., 1989; Hafez and Löhren, 1990; Jones et al., 1991; Gough et al., 1994; Lu et al., 1994; Maharaj et al., 1994; Tanaka et al., 1995; Mase et al., 2003; Al-Ankari, 2004).
It has been suggested that turkey isolates do not induce respiratory signs in broilers (Buys et al., 1989). On the other hand, Shin et al. (2000) demonstrated the reproducibility of respiratory signs in chickens following infection with subtype C aMPV originated from turkeys. aMPV field isolates from chickens show some antigenic variation to aMPVs of turkey origin (Prével, 1995). But sequence analysis of aMPV-subtype B isolates from turkey and chicken origin indicate that they have the same origin (Cook and Cavanagh, 2002). Although SHS induced by aMPV in broilers has been reported from many countries, the pathogenesis of aMPV infections and its role as a primary pathogen in broilers has not been fully understood. Our objective was to investigate pathogenesis of aMPV subtype A and B turkey isolates in commercial broiler-type chickens, and relate the outcome of the experimental results to field observations.

MATERIALS AND METHODS

Viruses

A virulent aMPV subtype A strain (BUT 8544) and a virulent Italian subtype B strain (kindly provided by Dr. R. C. Jones, Liverpool, UK), both isolated from turkeys, were propagated and titrated in chicken tracheal organ culture (TOC; Cook et al., 1976). Each bird received 10^4 ciliostatic doses $(CD)_{50}$ of aMPV oculonasally.
For virus neutralization (VN) test aMPV subtype A strain BUT 8544 (Wilding et al, 1986), which was adapted to chicken embryo fibroblasts (CEF), was used (Hafez, 1992).

Detection of aMPV in swabs and tissue samples

Detection of aMPV was done by subtyping nested RT-PCR as described before (Cavanagh et al., 1999).

ELISA and virus neutralization test

Sera were tested for aMPV-specific antibodies using a commercial aMPV-ELISA system (ART Ab Test Kit©, BioCheck B.V.). Furthermore, sera and tracheal washes were tested for aMPV-specific neutralizing antibodies following previously published procedures (Baxter-Jones et al., 1989; Obi et al., 1997).

Isolation and in vitro stimulation of splenic leukocytes

Leukocytes were isolated by density centrifugation (Archambault et al., 1976) from single cell suspensions of spleens. For in vitro culture, triplicates of 10^6 spleen cells/well were incubated in 96-well tissue culture plates with 5 μg Concanavalin A (ConA)/ml medium (RPMI 1640 supplemented with 10 % fetal bovine serum, antibiotics and L-glutamine) or medium without ConA. After 48 h of incubation at 41°C and at 5 % CO_2, supernatants were harvested and tested for IFN-γ using a commercially available ELISA-system (Cytosets©, BioSource). Samples were diluted 8- to 32-fold to obtain OD-values in the linear range of IFN-γ standard dilution series' regression curve. The IFN-γ-concentrations are expressed in pg/ml.

Assessment of Field Samples
Serum samples, tracheal samples and choanal swab samples from suspected cases in commercial broilers were collected and investigated for aMPV and aMPV antibodies by RT-PCR and ELISA test, respectively.

Experimental Trial
One hundred and twenty one-day-old commercial broilers (Ross Type) were kept in the isolation house, Clinic for Poultry, University of Veterinary Medicine Hannover. At 16-days of age, when birds were negative for maternally derived aMPV-antibodies detectable in the ELISA, the chickens were equally divided into three groups (group aMPV-A, aMPV-B, and virus-free controls) and housed in separate isolation units. Birds received feed and drinking water *ad libitum*. Group aMPV-A was inoculated oculonasally with 10^4 CD_{50} of aMPV subtype A and group aMPV-B with 10^4 CD_{50} of aMPV subtype B. Virus-free controls received virus-free tracheal organ culture (TOC) supernatant.
Clinical signs in the broilers were observed daily up to 24 days post infection (PI). The clinical score was determined according to the scoring system previously described by Jones et al. (1992) and Naylor and Jones (1994): Score 0: no clinical signs, score 1: clear nasal exudates, score 2: turbid nasal exudates, and score 3: swollen infraorbital sinus and/or frothy eyes.
Five chicks from each group were exsanguinated at 3, 6, 11, 14, 17, 20, and 24 days PI. Spleen, bursa cloacalis, lung, trachea, Harderian gland, and nasal turbinate were collected, pools from isolated total RNA were made per organ and group, and aMPV was detected by subtyping nested RT-PCR.
Tracheas were removed carefully, and tracheal washings were conducted thoroughly. Eight serum samples per group and 5 tracheal washes were investigated for VN and ELISA antibodies against aMPV.
Spleens were harvested for ex vivo culture.

Statistical analysis
Group responses of infected and virus-free birds were analyzed by student's T-test or analysis of variance (ANOVA). *P*-values < 0.05 were considered as significant.

RESULTS

Development of clinical signs
Clinical signs were monitored after infection of broilers with aMPV-A and aMPV-B. Both infected groups developed mild clinical signs such as depression, nasal exudates, swollen infraorbital sinus and frothy eyes beginning at 4 days PI. The peak was seen at 6 days PI, when 15 to 27 % of infected birds showed clinical signs (Figure 1). Overall, aMPV-A infected birds showed less lesions than aMPV-B infected broilers with a maximum clinical lesion score of 0.36 and 0.56 at 6 days PI, respectively (data not shown).

aMPV-detection by RT-PCR
aMPV was detected using a subtyping nested RT-PCR (Table 1). Both aMPV subtypes were detected in nasal turbinates and Harderian glands on day 3 and 6 PI. Both aMPV-subtypes were detected in the trachea on day 6 PI. Only aMPV-B was

detected in tracheal samples at 3 days PI. Furthermore, aMPV-B was also detected at 6 days PI in lung, spleen and bursa cloacalis. At 11 days PI, most tissues were negative for aMPV, but the trachea, where aMPV-B was still detected.

Induction of aMPV-antibodies
Both aMPV-infected groups developed significant serum VN (data not shown) and ELISA (Figure 2) antibody levels at 6 and 11 days PI, respectively. The titres increased up to 24 days PI (Figure 2). In tracheal washes VN-antibody titres increased significantly at 6 days PI, the titres peaked at 14 days PI, and then the VN-titres decreased up to 24 days PI (Figure 3).

ConA-stimulation of splenic T cells
Spleen cells of aMPV-infected and virus-free control birds were stimulated ex vivo with ConA. At 3 days PI, spleen cells from both infected groups released significantly higher IFN-γ levels after stimulation with ConA than spleen cells from virus-free birds (Figure 4). At 6 days PI, only spleen cells from aMPV-B-infected birds released significantly higher IFN-γ-levels in comparison to controls. Spleen cells of all groups released comparable IFN-γ-levels of an average of 914 pg/ml without ConA-simulation.

Field observations
Serum samples from various flocks were collected at different time points after hatch. Maternal antibody levels varied between flocks with titres of 8568 ± 2840 to 2343 ± 1186 at 7 days PI. Between 11 and 19 days post hatch more than 80 % of the tested broiler flocks (n = 15-30 serum samples/flock) had antibody levels below the cut-off value of the ELISA system. Based on the evaluation of the maternal antibody decline in 5 broiler flocks, a half life time of 3-4 days can be estimated for aMPV maternally derived antibodies.
Different field samples were tested for aMPV by subtyping nested RT-PCR and for serum aMPV-antibodies in the ELISA. In case one, samples were derived from a 37 day-old broiler flock, showing increased mortality rates and respiratory signs such as swollen heads. No aMPV-antibodies and no aMPV were detected in serum samples, swabs and tracheal tissue, respectively. No other primary pathogens such bacteria or other viruses were detected. In case two, serum and tracheal tissue were obtained from a 20 day-old broiler flock, showing increased mortality rates and polyserositis, *E. coli* was isolated from inner organs. Although this flock was not vaccinated against aMPV, serum samples had aMPV-antibody titres of an average of 10,897. No aMPV was detected by RT-PCR. In case three, samples were obtained from a broiler flock at 1 day, 17 days and 38 days post hatch. The farm had a history of swollen head syndrome in previous broiler placements. During the whole observation period, no clinical signs were observed. At 1 days post hatch, maternal antibody levels of an average of 1416 were detected, which is close to the cut-off level in the ELISA. At 17 days post hatch, the maternally derived antibodies had waned, all birds had antibody levels below the cut-off level (n =20; average antibody level of 433). At 38 days post hatch, antibody levels increased again, from 20 tested serum samples, 1 had antibody levels above the cut-off, 7 in the suspected range, and 12 were below the cut-off (average titre of all samples: 1131). At 1 and 38 days post hatch, aMPV-subtype B was detected by RT-PCR in tracheal samples of this flock.

DISCUSSION

In this study we compared the pathogenesis of aMPV-A and aMPV-B-turkey isolates in broilers. Both aMPV-subtypes induced only mild clinical lesions under experimental conditions, milder than in turkeys as it has been shown with the same virus strains before (Liman et al., 2004). aMPV-B infected birds showed slightly severer lesions than aMPV-A-infected birds, but the differences were not significant ($P > 0.05$). Both aMPV subtypes were detected by RT-PCR in tissues of the upper respiratory tract such as nasal turbinates and Harderian gland at 3 and 6 days PI. Both subtypes were detected in the trachea at 6 days PI. Only subtype B was already detected in the trachea as early as 3 days PI, and up to 11 days PI. Furthermore, only subtype B as detected in lung, spleen and bursa cloacalis. The differences in the detection of the two subtypes may be due to differences in the sensitivity of the RT-PCR. But the clinical data also indicate that this subtype B aMPV may be more virulent and possibly more invasive than the aMPV-A isolate under the described experimental conditions in broilers. This speculation may be supported by the significantly longer enhanced IFN-γ release of ex vivo stimulated spleen cells of aMPV-B infected birds. While both infected groups showed higher IFN-γ-release after ConA stimulation at 3 days PI than virus-free birds, spleen cells of aMPV-B infected birds still released significantly enhanced IFN-γ levels at 6 and slightly enhanced levels at 11 days PI.

Both subtypes induced serum antibodies, which were detected in the VN-test already at 6 and in the ELISA at 11 days PI. While the serum antibody levels increased up to 24 days PI, the last day of the experiment, VN-antibodies in tracheal washes increased up to 14 days PI, and then decreased again. This observation is interesting, because it indicates that aMPV may only induce short lasting VN-antibodies locally in broilers. This may explain why birds with high circulating antibody levels may still be susceptible for aMPV-infection, possibly due to insufficient local immunity (Le Gros et al., 1988; Williams et al., 1991a, b).

Field cases presented to our diagnostic facility were evaluated for the presence of aMPV infection in broilers. Despite the clinical observation of swollen head syndrome, often no aMPV was isolated, and aMPV antibodies were not always detected as shown in case 1. Considering the experimental aMPV infection and field observations our studies confirm previous results demonstrating that the window for aMPV-detection by RT-PCR is relatively small (Cook et al., 1991, Catelli et al., 1998). At 11 days post infection, no aMPV-A was detectable anymore, although minor clinical signs were still seen, and aMPV-antibodies may still be low.

In the second field case, high aMPV-antibody levels were detected in 20-day old broilers with a history of higher mortality and *E. coli* isolation (Majo et al., 1997). No aMPV was detected by RT-PCR. In this case, the infection may have progressed, aMPV had been cleared to undetectable levels and antibody production had been stimulated. The possibility of residual maternal antibodies in this case can be ruled out, because no or only low maternal antibodies are expected at 20 days post hatch as our field observations indicate.

Looking at the pathogenesis of aMPV in broilers and the observations in the field, our studies show that it is difficult to detect aMPV-infection under field conditions. The window for aMPV-detection by RT-PCR is small which coincides with previous observation (Catelli et al., 1998; Cook et al., 2002), and it may vary with the aMPV-

strain and subtype as our study demonstrates. In the field the development of a detectable humoral immunity may take place after the actual time for slaughter of broilers.

REFERENCES

AL-ANKARI, A. S., AL-RAMADAN, M. A., and EL-DEMERDASH (2004): Risk factors associated with prevalence of swollen head syndrome (SHS) in broiler chickens in eastern province-Saudi Arabia. Int. J. Poult. Sci. 3 (10): 646-650.^

ARCHAMBAULT, L. P., DUNLOP, W. R., CUCCHIARA, F. P., COLLINS, W. M., and CORBETT, A. C. (1976): A simplified technique for obtaining purified peripheral lymphocytes from chickens. Poult Sci. 55(5):1972-3.

BAXTER-JONES, C., GRANT, M., JONES, R. C., AND WILDING, G. P. (1989): A comparison of three methods for detecting antibodies to turkey rhinotracheitis virus. Avian Pathol. 1891-98.

BUYS, S. B., J. H. DU PREEZ and H. J. ELS (1989): Swollen head syndrome in chickens: a preliminary report on the isolation of a possible aetiological agent. J. South Africa. Vet. Ass. 60(4): 221-222.

CAVANAGH, D., MAWDITT, K., SHAW, K., BRITTON, P., and NAYLOR, C. (1997): Towards the routine application of nucleic acid technology for avian disease diagnosis. Acta Vet Hung. 45:281-98.

CAVANAGH, D., MAWDITT, K., BRITTON, P., and NAYLOR, C. J. (1999): Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathol. 28:593-605.

CATELLI, E., COOK, J. K. A., CHESHER, J., ORBELL, S. J., WOODS, M. A., BAXENDALE, W., and HUGGINS, M. B. (1998): The use of virus isolation, histopathology and immunoperoxidase techniques to study the dissemination of a chicken isolate of avian pneumovirus in chickens. Avian Pathol. 27, 632-640.

CATELLI, E., DE MARCO, M. A., DELOGU, M., TERREGINO, C., and GUBERTI, V. (2001): Serological evidence of avian pneumovirus infection in reared and free-living pheasants. Vet. Rec. 149:56-58.

COOK, J. K., DARBYSHIRE, J. H., and PETERS, R. W. (1976): The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 50(1-2):109-18.

COOK, J. K. A., ELLIS, M. M., and HUGGINS, M. B. (1991): The pathogenesis of turkey rhinotracheitis virus in turkey poults inoculated with the virus alone or together with two strains of bacteria. Avian Pathol. 20:155-166.

COOK, J. K. A., and CAVANAGH, D. (2002). Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathol. 31:117-132.

GOUGH, R. E., MANVELL, R. J., DRURY, S. E. N., and PEARSON, D. B. (1994): Isolation of an avian pneumovirus from broiler chickens. Vet. Rec. 134: 353-354.

HAFEZ, H. M., and LÖHREN, U. (1990): Swollen head syndrome: clinical observations and serological examination in West Germany. Dtsch. Tierärzt. Wschr. 97: 322-324.

HAFEZ, H. M. (1992): Rhinotracheitis der Puten (TRT) und Swollen Head Syndrom der Hühner (SHS): Ein Beitrag zur Klinik, Ätiologie; Epidemiologie und Bekämpfung. Habilationsschrift, LMU-University, Munich, 1994.

JONES, R. C., C. J: NAYLOR, J. M. BRADBURY, C. E. SAVAGE, K. WORTHINGTON and R. A. WILLIAMS (1991): Isolation of a turkey rhinotracheitis-like virus from broiler breeder chickens in England. Vet. Rec. 129: 509-510.

JONES, R. C., NAYLOR, C. J., AL-AFALEQ, A., WORTHINGTON, K. J., and JONES, R. (1992): Effect of cyclophosphamide immunosuppression on the immunity of turkeys to viral rhinotracheitis. Res Vet Sci. 53(1):38-41.

LE GROS, F. X., MAGAND, D., GIRAUD, P., BOUQUET, J. F., GUITTET, M., and BENNEJEAN, G. (1988): Turkey rhinotracheitis. Field results of the vaccination of day old turkey with a live attenuated virus. Proceedings of the 37the Western Poultry Disease Conference, Davis, California, 1988, 63-66.

LIMAN, A. and RAUTENSCHLEIN, S. (2004): Evaluation of local immune reactions following inoculation of turkeys with avian pneumovirus. In: IV. International Symposium on avian corona- and pneumovirus infections. Rauischholzhausen, Deutschland, 20.-23.6.2004; pp. 322-328.

LU, Y. S., SHIEN, Y. S., TSAI, H. J., TSENG, C. S., LEE, S. H., and LIN, D. F. (1994): Swollen head syndrome in Taiwan-isolation of an avian pneumovirus and serological survey. Avian Pathol. 23: 169-174.

MAHARAJ, S. B., THOMSON, D. K., and DA GRACA, J. V. (1994): Isolation of an avian pneumovirus like agent from broiler breeder chickens in South Africa. Vet. Rec. 134: 525-526.

MAJO, N., GIBERT, X., VILAFRANCA, M., O'LOAN, C. J., ALLAN, G. M., COSTA, L., PAGES, A., and RAMIS, A. (1997): Turkey rhinotracheitis virus and Escherichia coli experimental infection in chickens: histopathological, immunocytochemical and microbiological study. Vet Microbiol. 57(1):29-40.

MASE, M., YAMAGUCHI, S., TSUKAMOTO, K., IMADA, T., IMAI, K., and NAKAMURA, K. (2003): Presence of Avian Pneumovirus Subtypes A and B in Japan. Avian Dis. 47: 481-484.

MORLEY, A. J., and THOMSON, D. K. (1984): Swollen head syndrome in broiler chickens. Avian Dis. 28 (1): 238-243.

NAYLOR C. J., and JONES, R. C. (1994): Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. Vaccine. 12(13):1225-30.

OBI, T., KOKUMAI, N., IBUKI, A., TAKUMA, H., and TANAKA, M. (1997): Antigenic differentiation of turkey rhinotracheitis virus strains using monoclonal antibodies and polyclonal antisera. J. Vet. Med. Sci. 59(9):795-9.

O'BRIEN, J. D. P. (1985): Swollen head syndrome in broiler breeder. Vet. Rec. 117: 619-620.

PATTISON, M., CHETTLE, N., RANDALL, C. J., and WYETH, P. J. (1989): Observations on swollen head syndrome in broiler and broiler breeder chickens. Vet. Rec. 125(9):229-31.

PERELMAN, B., MEROZ, M., and SAMBERG, Y. (1988): Swollen head syndrome in broiler breeder in Israel. Vet. Rec. 123: 135.

PICAULT, J. P., GIRAUD, P., DROUIN, P., GUITTET, M., BENNEJEAN, G., LAMANDE, J., TOQUIN, D., and GUEGUEN, C. (1987): Isolation of a TRTV-like virus from chickens with swollen head syndrome. Vet. Rec. 121: 135.

PREVEL, P. (1995): Differentiation of avian pneumovirus-isolates by monoclonal antibodies to detect turkey rhinotracheitis-antibodies in ELISA. Doctoral thesis, Veterinary School Hannover, Germany, 1995.

SHIN, H. J., MCCOMB, B., BACK, A., SHAW, D. P., HALVORSON, D. A., and NAGARAJA, K. V. (2000): Susceptibility of broiler chicks to infection by avian pneumovirus of turkey origin. Avian Dis. 44(4):797-802.

TANAKA, M., TAKUMA, H., KOKUMAI, N., OISHI, E., OBI, T., HIRAMATSU, K., and SHIMIZU, Y. (1995): Turkey rhinotracheitis virus isolated from broiler chicken with swollen head syndrome in Japan. J. Vet. Med. Sci. 57 (5): 939-941.

WILDING, G. P., BAXTER-JONES, C., and GRANT, M. (1986): Ciliostatic agent found in rhinotracheitis. Vet. Rec. 118(26):735.

WILLIAMS, R. A., SAVAGE, C. E., and JONES, R. C. (1991a): Development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathol. 20:45-55.

WILLIAMS, R. A., SAVAGE, C. E., WORTHINGTON, K. J., and JONES, R. C. (1991b): Further studies on the development of live attenuated vaccine against turkey rhinotracheitis. Avian Pathol. 20:585-596.

WYETH, P. J., CHETTLE, N. J., GOUGH, R. E., and Collins, M. S. (1987): Antibodies to TRT in chickens with swollen head syndrome. Vet. Rec. 120(12):286-7.

ZELLEN, G. K. (1988): Case report: Swollen head syndrome in broiler chickens. Proceedings of the 37th Western Poultry Disease Conference, Davis, California 1988:139.

Table 1: Detection of aMPV in different tissue by nested RT-PCR

Tissue	3 days PI		6 days PI		11 days PI	
	aMPV-A	aMPV-B	aMPV-A	aMPV-B	aMPV-A	aMPV-B
Nasal turbinate	+	+	+	+	-	-
Harderian Gland	+	+	+	+	-	-
Trachea	-	+	+	+	-	+
Lung	-	-	-	+	-	-
Spleen	-	-	-	+	-	-
Bursa cloacalis	-	-	-	+	-	-

At 3, 6, and 11 days post aMPV-infection, different tissues were harvested, pooled per organ and group, and investigated for aMPV by nested RT-PCR.
+ = aMPV-positive RT-PCR result; - = aMPV-negative RT-PCR result.

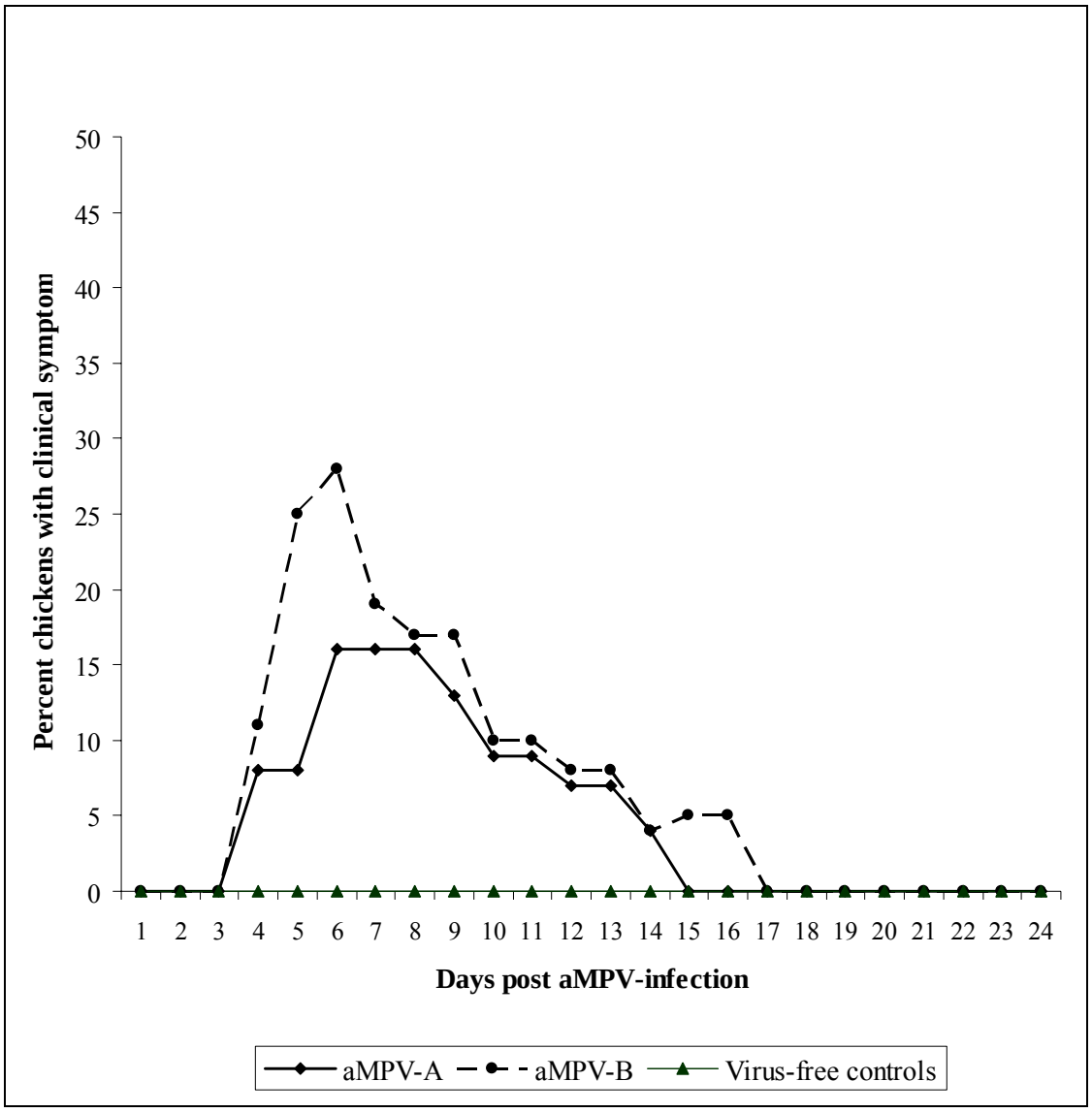

Figure 1. Induction of clinical signs following infection of commercial broilers with aMPV-A or aMPV-B.

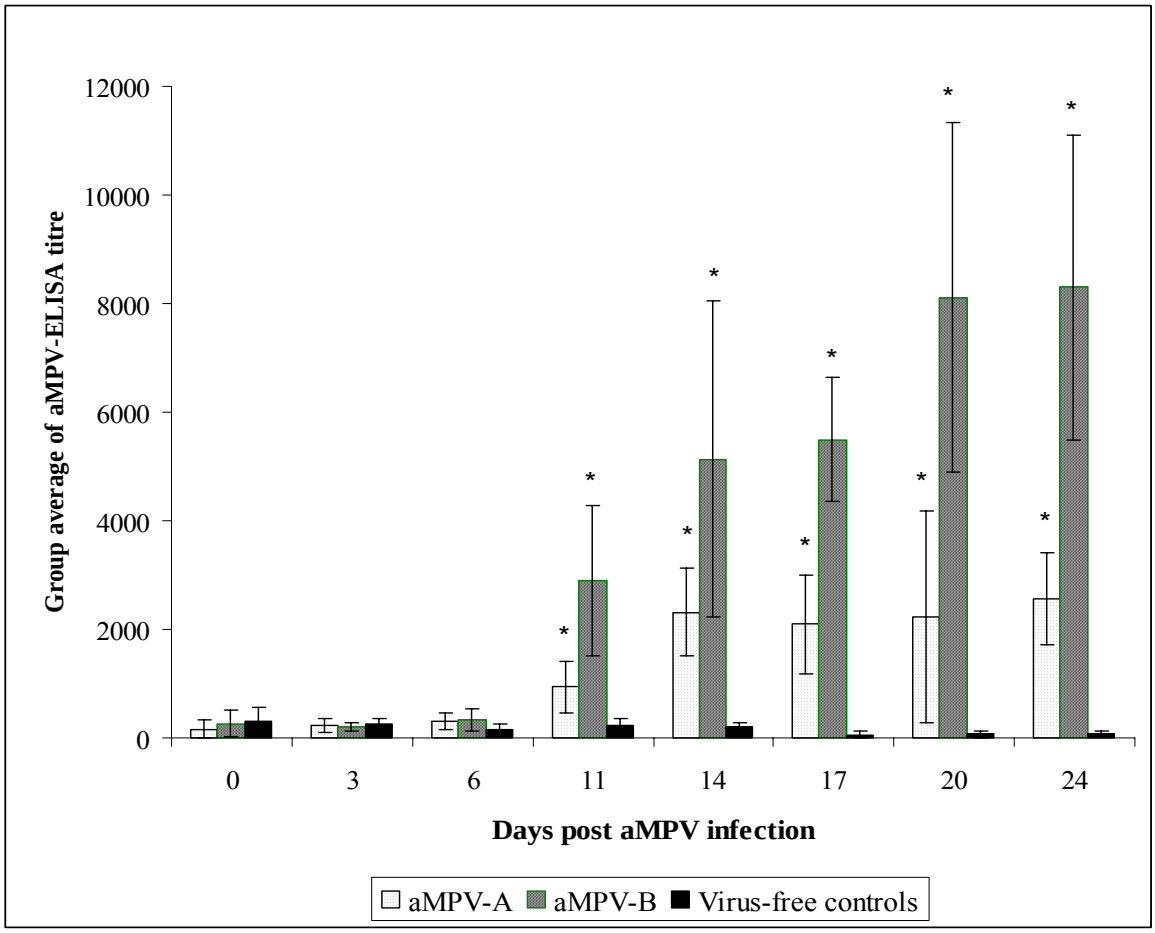

Figure 2: Development of aMPV-ELISA serum antibodies in broilers infected with aMPV-A or aMPV-B. *Significantly different to ELISA-titres of virus-free birds ($P < 0.05$; Student's t-test).

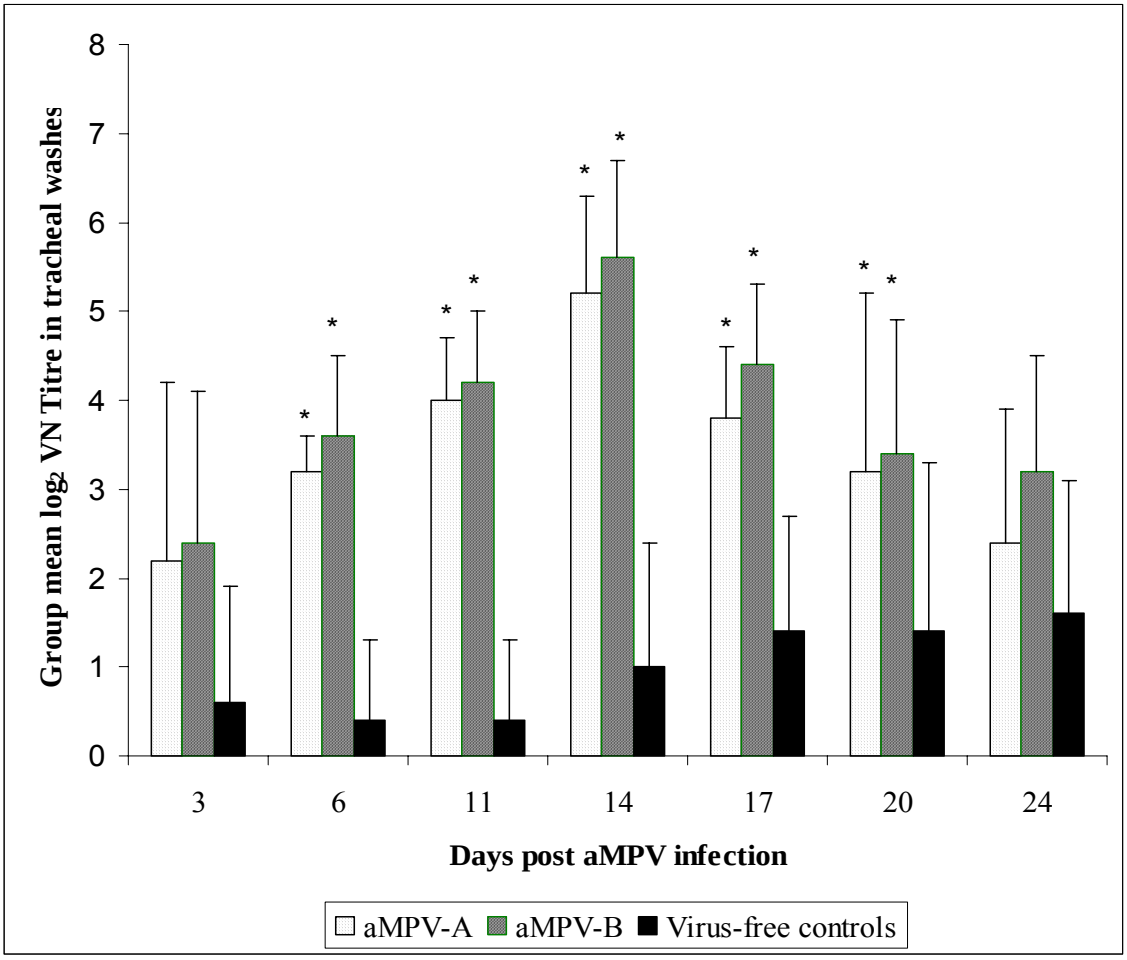

Figure 3: Induction of VN-antibodies in tracheal washes following infection of commercial broilers with aMPV-A or aMPV-B. *Significantly different to VN-titres of virus-free birds ($P < 0.05$; Student's t-test).

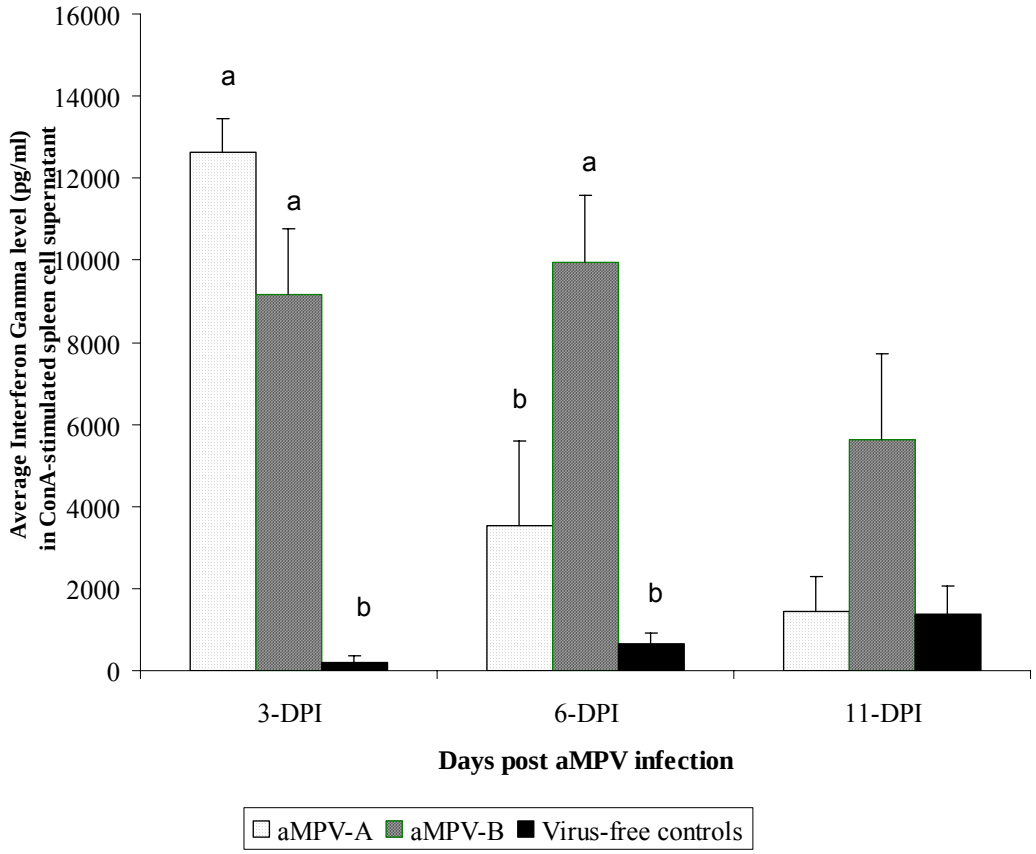

Figure 4. ConA-stimulation of spleen cells of aMPV-A and aMPV-B-infected broilers and measurement of IFN-γ release by ELISA. Different superscript letters indicate significant differences between infected and virus-free birds ($P < 0.05$; ANOVA).

COMPARED SUSCEPTIBILITY OF SPF DUCKLINGS AND SPF TURKEYS TO THE INFECTION BY AVIAN METAPNEUMOVIRUSES BELONGING TO THE FOUR SUBGROUPS

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SUMMARY

Avian metapneumoviruses (AMPV) are known worldwide as responsible of respiratory symptoms and/or egg drop in different avian species such as turkeys, chickens, guinea fowl, Muscovy or Pekin ducks or wild birds as Canada geese. In this study, SPF Muscovy ducklings and SPF turkeys were inoculated with AMPV belonging to the different subgroups (A, B, C and D) and originating from turkey or duck. On the one hand, in ducks inoculated with subgroups A, B and D viruses originating from turkey, no or very little symptoms, no virus reisolation and no seroconversion were observed, which is evocative of a resistant status of muscovy ducklings to those AMPV subgroups. On the other hand, when muscovy ducklings were inoculated with two AMPV subgroup C isolates, very variable symptoms were observed depending on the AMPV isolate, viral isolation was possible, quantitative real time RT-PCR (RRT-PCR) was positive and seroconversion was observed with the subgroup C-derived antigen, which results confirm the duck sensitivity to AMPV-C. In SPF turkeys inoculated with AMPV subgroup A, B and C originating from turkey, symptoms and seroconversion were variable but positive with the homologous AMPV derived antigen. RRT-PCR from tracheal swabs was also positive from day 3 post inoculation (PI) to day 7 and negative at day 10 PI with AMPV-B and -C (AMPV-A is in progress). With AMPV-C originating from duck, very slight symptoms were registered at day 7 PI, low seroconversion and no detection with RRT-PCR from tracheal swabs were observed, which results are evocative of a lower susceptibility of turkey to AMPV-C originating from ducks. Further studies with other AMPV strains are in progress.

INTRODUCTION

Since the eighties, avian metapneumoviruses have been evidenced in several avian species and notably in turkeys, chickens, guinea fowls and ducks exhibiting respiratory symptoms and/or egg drops (Cook, 2000). Antigenic and molecular characterizations of these AMPV strains have allowed to define four subgroups named A, B, C and D (Cook & Cavanagh, 2002). Subgroups A and B were described in Europe , Africa, Chile, Brazil and Japan (Cook, 2000), subgroup D only in France (Bayon-Auboyer et al., 2000). In turkey flocks, those strains were isolated from birds exhibiting characteristic rhinotracheitis and sinusitis. Subgroup C was isolated and defined in the United States of America from turkey with rhinotracheitis (Senne et al., 1997) and wild Canada Goose without symptom (Bennett et al., 2002), or in France from Muscovy or Pekin duck breeders exhibiting egg drops after mild respiratory signs (Toquin et al., 1999). A phylogenetic study of subgroup C AMPV based on the sequences of the G gene, which is the most variable gene in AMPV, evidenced different lineages between the AMPV-C strains isolated from turkey, goose or duck (Toquin et al., 2006a). The aim of this study was to compare the susceptibility of Specific Pathogen Free (SPF) Muscovy ducklings and SPF turkeys to those different AMPV strains belonging to the four subgroups and originating either from duck or turkey.

MATERIALS and METHODS

Virus

All strains belonging to the four subgroups, except the Colorado strain (AMPV-C isolated from turkey, kindly provided by Dr Senne), were isolated in the author's laboratory and cloned 3 times in Vero cells before use. Virus titres were determined according to the Reed and Muench method (Reed & Muench, 1938) in Vero cells and were expressed as median tissue culture infective doses ($TCID_{50}$) per ml of virus suspension. Strains 85051 (subgroup A), 86004 (subgroup B) and 85035 (subgroup D) were isolated from turkeys (Bayon-Auboyer et al., 2000). Strains 99178 (duck1) and 99350 (duck2) (subgroup C) were isolated respectively from Muscovy and Pekin ducks (Toquin et al., 1999).

Animals

For each virus, twenty 18-day-old SPF Muscovy ducklings and eleven 28-day-old SPF turkeys (both from AFSSA Ploufragan) were housed separately in high security negative-pressure filtered-air containment units. The birds were inoculated intranasally with $10^{3.50}$ to $10^{3.70}$ $TCID_{50}$ / bird of the different AMPV strains.

Symptoms

All Muscovy ducklings were clinically observed daily between 3 and 14 day post inoculation (PI), and SPF turkeys between 3 and 10 days PI. All symptoms were registered and quantified according to a clinical score index established for turkey and duck experimentation including mild (score = 1) or serious (score = 2) nasal discharge, nasal crust on one (1) or the two nostrils (2) and mild (1) or serious (2) cough or rale. The cumulative maximum score value was 6.

Virus isolation
Tracheal swabs were taken on 5 birds at days 4 and 7 PI for ducklings or at days 3 and 7 PI for turkeys. Virus isolation was performed in Vero cells. Negative cultures were subcultivated so as to perform three serial passages.

Molecular detection
Twenty tracheal swabs were tested on days 3 – 5 – 7- 11 PI for ducks and 11 tracheal swabs at days 3 – 5 – 7 – 10 PI for turkeys. Molecular detection was performed with Quantitative Real-time one-step RT-PCR tests (RRT-PCR) (TaqVet™ APV kit, LSI laboratory, FRANCE) (Lemière et al., 2005) (Guionie et al. 2006) according to the manufacturer's recommendations.

Serology
All birds were blood sampled before experiment and at day 28 PI for ducks or at day 18 PI for turkeys. The individual sera were tested with an ELISA test using the antigens derived from the different AMPV strains (Eterradossi et al., 1992).

RESULTS

SPF Muscovy ducklings susceptibility to AMPVs
No respiratory symptoms were observed in Muscovy ducklings used as controls or inoculated with AMPV-A or -B. With AMPV–D, slight clear nasal discharge was noticed at day 4 and 5 PI in respectively 3 and 1 out 20 ducklings. No re isolation of AMPV-A, -B and –D viruses was possible at days 4 and 7 PI. No seroconversion was observed with the AMPV-A, -B and –D antigens used before and after inoculation, neither in the control group nor in the groups inoculated with AMPV-A, -B and –D. All Optical density (OD) average values were not significantly different from those obtained with the control sera (Table 1).
Regarding AMPV-C, no symptom was observed following the inoculation of the Colorado strain originating from turkey, whereas the 99178 strain originating from duck induced respiratory symptoms (tracheal rales) between 4 and 10 PI. Virus isolation was positive at day 4 PI respectively from 4 out of 5 ducks inoculated with the Colorado strain and from 5 out of 5 ducks inoculated with the 99178 strain. At day 7 PI, 2 out of 5 Colorado-inoculated ducks were still positive for virus isolation but none of the 99178-inoculated birds was found positive. In the latter group, all ducklings were found highly positive with the RRT-PCR at day 3 PI; the number of positives decreased at day 5 PI and all animals were negative at days 7 and 11 PI (RRT-PCR for the Colorado-inoculated ducks is in progress). The average OD values obtained with sera from AMPV-C inoculated ducklings were positive with the AMPV-C antigens and differed significantly from those in control birds (table 1).

SPF turkeys susceptibility to AMPVs
No respiratory symptom were registered in the control group. All turkeys inoculated with the AMPV-A, -B and $-C_{Colorado}$ strains developed respiratory symptoms (nasal discharge, nasal crusts) between 3 to 10 days PI, with maximum signs at days 5 to 7 PI (as also observed in AMPV-D, data not shown). With the 99350 AMPV-C strain originating from Pekin ducks, no symptom was registered from days 3 to 10 PI, except at day 7 PI with 1 bird out of 11 showing slight nasal discharge (table 2).

Tracheal swabs taken from birds inoculated with AMPV-B and $-C_{Colorado}$ tested positive at day 3 PI by RRT-PCR, positive with increased values at day 5 PI, weakly positive or negative at day 7 PI and negative at day 10 PI. All tracheal swabs from turkeys inoculated with the AMPV-C duck strain were found negative from day 3 PI to day 10 PI by RRT-PCR (table 3). All sera were found negative before inoculation as well as in the control group. After inoculation, all sera from the turkeys inoculated with AMPV-A, -B and -C $_{Colorado}$ were highly positive against their homologous subgroup antigens. Slight cross reactivity was observed between AMPV-A and –B, as documented previously. The average OD value obtained with sera from turkeys inoculated with AMPV-C duck (0.139) was below the threshold value (0.200), however the OD values of 4 sera out of 11 were higher than 0.200 (with 0.279 as the maximum obtained value) (table 4), which result suggests that some of the inoculated turkeys had indeed been infected without symptoms.

DISCUSSION

Avian metapneumoviruses are frequently isolated from various avian species in many countries, but very few data have been published on the cross susceptibility of these avian species to AMPV belonging to different subgroups. In our study, after inoculation of SPF ducklings with AMPV strains isolated from turkeys and belonging to the subgroups A, B and D, no seroconversion, no virus isolation and no seronconversion were observed which is evocative of a resistant status of ducks to these AMPVs. This suggests that the role of ducks in spreading AMPV-A and -B in turkey flocks should hence be considered as negligible and calls into question the use of commercial live AMPV-A or -B vaccines to protect ducks against AMPV-C infection, although cross protection induced with AMPV-A and -B attenuated vaccines against AMPV-C virulent challenge has been documented in turkeys (Cook et al., 1999). On the other hand, Muscovy ducklings inoculated with the Colorado isolate belonging to AMPV subgroup C but originating from turkey did not develop respiratory signs, but were serologically positive and viral isolation was possible at days 5 and 7 PI. Protection against a challenge AMPV-C isolate originatingfrom duck still has to be tested, however, as the Colorado strain is virulent for turkeys, the use of such a turkey virulent AMPV as a vaccine for ducks is not an issue to be considered.

Experimental inoculation of SPF turkeys with AMPV-A, -B and –C Colorado has confirmed the pathogenicity of those strains, the induced humoral responses and the virus replication in the trachea at day 3 to 7 PI. On the other hand, inoculation with an AMPV-C strain originating from duck demonstrated that this strain was quite apathogenic. No virus detection was possible in the trachea at day 3 to day 10 PI and only 4 out of 11 inoculated turkey were positive with low OD values in ELISA. Further studies are necessary to explain these results which could be related to a very low replicative efficiency of the duck AMPV-C isolate in turkeys, possibly combined with a lack of replication of this virus in the upper respiratory tract of turkeys - as suggested by the lack of detection by RRT-PCr from tracheal swabs. Additional studies with the subgroup D strain (ref 85035) and with the subgroup C duck1 stain (ref 99178) are in progress.

The results reported here following inoculation of a duck AMPV-C isolates to turkeys are reminiscent of results recently published with a subgroup C virus isolated in a

wild Canada goose (Bennett et al., 2005). This strain was described as apathogenic for day-old or 15-day-old turkeys, replicated well in the trachea (but less in 15-day-old than in day-old birds), it induced an humoral response and was protective against virulent AMPV-C challenge performed with a turkey AMPV-C isolate. The goose AMPV-C isolate was consequently proposed by the authors as a possible vaccine for turkeys. However, a lower susceptibility of older turkey (as used in our schedule : 28-day-old) to the goose strain was not investigated and no data about the potential pathogenicity of this wild goose strain for other avian species than turkeys (such as ducks) has been published.

The first phylogenetic study based on the G gene of Metapneumoviruses has shown that the US goose AMPV-C strain grouped together with the other North-American isolates rather than with the French duck isolates, a result that suggested that genetic lineages among AMPV-C may be more related to the geographical origin than to the host species (Toquin et al., 2006a). However further studies with more G gene sequences of other subgroup C isolates from both turkeys and ducks are required for a better understanding of the molecular epidemiology of subgroup-C AMPVs in Europe and the United States. Similarly, the isolation and sequencing of the G gene of possibly detected AMPV isolates that do not fit any of the previously recognised subgroups remains necessary to follow the potential evolution of AMPV strains and the possible emergence of new AMPV subgroups or sub lineages.

Further experimental studies are ongoing in the authors laboratory to establish whether, as suggested by the results presented here, AMPV subgroups A, B and D can be considered more specifically as turkeys (and chickens) pathogens, whereas the susceptibility of turkeys to AMPV subgroup C viruses seems to be linked to the animal origin these isolates. As experienced in the presented experiments, RRT-PCR will prove a useful and rapid new tool for the control of viral excretion in such studies.

ACKNOWLEDGEMENTS

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REFERENCES

Bayon-Auboyer, M.H., Arnauld, C., Toquin, D. and Eterradossi, N., 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J Gen Virol 81, 2723-33.

Bennett, R.S., Larue, R., Shaw, D., Yu, Q., Nagaraja, K.V., Halvorson, D.A. and Njenga, M.K., 2005. A wild goose metapneumovirus containing a large attachment glycoprotein is avirulent but immunoprotective in domestic turkeys. J Virol 79, 14834-42.

Bennett, R.S., McComb, B., Shin, H.J., Njenga, M.K., Nagaraja, K.V. and Halvorson, D.A., 2002. Detection of avian pneumovirus in wild Canada (Branta canadensis) and blue-winged teal (Anas discors) geese. Avian Dis 46, 1025-9.

Cook, J.K., 2000. Avian rhinotracheitis. Rev Sci Tech 19, 602-13.

Cook, J.K. and Cavanagh, D., 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathol 31, 117-32.

Cook, J.K.A., Huggins, M.B., Orbell, S.J. and Senne, D.A., 1999. Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. Avian Pathol 28, 607-617.

Eterradossi, N., Toquin, D., Guittet, M. and Bennejean, G., 1992. Discrepancies in turkey rhinotracheitis ELISA results using different antigens. Vet Rec 131, 563-4.

Guionie, O., Toquin, D., Sellal, E., Bouley, S., Zwingelstein, F., Allee, C., Bougeard, S., Lemiere S. and Eterradossi, N. 2006. Laboratory evaluation of a quantitative real-time reverse transcription PCR assay for the detection and identification of the four subgroups of avian metapneumovirus. 5th International Symposium on avian Corona- and Pneumoviruses and complicating Pathogens. Rauischholzhausen, Germany. 14-16 May 2006.

Lemière, S., Sellal, E., Bouley, S., Toquin, D., Guionie, O. and Eterradossi, N., 2005. Development of real time PCR for diagnosis of avian metapneumovirus (APV) infections. 14th WVPC, 612.

Reed, L.J. and Muench, H., 1938. A simple method of estimating 50 per cent end points. American Journal of Hygiene 27, 493-497.

Senne, D.A., Edson, R.K., Pedersen, J.C. and Panigrahy, B., 1997. Avian pneumovirus update. 134th Annual Congress American Veterinary Medical Association, Vol. July 1997, 190.

Toquin, D., Bayon-Auboyer, M.H., Jestin, V., Eterradossi, N. and Morin, H., 1999. Isolation of a pneumovirus from a Musckovy duck. Vet. Rec. 145, 680.

Toquin, D., Guionie, O., Jestin, V., Zwingelstein, F., Allee, C. and Eterradossi, N., 2006a. European and American Subgroup C Isolates of Avian Metapneumovirus belong to Different Genetic Lineages. Virus Genes 32, 97-103.

Table 1: AMPV ELISA results : Mean optical densities obtained from Muscovy ducklings inoculated with AMPV belonging to different subgroups

Duck Inoculated with	A 85051 (F/85)	B 86 004 (F/86)	C Colorado (USA/96)	C 99178 (F/99)	D Fr/85035 (F/85)
AMPV-A	0.077*	0.025	-0.008	-0.002	0.025
AMPV-B	0.046	0.063	0.006	0.002	0.032
AMPV-Col	0.041	0.032	0.418	0.362	0.035
AMPV-Cduck1	0.073	0.051	0.544	0.508	0.059
AMPV-D	0.028	0.035	0.003	0.000	0.024
Control	0.033	0.019	0.000	0.004	0.026

*boxed value = homologous value

TABLE 2 : Percentage of turkeys exhibiting respiratory symptoms after inoculation with AMPVs and cumulated morbidity during the experiment.

	D0	3 DPI	5 DPI	7 DPI	10 DPI	Cumulated Morbidity
Control	0%	0%	0%	0%	0%	0%
AMPV-A	0%	9%	18%	27%	0%	36%
AMPV-B	0%	27%	91%	82%	9%	91%
AMPV-Col	0%	36%	40%	50%	0%	73%
AMPV-CDuck2	0%	0%	0%	9%	0%	9%

TABLE 3: Percentage of positive tracheal swabs from inoculated turkeys, as detected with the Quantitative real time RT-PCR test.

	D0	3 DPI	5 DPI	7 DPI	10 DPI
Control	In Progress				
AMPV-A	In Progress				
AMPV-B	ND	100%	100%	91%	0%
AMPV-Col	ND	90%	100%	20%	0%
AMPV-CDuck2	0%	0%	0%	0%	0%

TABLE 4 : AMPV ELISA results : Mean optical densities obtained from turkeys inoculated with AMPV belonging to different subgroups

Turkeys Inoculated with	A 85051 (F/85)	B 86 004 (F/86)	C Colorado (USA/96)	C 99178 (F/99)
AMPV- A	1.379*	0.202	In progress	0.203
AMPV-B	0.493	0.774	In progress	0.230
AMPV-Col	0.044	0.033	In progress	0.846
AMPV-Cduck2	0.016	0.003	In progress	0.139

* boxed value = homologous value

ESTABLISHMENT OF CHRONICALLY INFECTED CELLS WITH AVIAN METAPNEUMOVIRUS AND THEIR POTENTIAL USE AS A SOURCE FOR A VACCINE

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SUMMARY

The three main subtypes of avian Metapneumovirus (aMPV) are A, B and C, based on their different antigenic and genetic properties. Despite their cytotoxic nature, we were able to establish for each subtype a cell line that chronically produces the virus without apparent cytopathic effect and that could easily be maintained for many passages. We named these cultures "chronically infected cells"; CIC-A, -B or -C, according to the virus subtype used for their generation. Interestingly, the three kinds of CIC produced infectious viruses that retained their cytopathic potential as determined by infection of naïve Vero cells. We tested one of the CIC generated viruses as a candidate for vaccination of turkeys against aMPV infection. Preliminary experiments have demonstrated the efficiency of such a vaccine in protecting against a heterologous virulent isolate.

INTRODUCTION

aMPV is a paramyxovirus that is classified in the *Metapneumovirus* genus, within the subfamily *Pneumovirinae* of the *Paramyxoviridae* family „Pringle et al. (1998)". This genus also includes the recently discovered human metapneumovirus (hMPV) that causes mild to severe respiratory tract disease in humans „van den Hoogen et al. (2001)". The aMPV genome consists of about 13 kb of a non-segmented, linear, negative-sense strand RNA, which encodes for eight genes: nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), surface glycoprotein (G), and RNA-dependent RNA polymerase (L). The absence of two non-structural (NS) proteins found in related pneumoviruses, and the unique gene order, 3' N-P-M-F-M2-SH-G-L 5' distinguish aMPV and hMPV as metapneumoviruses „Yu et al. (1992), Ling et al. (1992), Lamb et al. (2001)". Although, aMPV was isolated some twenty years ago, relatively little is known on the

biology of this virus. An important resurgence of interest occurred with the isolation of the hMPV in 2000 which further highlighted the importance of studying this genus of.

Two aMPV subgroups, A and B, were initially recognized based on neutralization tests using monoclonal antibodies against the surface G glycoprotein „Cook et al. (1993), Collins et al. (1993)" and sequence variability in the G gene „Juhasz et al. (1994)". Later on, two additional subgroups, C „Cook et al.,(1999), Seal et al. (1998)" and D „Bayon-Auboyer et al. (2000)" were discovered. An additional aMPV isolate has been recovered from Muscovy ducks but to date remains unclassified „Toquin et al. (1999)". The aMPV subgroups not only differ by sequence and immunological criteria, but also by their geographical distribution and prevalence. For example, whereas subgroup A and B are found in many parts of the world, including Europe and the Middle East, subgroup C virus has been identified to date only in the US, and aMPV-D only in France. It seems that differences exist also in the biological features of different aMPV subgroups. For example the aMPV-A and B cause ciliostasis in tracheal organ culture (TOC) while aMPV-C does not „Cook et al. (2002)". In addition, whereas aMPV-A and B infection in turkeys causes loss of the nasal and tracheal ciliated epithelium, this epithelium remains intact in aMPV-C infected turkeys „Jirjis et al. (2002)". Differences between subgroups also appear to exist in their host range. Whereas aMPV-A and B have been isolated from chickens with swollen head syndrome (SHS), aMPV-C has to date not been isolated from chickens „Cook et al. (2000)".
The occurrence of persistency in paramyxoviruses infection and the establishment of persistently infected cells with paramyxovirus and pneumoviruses has been described. Recently, it was observed that hMPV can persist in the lungs of mice for several weeks „Alvarez et al. (2004)". Here we report the establishment of long lasting persistent infections by three subtypes of aMPV in tissue culture, and the use of this system as a source for vaccinal particules.

MATERIALS and METHODS

Establishement of the chronically infected cells (CIC).

To investigate the possibility that the three main types of aMPV are able to establish persistent infection in cell culture, we repeatedly passaged Vero cells initially infected with aMPV-A, -B and –C. Specifically, the following isolates were used: #F83 (aMPV-A) and #247/11 (aMPV-C) which were kindly provided by the Veterinary Laboratory Agency, Weybridge, UK and #1708 (aMPV-B; an Israeli field isolate which was passaged twice in tracheal organ culture [TOC] prior to it's adaptation to Vero cells; genebank # AY728268). All infections in this study were done on cells in monolayer (split previous day), from which the media was removed and replaced with the inoculum for 30min at room temperature. These cells were then washed and fresh media was added. Cells were observed daily for appearance of cytopathic effect (CPE). Growth rates of the cells were determined by counting the number of cells at daily intervals.
Virus titration was performed by end-point dilution assay on naïve Vero cells. Briefly, 96 wells plates were seeded with naïve Vero cells and inoculated the next day.

RT-PCR
Viral RNA was extracted from the supernatants of infected cultures using QIAamp viral RNA kit (QIAGEN) according to the manufacters' protocol, and tested by RT-PCR using Reddy Mix Reverse-iT One Step kit (Abgene). Subtype-specific primers were used as follows: primers Ga, G2, G12; „Bayon-Auboyer et al. (1999)" for aMPV-A and -B and primers Cf (5'-TGCTGGAGAGAGGAGGAAAA-3') and Cr (5'-TGTTGCCTGTCTGTTCTTGG-3') for aMPV-C ..

Immunostaining
To determine the extent of the infection, the cells were immunostained with hyperimmune sera against aMPV #1708 The antisera used were obtained from chickens (n=6) infected at 2 and 4 weeks of age with aMPV-B isolate #1708, by eye drops of undiluted TOC-conditioned media. The antisera were collected two weeks after the second inoculation. The second bleed was used for the immunostaining. Briefly, the cells were plated on 60mm plates with a glass cover slip. The cover slip was removed after 48h and the cells fixed with cold acetone. Some of the hyper-immune sera (diluted 1/200) were reacted with the cells for one hour at room temperature and then incubated in the same conditions with FITC-labeled anti-chicken immunoglobulin. Counter-staining was performed with Evans blue. Slides were observed in a UV microscope.

Vaccination with CIC-A generated virions.
One day old commercial turkeys (Yafe Hod) were separated into four groups of 50 poults each, in regular poultry houses. Food and water were provided *ad libidum* and all poults were vaccinated against Newcastle Disease Virus on day 7. All poults were vaccinated by eye drops with live vaccines on day one. The groups were vaccinated as follows: the first group with a commercial vaccine of subtype A; the second group with a commercial vaccine containing a mixture of subtype A and B; the third group with the CIC-A conditioned media (passage 33); and the fourth group was unvaccinated control group. On day 14, all the groups except the control were boosted with a second dose of the initial aMPV vaccine. On day 25, each group was further divided into two separate houses; one group serving as a negative control and the second as a challenged group. The challenged groups were inoculated by eye drop with aMPV-B #1708 (TOC conditioned media, passage 10).. Blood samples and tracheal swabs were taken according to the timeline indicate in Figure 1. Half of the birds were sacrificed on day 31, trachea, lungs and turbinates were removed in a sterile manner for further analysis. The experiment was ended on day 43 when all birds were euthanized and sample collection was completed.
Sero-conversion and the presence of viral RNA were used to evaluate the vaccine efficacy. Antibody levels were tested with a commercial ELISA kit (Idexx). The presence of viral RNA was tested by RT-PCR on the tracheal swab samples and on the organ samples as previously described „Banet-Noach et al. (2005)".

RESULTS

Establishment of the chronically infected cells
Vero cells, infected with aMPV-A, B or C were incubated until CPE was observed. The media were then replenished to allow the recovery of survival cells (estimated to

be about 10% of the initial number of cells). After the 4th passage of the surviving cells, the culture media were tested for the presence of aMPV RNA by RT-PCR .This analysis indicated that the cultures were infected and continued to produce aMPV virions (data not shown). Immunohistochemistry of these cultures at their 8th passage with sera against aMPV-B revealed a uniform staining of all the cells in culture (Figure 2). In addition, titration of the virus in supernatants of these cultures showed titers that matched those produced by the acutely infected cells (Table 1). All of the chronically infected cells (CIC) could easily be passaged for an excess of 40 times, despite a reduced rate of cell division (Table 1). This reduction in growth rate might be indicative of competition by the viral replication complexes for factors required for cell division. Remarkably, the CIC exhibited minimal cytopathic effect (CPE) compared to naïve cells infected with the three subtypes (Table 1).

Vaccination experiment

The persistent replication of aMPV in cultures may generate attenuated viruses, which in turn may serve as potential vaccines. To test this we performed preliminary vaccine experiments with one of the CIC-generated subtypes (CIC-A) as described in the Material and Methods section. Following vaccination the poults were challenged with an infectious isolate of aMPV-B. This isolate is known to cause clinical symptoms, however, inconsistently, in line with other aMPV infections . In this experiment clinical signs were not observed, thus leading us to monitor viral infection by detection of the genomic viral RNA in swabs and tissue samples and by detection of antibody levels in the blood of infected birds. Low mortality rates were observed in all groups independent of the treatment. The one day-old poults already had high levels of maternal antibodies against aMPV reflecting the prevalence and high exposure of turkeys to this virus, in Israel. The titer reduced over time, reaching basal levels by day 17. The antibody titer increased again but only in poults challenged by an aMPV-B infectious isolate, suggesting adequate infection of these poults.

After the first vaccination, the vaccine strain was detected in tracheal swabs in a portion of the birds for up to 10 days (table 2) and was absent both after the second vaccination and at the time of challenge.

After inoculation with an infectious field isolate of aMPV-B, all challenged groups were positive for viral RNA (aMPV-B), as detected in tracheal swabs for up to 8 days (time of the last sampling) (Table 3). As expected, the group vaccinated and challenged with an homologous virus showed a smaller number of birds positive for aMPV-B, whereas the control unvaccinated group had the highest incidence of viral RNA in tracheal swabs. Birds vaccinated with aMPV-A, including with CIC-A media, demonstrated a lower incidence of the challenge virus, aMPV-B, compared to control groups.

Furthermore, we analyzed the presence of aMPV-B viral RNA in different organs, including trachea, turbinate and lungs, and found a limited distribution of the viral aMPV-B RNA in the airways of the CIC-A vaccinated group compared to the group vaccinated with the commercial A vaccine (Table 4). In CIC-A vaccinated group viral RNA was only detected in the turbinate and neither in the trachea nor the lungs.

DISCUSSION

The establishment of the CIC was accomplished for subtypes A, B and C of aMPV. The fact that large number of cells survived the initial infection indicates that no selection for rare cell mutants was involved. Moreover, it appeared that a selection for noncytopathic viruses did not take place as CIC supernatants contained viruses that caused CPE upon infection of naïve Vero cells. Overall, it is clear that establishment of the chronic infection is relatively rapid, does not include the selection of rare cell or virus mutants and applies for all of the three tested subtypes. This result is in line with the establishment of persistent infection in cell cultures observed for other paramyxoviruses for example: Respiratory Syncytial Virus (Guerrero-Plata et al (2004), Martinez et al. (2001), Valdovinos et al. (2003)), Measles (Sheshberadaran et al. (1985), Carter et al. (1983), Viola et al. (1978), Young et al. (1985), Goldman et al. (1995)), PI-3 (Murphy et al. (1990), Moscona (1991), Moscona & Galinski,(1990)). To date however, the mechanism of this persistency is unknown and remains to be illucidated.
The viral particles generated by CIC may be somewhat attenuated to enable chronic infection, hence these virions may be a good source for a vaccine. We tested the efficacy of CIC-A generated virions as a vaccine and compared it to commercial vaccines. In these experiments we challenged with aMPV-B to facilitate distinction between the vaccine and challenge viral RNA. The vaccine RNA (aMPV-A) was present in tracheal swabs of all vaccinated groups. The challenge RNA (aMPV-B) was also detected in tracheal swabs of all challenged groups, however the level of detection was dependent on the vaccination status. CIC-A vaccinated birds demonstrated an equivalent rate of detection of aMPV-B compared to the commercial A vaccinated group, and in tissue analysis of RNA was found to have a more limited tissue distribution of the virus, suggesting better clearance of the virus. These preliminary data show that CIC-generated viruses may serve as a vaccine in at least equal efficacy as matched commercial vaccine. More work has to be performed to test CIC-generated viruses as vaccines against homologous challenge, and to evaluate the reproducibility of the results.

REFERENCES

Alvarez, R., K. S. Harrod, W. J. Shieh, S. Zaki, and R. A. Tripp. 2004. Human metapneumovirus persists in BALB/c mice despite the presence of neutralizing antibodies. J Virol 78:14003-14011.

Banet-Noach, C., L. Simanov, and S. Perk. 2005. Characterization of Israeli Avian Metapneumovirus strains in turkeys and chickens. Avian Pathology 34: (3) 220-6 .

Bayon-Auboyer, M. H., V. Jestin, D. Toquin, M. Cherbonnel, and N. Eterradossi. 1999. Comparison of F-, G- and N-based RT-PCR protocols with conventional virological procedures for the detection and typing of turkey rhinotracheitis virus. Archives of Virology 144:1091-109.

Bayon-Auboyer, M. H., C. Arnauld, D. Toquin, and N. Eterradossi. 2000. Nucleotide sequences of the F, L and G protein genes of two non-A/non-B avian pneumoviruses (APV) reveal a novel APV subgroup. J Gen Virol 81:2723-2733.

Carter, M. J., M. M. Willcocks, S. Loffler, and V. Ter Meulen. 1983. Comparison of lytic and persistent measles virus matrix proteins by competition radioimmunoassay. J Gen Virol 64 (Pt 8):1801-5.

Collins, M. S., R. E. Gough, and D. J. Alexander. 1993. Antigenic differentiation of avian pneumovirus isolates using polyclonal antisera and mouse monoclonal antibodies. Avian Pathology 22:469-479.

Cook, J. K. A., B. V. Jones, M. M. Ellis, L. Jing, and D. Cavanagh. 1993. Antigenic differentiation of strains of turkey rhinotracheitis virus using monoclonal antibodies. Avian Pathol 22:257-273.

Cook, J. K. A. 2000. Avian pneumovirus infections of turkeys and chickens. Vet J 160:118-125.

Cook, J. K. A., M. B. Huggins, S. J. Orbell, and D. A. Senne. 1999. Preliminary antigenic characterization of an avian pneumovirus isolated from commercial turkeys in Colorado, USA. Avian Pathology 28:607-617.

Cook, J. K. A. and D. Cavanagh 2002. Detection and differentiation of avian pneumoviruses (metapneumoviruses). Avian Pathology 31(2): 117-32.

Goldman, M. B., D. J. Buckthal, S. Picciotto, T. A. O'Bryan, and J. N. Goldman. 1995. Measles virus persistence in an immortalized murine macrophage cell line. Virology 207:12-22.

Guerrero-Plata, A., E. Ortega, V. Ortiz-Navarrete, and B. Gomez. 2004. Antigen presentation by a macrophage-like cell line persistently infected with respiratory syncytial virus. Virus Res 99:95-100.

Jirjis, F. F., S. L. Noll, D. A. Halvorson, K. V. Nagaraja, and D. P. Shaw. 2002. Pathogenesis of avian pneumovirus infection in turkeys. Vet Pathol 39:300-310.

Juhasz, K., and A. J. Easton. 1994. Extensive sequence variation in the attachment (G) protein gene of avian pneumovirus: evidence for two distinct subgroups. J Gen Virol 75:2873-2880.

Lamb, R. A., and D. Kolakofsky. 2001. *Paramyxoviridae*: The viruses and their replication., p. 1305-1340. *In* P. M. Howley (ed.), Fields Virology. LIPPINCOTT WILLIAMS & WILKINS.

Ling, R., A. J. Easton, and C. R. Pringle. 1992. Sequence analysis of the 22K, SH and G genes of turkey rhinotracheitis virus and their intergenic regions reveals a gene order different from that of other pneumoviruses. J Gen Virol 73:1709-1715.

Martinez, I., J. Bustos, and J. A. Melero. 2001. Reduced expression of surface glycoproteins in mouse fibroblasts persistently infected with human respiratory syncytial virus (HRSV). Arch Virol 146:669-83.

Moscona, A. 1991. Defective interfering particles of human parainfluenza virus type 3 are associated with persistent infection in cell culture. Virology 183:821-4.

Moscona, A., and M. S. Galinski. 1990. Characterization of human parainfluenza virus type 3 persistent infection in cell culture. J Virol 64:3212-8.

Murphy, D. G., K. Dimock, and C. Y. Kang. 1990. Viral RNA and protein synthesis in two LLC-MK2 cell lines persistently infected with human parainfluenza virus 3. Virus Res 16:1-16.

Pringle, C. R., J. S. Randhawa, A. C. Marriott, A. J. Easton, S. D. Wilson, K. P. Tolley, and D. Cavanagh. 1998. Virus taxonomy-San Diego. Arch Virol 143:1449-1459.

Sheshberadaran, H., E. Norrby, and K. W. Rammohan. 1985. Monoclonal antibodies against five structural components of measles virus. II. Characterization of five cell lines persistently infected with measles virus. Arch Virol 83:251-68.

Seal, B. S. 1998. Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains. Virus Res 58:45-52.

Toquin, D., M. H. Bayon-Auboyer, Senne, D. A., Eterradossi, N. 2000. Lack of antigenic relationship between French and recent North American non-A/non-B turkey rhinotracheitis viruses." Avian Dis 44(4): 977-82.

Valdovinos, M. R., and B. Gomez. 2003. Establishment of respiratory syncytial virus persistence in cell lines: association with defective interfering particles. Intervirology 46:190-8.

van den Hoogen, B. G., J. C. de Jong, J. Groen, T. Kuiken, R. de Groot, R. A. Fouchier, and A. D. Osterhaus. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719-724.

Viola, M. V., C. Scott, and P. D. Duffy. 1978. Persistent measles virus infection in vitro and in man. Arthritis Rheum 21:S47-51.

Young, K. K., B. E. Heineke, and S. L. Wechsler. 1985. M protein instability and lack of H protein processing associated with nonproductive persistent infection of HeLa cells by measles virus. Virology 143:536-45.

Yu, Q., P. J. Davis, T. D. Brown, and D. Cavanagh. 1992. Sequence and in vitro expression of the M2 gene of turkey rhinotracheitis pneumovirus. J Gen Virol 73:1355-1363.

Table 1: Characteristics of the aMPV infected cells and virus subtypes.

Cell type	Infected with	CPE*	Cell division/day†	Titer production‡ (ID_{50}/ml)
Naïve Vero cells			2.2	
Acutely infected Vero cells	aMPV-A	Small syncytia, partial rounding & detachment	-	$5x10^3$
	aMPV-B	Partial rounding & detachment	-	$3.2x10^3$
	aMPV-C	Very large syncytia	-	$1.4x10^3$
Chronically infected Vero cells	aMPV-A	Very few, small-sized syncytia	1.5	$2.7x10^3$
	aMPV-B	Very few floating cells	1.4	$3.2x10^3$
	aMPV-C	Very few, small-sized syncytia	1.6	$0.6x10^3$

* Syncytia were first observed at day 3, evolved to either rounding and detachment of the cells or to larger syncytia, as monitored until day 7.

†Cell division rate was deduced from daily counting of the number of cell over a 4 day period. The rate was deduced from the log phase of growth.

‡Viral titers were determined on Vero cells by end-point dilution assay. Figure 1: Timeline of the experimental design. B: blood samples, S: tracheal swabs, Or: organs (lungs, trachea, turbinates)

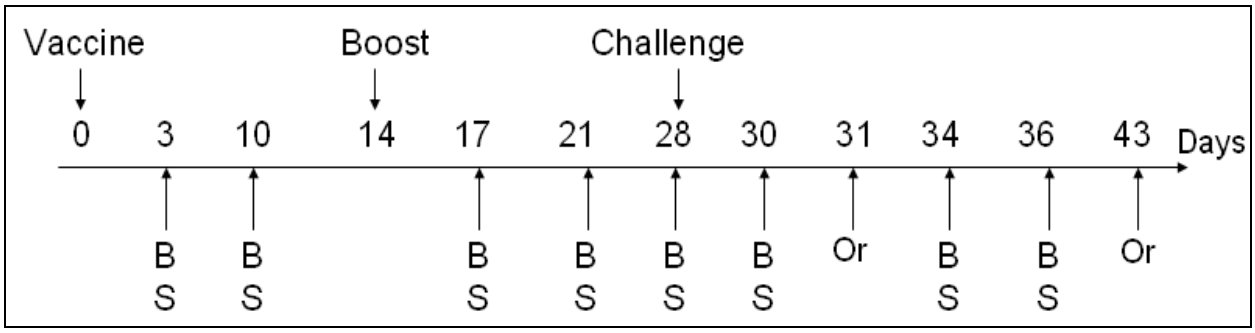

Figure 2: Immunostaining of CIC cultures. CIC, at their 8th passage were fixed and stained with sera against aMPV-B as a primary antibodies and FITC-conjugated anti-chicken antibodies as secondary antibodies. Naïve cells (Vero) served as a negative control.. CIC-C demonstrated lower staining intensity likely to be due to weaker cross-reactivity of the Ab to aMPV-C.

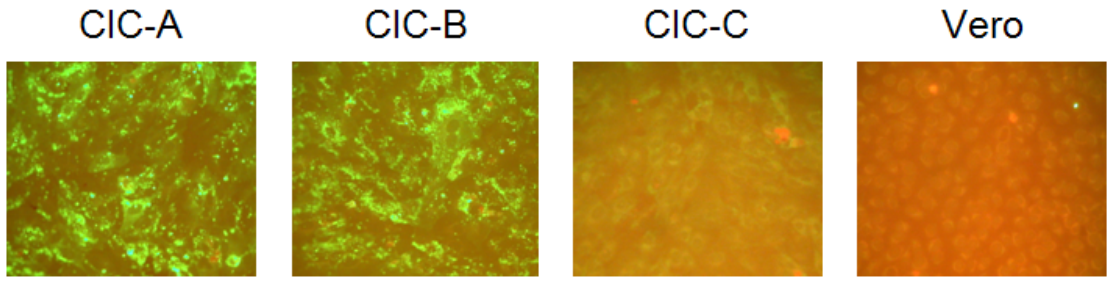

Table 2: Comparable replication rates of the CIC-A with the matched commercial vaccine. The vaccine strain was detected by RT-PCR in RNA extracted from tracheal swabs sampled prior to the challenge. Values represent the number of positive samples compared to the total number of tested samples.

	Day 3	Day 10	Day 17	Day 28
Non vaccinated	0/4	0/4	0/4	0/8
CIC-A	6/12	1/4	0/4	0/8
Commercial A	3/12	1/4	0/4	0/8
Commercial A + B	10/12	2/4	0/4	0/8

Table 3: CIC-A performs as well as a commercial A vaccine. RNA from the challenge virus, aMPV-B, was detected by RT-PCR in swabs of all groups at day 30 but was significantly reduced in vaccinated birds as compared to control. Values represent the number of positive samples as a ratio of the total number of tested samples.

	Day 30	Day 36
Non vaccinated	7/12	12/12
CIC-A	7/12	1/11
Commercial A	8/12	2/13
Commercial A + B	3/12	2/12

Table 4**:** Limited distribution of the challenge virus in the airways of CIC-A vaccinated birds. RNA was extracted from trachea and turbinate following challenge with an infectious aMPV-B isolate. aMPV-B RNA as tested by RT-PCR was not detected in trachea and to a lower extent in turbinate of CIC-A vaccinated birds as compared to either control or commercial A vaccinated birds. ,Samples were collected 3 days after challenge, and the number of positive samples is indicated as a ratio of the total number of samples tested.

	Trachea	Turbinate
Non vaccinated	6/12	12/12
CIC-A	0/10	6/10
Commercial A	4/12	9/12
Commercial A + B	0/13	1/13

UNSTABLE ATTENUATION OF LIVE AVIAN PNEUMOVIRUS VACCINES

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SUMMARY

Since the 1980s a number of live attenuated type A and B Avian Pneumovirus (APV) vaccines have been developed in Europe and these have generally afforded good protection. All have been produced by repeated passages of field virus in a laboratory culture system to achieve various degrees of attenuation. They are widely used in commercial growing turkeys and to prime future layers and breeders. When tested under experimental conditions, these empirically derived vaccines were shown to be fully protective whilst not causing detectable disease themselves. However, they have not performed as well when used in the field and unstable attenuation has been considered to be a possible factor. This paper describes the first evidence of reversion of an APV vaccine in the field. First, systematic evidence of extended vaccine virus persistence in the field arose from longitudinal studies performed in the UK in 1995. Secondly, from an outbreak of Turkey Rhinotracheitis (TRT) in an Italian turkey flock previously vaccinated at day old, we isolated an APV which proved to be a vaccine derivative by sequencing. Finally, in order to determine whether vaccine virus or a derivative of increased virulence had been isolated, the virus was applied to 1 day old poults in secure isolation conditions. The vaccine derivative virus was shown to be able to cause disease with a severity similar to that caused by virulent field virus.

INTRODUCTION

During early 1990s a number of live attenuated A and B type APV vaccines were developed in Europe which generally afforded good protection. All were produced by passage of virulent virus in a laboratory culture system and these have resulted in a range of protective empirical vaccines (Giraud et al., 1987; Cook et al., 1989a, 1989b; Williams et al., 1991a, 1991b). They are widely used in growing turkeys, broilers and to prime future layers and breeders. When tested in experimental

conditions, European vaccines have been shown to be fully protective whilst only causing minimal or no disease themselves (Cook et al. 1989a; Williams et al., 1991b) whereas the vaccines did not perform as well when used in the field (Pattison, 1998; Banet-Naoch et al., 2005). One underlining reason for observed instances of post vaccinal disease may be instability of the vaccine. In experimental conditions, disease has been seen after 4 to 10 back passages of vaccine in naïve turkeys (Naylor & Jones, 1994). It is now widely believed that in the field, poor vaccine application may allow shed virus to pass to unvaccinated birds. This passage may be repeated until significant virulence is regained.

First indications of vaccine persistence in turkeys flocks was suggested by UK field studies involving seven different farms (Naylor et al., 1997). This showed that virus detected up to about 5 weeks of age was of the same type as the vaccine virus (A or B) that had been applied. The authors concluded that viruses detected were likely to be either vaccine or vaccine-derived but at that time they were not able to prove this assumption. We now report a further study in which vaccine was applied to poults and where genome sequencing was used to clearly identify the origin of the detections. In Italy, virus isolation was attempted from vaccinated birds showing TRT-type clinical signs and one isolate was sequenced in known marker regions to determine whether it was vaccine derived. The virulence of the isolate was assessed by looking for clinical effects after inoculation of 1 day old poults in secure isolation conditions.

Study 1 – Evidence of vaccine persistence on farms

MATERIALS AND METHODS

Six UK farms were studied and on all, oro-pharyngeal swabs were collected for later APV RNA detection. In all cases one day old poults were vaccinated by coarse spray in the hatchery with live APV B type vaccine. Swabs were taken then air dried at ambient temperature prior to testing by RT-PCR. This was done twice a week until turkeys were 10 weeks old. The methodology followed was that described by Cavanagh et al. (1999). A specific RT-nested PCR, based on G gene sequence and able to differentiate A and B types, was used to detect APV RNA in the dry swabs. RNA was extracted from pools of 10 swabs, cDNA was prepared in an RT reaction and this was amplified in a nested PCR. When type B APV was detected, the differentiation between vaccinal and field APV was based on the F and G gene analysis described in detailed by Cavanagh et al.(1999). When numerous B type field strains were compared, the presence of an A residue in the F gene nucleotide at position 979 and nucleotides G, A and C in positions 445, 464 and 525 respectively in the G gene were found to be unique to the B type vaccine.

RESULTS

The results of APV genome detection by RT-PCR are shown in Figure 1. All the detections, except one at 53 days of age were of B type. As previously described by Cavanagh et al. (1999), the F and G genes were sequenced and specific residues at nucleotide 979 of the F gene and 445, 464 and 525 of the G gene were compared to reference strains to determine which detections had originated from the vaccine.

Details of sequences at these positions can be found on Table 1. All the early detections (from 2 to 6 weeks of age) had vaccine sequence, while the later detections (more than 5 weeks of age) were not vaccine. The vaccine had been shown to persist for a much longer period than in experimental conditions.

Study 2 - Isolation and characterisation of a vaccine-derived APV strain.

In study 2 we isolated and characterized a vaccine derived APV strain.

MATERIALS AND METHODS

Swabs were collected from 18-day old turkeys showing respiratory signs. The poults has been given live APV type A vaccine at 1 day old in the hatchery. Swabs taken from the choanal cleft of 10 birds were immediately immersed in transport medium with antibiotics to suppress bacterial and mycoplasma growth and kept on ice until inoculation of chicken embryo tracheal organ cultures (TOC). Swabs were pooled and the supernatant was used after filtration through 0,45μm membrane filters to inoculate TOCs (Cook et al., 1976). Ciliostasis was taken as a preliminary indicator of APV detection. APV identification was determined by RT-PCR using the method of study 1 except on this occasion the RNA was extracted from the inoculated medium rather than dry swabs. The genome of isolated virus was sequenced to cover 9 specific RNA base substitutions which had occurred when the vaccine was generated from its progenitor field strain.

RESULTS

An APV type A, designated 309/04 was isolated from the flock. The sequencing of the 9 vaccine characterising genome regions gave the unique vaccine base in every case so showed unequivocally that the isolated virus had derived from the applied vaccine as illustrated in Table 2. Because live virus was isolated, this presented the opportunity to determine whether virulence had increased

Study 3 - Virulence assessment of 309/04 APV strain

MATERIALS AND METHODS

Sixty 1-day-old commercial turkeys were housed in 3 positive pressure isolators with 20 poults in each. Poults in isolator 1, 2 and 3 were respectively inoculated with 309/04 APV, vaccine or uninoculated TOC medium. For inoculation 309/04 and vaccine were used at doses of 2.8 and 4.5 Log $_{10}$ ID50% per poult respectively. The vaccine dose represented a 10 times overdose compared to the manufacturer's recommendation, this was to ensure that any disease differences could not be caused by dose effect. Ten birds per group were identified using leg labels and clinical signs were observed daily up to 13 days post infection (d.p.i.). Between 3 and 13 d.p.i two birds were humanely killed, by dislocation of cervical vertebrae, and sampled in each two day period and examined for histological lesions. Nasal cavity, infra-orbital sinuses and the upper portion of the trachea were removed and fixed in 10 % formalin. At 13 d.p.i. all birds were killed.

Clinical signs were scored as previously described (Naylor & Jones, 1994) thus: 0, no signs; 1, clear nasal exudate; 2, turbid nasal exudate; 3, swollen infra-orbital sinuses and /or frothy eyes. The differences in the clinical scores among the three groups were firstly tested using the Kruskal-Wallis non parametric one-way ANOVA. The differences between groups were tested using the Mann-Whitney test. A *p* value<0,05 was considered statistically significant. Tissue samples collected for histology were fixed in 10% formalin, processed by conventional methods and embedded in paraffin wax. Sections were stained by haematoxylin and eosin (H&E).

RESULTS

The daily mean clinical scores, for each group, are reported graphically in Figure 2. Virus 309/04 resulted in severe clinical signs in the inoculated birds from day 6 including swollen sinuses and frothy eyes. Birds in the other two groups showed only minor signs. A difference statistically significant in clinical scores was found between 309/04 inoculated group and vaccine group (U=3967; $p < 0.001$) and between 309/04 inoculated group and control group (U=4275; $p < 0.001$). Difference between vaccine group and control group was not significant ($p = 0.162$). From day 4 the histological sections showed deciliation of the respiratory epithelium lining the turbinates and the nasal cavity in those birds infected with 309/04. This was associated with mononuclear cell infiltration of the lamina propria and oedema. The early microscopic changes were observed in the nose, while the sinuses were affected from day 7 p.i. Respiratory lesions were not seen in tissues of poults receiving vaccine or in the negative control group.

DISCUSSION

Previous studies have shown that APV vaccine virus could only be isolated between approximately 3 to 8 days after its application (Naylor, 1993; Ganapathy et al., 2005) and the latter workers also showed that the use of RT-PCR only extended this period by a small margin. In the current field study, vaccine-derived virus was found later at 18 days post vaccination and unlike in experimental conditions, this was associated with disease. These results clearly support our first studies showing that vaccine virus can persist in field conditions much longer than in experimental conditions. We have extended those studies so, for the first time, we have shown that the virulence of the virus has increased. The virus is clearly derived from vaccine but it has changed so that it now causes disease. It is widely believed that poor vaccine application leads to disease problems in poults. For the first time we have shown that this disease is caused by reverting vaccine virus. It would be interesting to fully sequence the whole virus because if, as expected, other mutations are present these might give an indication of changes leading to virulence. In the future it would be desirable to have vaccines which are more stable. Some work has been done with DNA (Kapczynski & Sellers; 2003; Naylor et al., 2002) and vector based vaccines (Quingzhong et al., 1994). However the recent development of an APV reverse genetics system (Naylor et al., 2004) should allow fully stable live vaccines to be made. In the meantime these results emphasize the importance of making sure that each bird receives a full does of vaccine.

REFERENCES

Banet-Naoch C, Simanov L, Perk S. Characterization of Israeli avian metapneumovirus strains in Turkeys and Chickens. Avian Pathol 2005; 34(3):220-226.

Cavanagh D, Mawditt K, Britton P, Naylor CJ. Longitudinal field studies of infectius bronchitis virus and avian pneumovirus in broiler using type-specific Polymerase chain reactions. Avian Pathol 1999; 28:593-605.

Cook JKA, Darbyshire JH, Peter RW. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch Virol 1976; 50:109-118.

Cook JKA, Ellis MM, Dolby CA, Holmes HC, Finney PM, Huggins MB. A live attenuated turkey Rhinotracheitis Virus Vaccine.1. Stability of the Attenuated Strain. Avian Pathol 1989a; 18:511-522.

Cook JKA, Holmes HC, Finney PM, Ellis MM, Huggins MB, Dolby CA. A live attenuated turkey Rhinotracheitis Virus Vaccine. 2. The use of the attenuated strains as an experimental vaccine. Avian Pathol 1989b; 18:523-534.

Ganapathy K, Cargill PW, Montiell E., Jones RC. Interaction between live avian Pneumovirus and Newcastle Disease virus vaccines in SPF chickens. Avian Pathol 2005; 34(4):297-302.

Giraud P, Guittet M, Toquin D, Bennejean G. Rhinotracheité infectieuse de la dinde (RTI). Progrés récents dans l'étiologie et la prévention. L'Aviculteur 1987; 477:47–53

Kapczynski DR, Sellers HS. Immunization of turkeys with a DNA vaccine expressing either the F or N gene of avian metapneumovirus. Avian Dis 2003; 47(4):1376-1383.

Naylor C, Shaw K, Britton P, Cavanagh D. Appearance of type B avian pneumovirus in Great Britain. Avian Pathol 1997; 26:327-338.

Naylor CJ, Brown PA, Edworthy N, Ling R, Jones RC, Savage CE, et al. Development of a reverse-genetics system for Avian pneumovirus demonstrates that the small hydrophobic (SH) and attachment (G) genes are not essential for virus viability. J Gen Virol 2004; 85:3219-3227.

Naylor CJ, Cavanagh D, Jones RC. Live TRT vaccination in the UK: past present and future. In: Hafez HM, editor. Proceedings of the 4th International symposium on turkey diseases; 2002 May 15-18; Berlin Germany. DVG-Service-GmbH: Giessen; 2002. p. 151-153.

Naylor CJ, Jones RC. Demonstration of a virulent subpopulation in a prototype live attenuated turkey rhinotracheitis vaccine. Vaccine 1994; 12(13):1225-1230.

Naylor CJ. Studies on avian pneumovirus including development of a vaccine. [PhD Thesis]. Liverpool (UK): Liverpool Univ.; 1993.

Pattison M. TRT in the Field: Field situation and control. In Clark SR, Ginsburg LM editors. Proceedings & Technical Supplements of the Roche Avian Pneumovirus Workshop. 1998 June 30-July 1, St. Cloud, Minnesota, USA. Parsippanny NJ RCD 9713: Roche Vitamins Inc; 1998. p 43-49.

Qingzhong Y, Barrett T, Brown TDK, Cook JKA, Green P, Skinner MA, et al. Protection against turkey rhinotracheitis pneumovirus (TRTV) induced by a fowlpox virus recombinant expressing the TRTV fusion glycoprotein (F). Vaccine 1994; 2:569-573.

Williams RA, Savage CE, Jones RC. Development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathol 1991a; 20:45-55.

Williams RA, Savage CE, Worthington KJ, Jones RC. Further studies on the development of a live attenuated vaccine against turkey rhinotracheitis. Avian Pathol 1991b; 20:585-596.

Table 1: Study 1 - differentiation of Type B vaccinal strains from Type B field strains.

F and G gene nucleotides at positions	Vaccine progenitor Fr/PR8602 /86[a]	Vaccine [a]	0-6 weeks detections	5-10 weeks detections	European Field strains [a]			
			7 strains	4 strains	NL/21 78/90	Hungary /89	Italy /88	Spain /88
F 979	G	**A**	**A**	G	nd [b]	G	G	G
G 445	**G**	**G**	**G**	**G**	A	**G**	**G**	**G**
G 464	**A**	**A**	**A**	G	**A**	**A**	**A**	**A**
G 525	**C**	**C**	**C**	A	**C**	A	A	A

[a] sequences reported by Cavanagh et al. (1999)
[b] not done

Figure 1: Study 1 - APV detection by RT-PCR in six turkey farms. Day old poults were vaccinated with live B type APV vaccine. (-) negative for APV. (B•) positive for APV type B-vaccine derived. (B○) positive for APV type B-wild strain. (A) positive for APV type A.

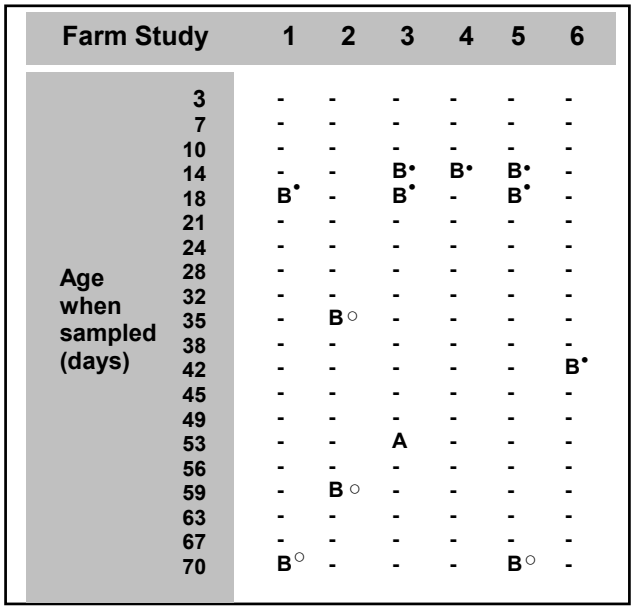
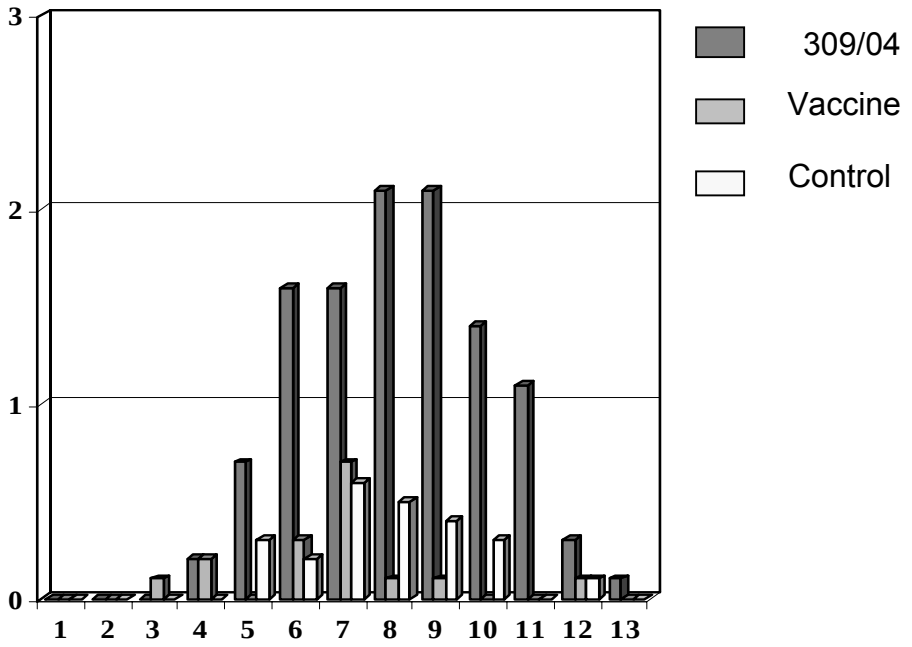


Table 2: Study 2 - Characterisation of the vaccine-derived 309/04 isolate.

Positions [a]	Nucleotides		
	Vaccine progenitor# 8544	Vaccine	309/04 strain
2941	G	**A**	**A**
3553	T	**C**	**C**
3825	G	**A**	**A**
5055	A	**G**	**G**
5140	T	**C**	**C**
5929	A	**G**	**G**
6358	T	**C**	**C**
10022	T	**G**	**G**
11624	T	**C**	**C**

[a] in antigenome

Figure 2: Study 3 - Mean daily clinical scores after inoculation of isolate 309/04 or ten times overdose of vaccine, compared to an uninoculated group.



PLAQUE PURIFICATION OF AN APV CANDIDATE VACCINE SHOWS THAT MUTATIONS ASSOCIATED WITH THE ATTACHMENT PROTEIN GENE CAN AFFECT PROTECTION

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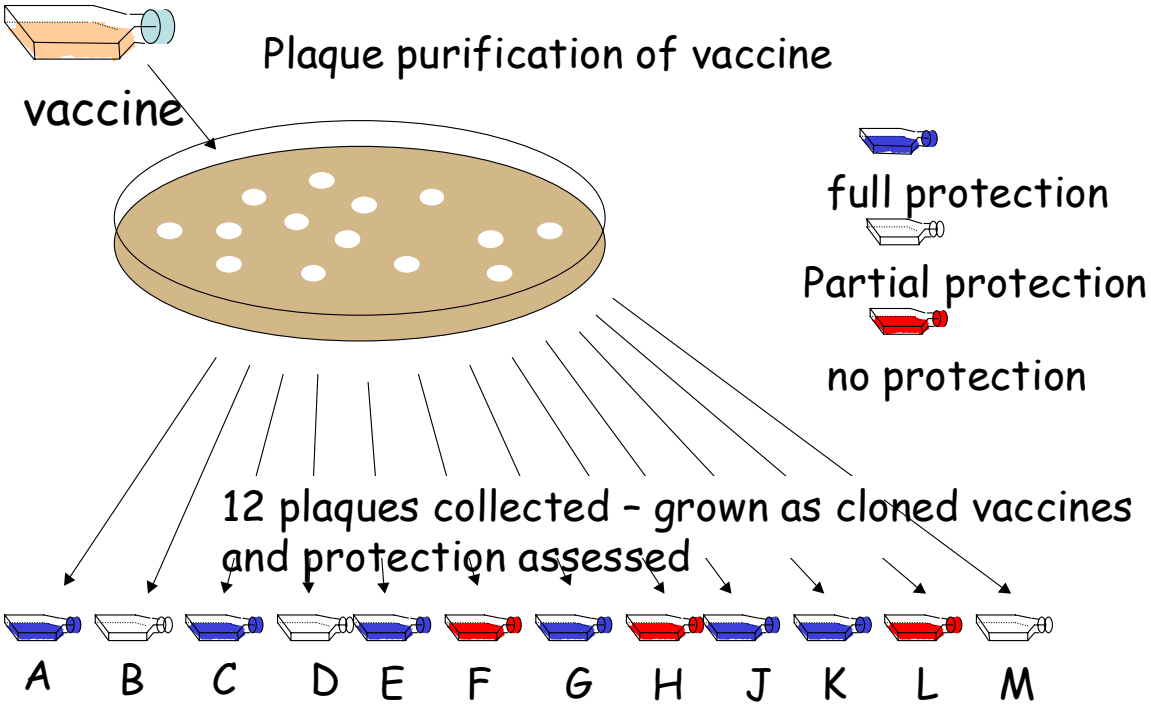
A prototype live APV vaccine was found to give complete protection when against virulent challenge. This was assessed by absence of clinical signs, inability to reisolate tracheal virus and lack of APV immunofluorescence in tracheas of killed birds. However when tested in reversion to virulence trials, the disease was found which was shown to be due in part to tiny subpopulation. To remove this cloned vaccines were prepared by plaque purification. Cloned vaccines displayed reduced reversion but some were also found to have lost their ability to protect.



Sequencing of 2 fully protective and 2 unprotective clones (F,H) showed that the only differnces were found in the SH-G intergenic region. These may have prevented termination of the SH gene transcript and led to it including the attachment protein (G)gene. Such dicistronic messages would be expected to reduce G expression and this was supported by Western blots of viral proteins which showed a less intense band at the size corresponding to G.

The remaining unprotective clone (L) was found to have an unaltered SH-G intergenic region. In this instance the G protein was found to contain approximately 30 coding mutations which might be expected to significantly alter the proteins anitgenicicty.

A virus lacking a G protein was constructed using reverse genetics which showed reduced protection compared to virus with a complete genome.

These results show that live APV vaccines may contain a range of populations and that these may have significantly different properties. They also show that the attachment protein is needed for full protection against virulent challenge. This might explain why cross protection between subtypes, where the differences occur principally in the G gene, may be inferior to homologous protection.

A METHOD OF EXCHANGING RNA SEQUENCES BETWEEN PROTECTIVE AND UNPROTECTIVE CANDIDATE APV VACCINES REVEALS SOME REGIONS IMPORTANT IN STIMULATING PROTECTION

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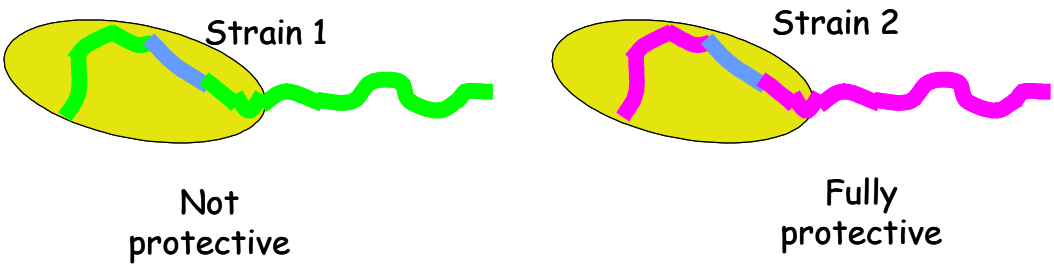
When two strains of APV were tested as candidate vaccines, neither produced post vaccinal disease but only 1 (strain 2) conferred protection.

When the 2 viruses were fully sequenced, the main differnces were seen in the first half of the genome and the highest density was in the fusion protein gene.

Genomes of both viruses fully sequenced

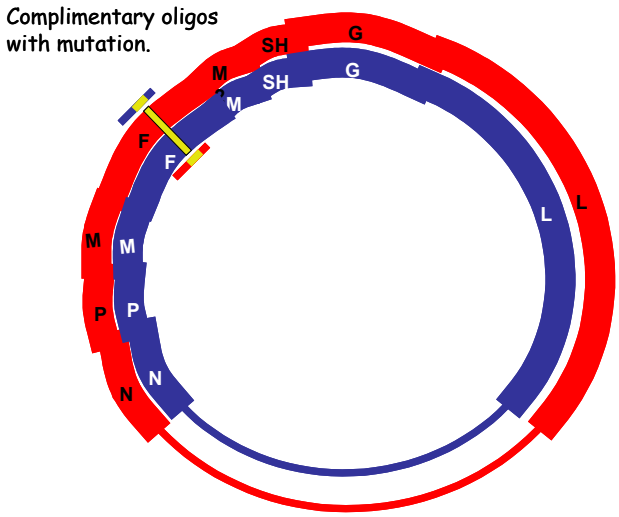


About 15 mutations found from N to M2

Highest density in Fusion protein gene

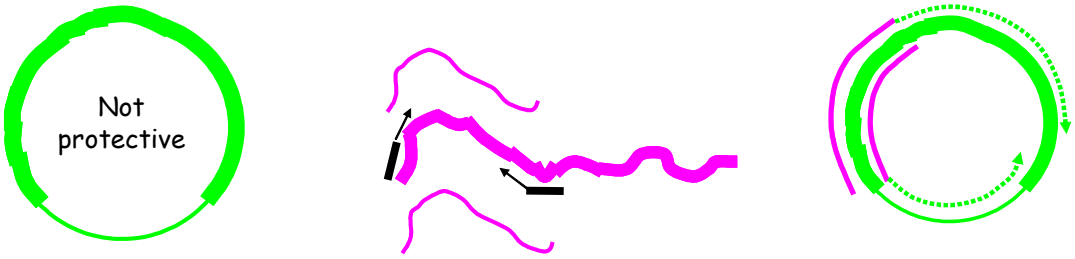
A method was required capable of exchanging sequences between the viruses. Commercial kits are available for introducing point mutations in plasmids such as those used to generate full viral genomes in reverse genetics systems

Commercial kits available for point mutations



However these are not suitable for making alterations to large regions, mainly due to the difficulty of constructing primers containing 100s of nucleotides.

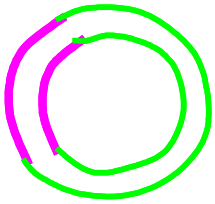
Long oligonucleotides s were produced by an alternative method of blunt ended PCR copying of sequence from strain 2 using pfu polymerase.



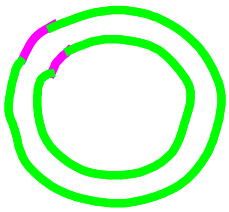
Long oligos not possible BUT blunt ended RT-PCR makes the same !!!

These were used to modify a cloned full length viral genome copy which had been based on unprotective strain 1.

Two plasmids constructed

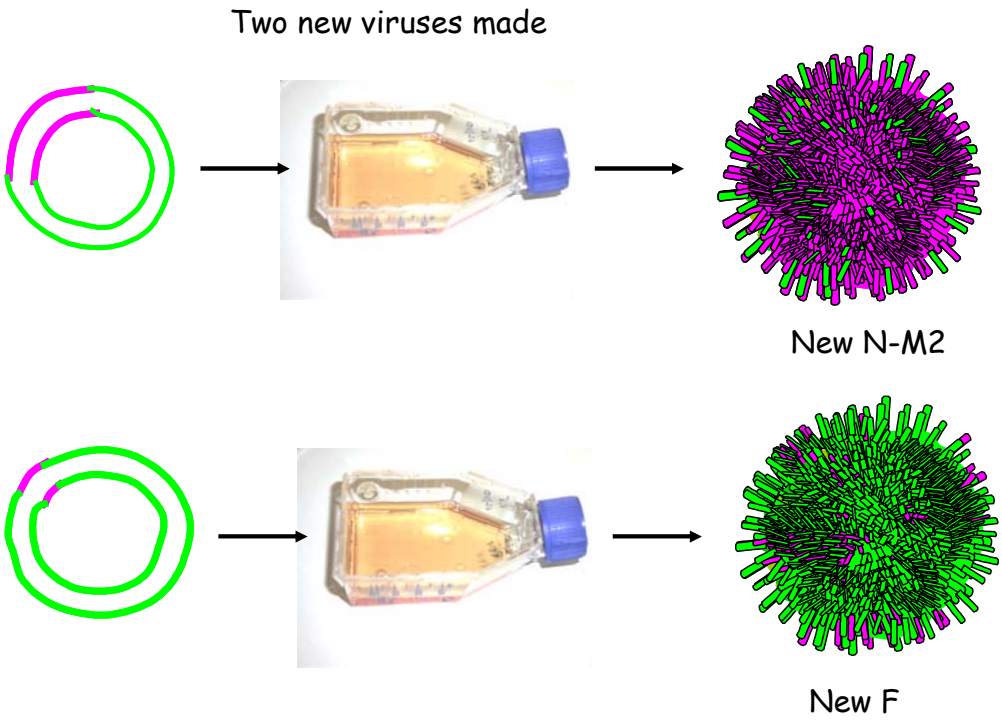


N to M2 substituted (5000 bases)

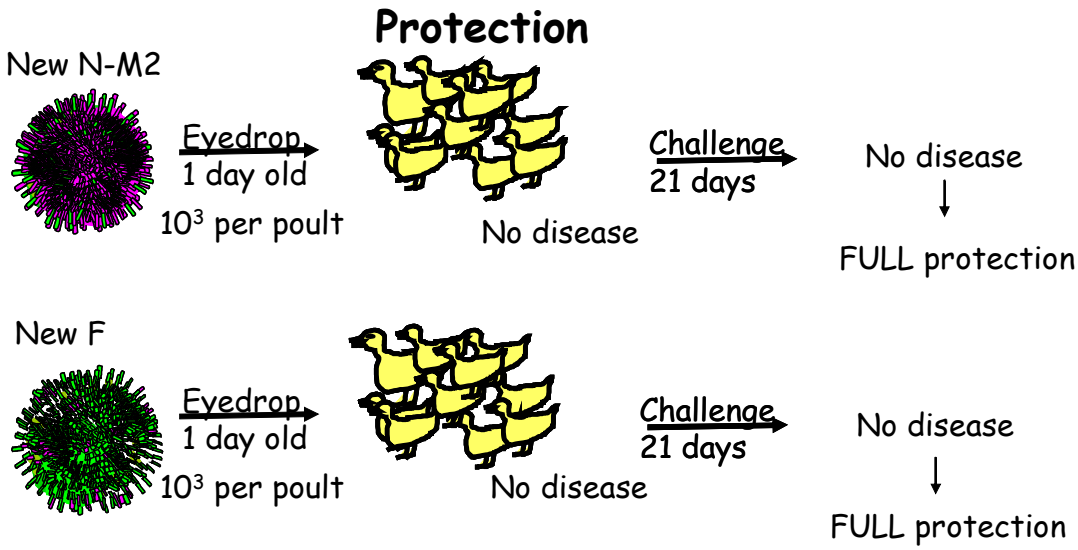


Complete fusion protein gene (F)
substituted (1600 bases)

Reverse genetics was used to create viruses with these altered sequences.



Challenge after inoculation of either of these viruses did not result in any clinical disease. Turkeys had been fully protected.



In this instance, altering only the fusion protein was sufficient to convert an unprotective virus into one which conferred full protection.

The novel mutagenesis method had proved capable of altering the desired long sequence. Furthermore, amplification was based on a linear copying method (not PCR) so more than 90% of copies would be expected to be error free.

TRUE INCIDENCE RATE OF INFECTIOUS BRONCHITIS, NEWCASTLE DISEASE AND MYCOPLASMOSIS IN BACKYARD CHICKENS FROM EIGHT RURAL COMMUNITIES IN THE STATE OF YUCATAN, MEXICO

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SUMMARY

A system for monitoring diseases in backyard chickens using sentinels was established. Eight rural communities were selected by convenience. On each one, six houses were selected and a package of four Creole birds of at least 6 weeks of age, free of the diseases under study and identified with plastic strips on the bottom of the leg was delivered to each house. Blood samples were collected every two months during a 6 month period. The true incidence rate (TIR) for Infectious Bronchitis (IBV), Newcastle disease (NDV), *Mycoplasma gallisepticum (Mg)* and *Mycoplasma synoviae (Ms)* through seroconvertion using the haemagglutination inhibition test for both viruses and the slide agglutination test for mycoplasmas, were determined. The TIR for IBV, NDV, *Mg* and *Ms* were 0.65; 0.002; 0.097 and 0.046 bird-two month risk respectively. The importance of IBV for backyard chickens in Yucatan was confirmed. The first evidence of mycoplasmosis as part of the respiratory disease syndrome observed in these animals was obtained. NDV does not seem to be present in the communities under study supporting the status of the State as free of the velogenic form of the disease.

INTRODUCTION

In Yucatan two important animal production systems exist. On one hand there is the modern commercial system and on the other there is the backyard production system that is considered poorly productive compared to the commercial system but very important for the families in the rural communities. (Rejon-Avila *et al.,* 1991).

The backyard system represents an important source of animal protein (eggs, meat and milk). The specie reported with most frequency are poultry being present in up to 85% of the houses that raise animals in some communities in the state (Rodríguez and Berán, 1988).

Because this system has been considered non productive there has been little

interest by the researchers who consider that because of the variety of species that participate and the dynamics within, it is difficult to study and transform (Honhold *et al*., 1993; Franco and Santamaría, 1994).
Few studies have been carried out reporting the sanitary situation of the animals raised in this system, although diseases not only affect the animals raised in the backyards but also those raised in the commercial farms with movement of diseases in both directions (Gutierrez-Ruiz, 2004).
Diseases are one of the most important factors having a major impact on the productivity of this system (Honhold *et al*., 1993, Rodríguez *et al*., 1996; Acosta-Casanova, 2004).
Few studies have been carried out in the state buy they describe the presence of respiratory and digestive syndromes without the determination of the cause or causes of these (Honhold *et al*., 1993, Rivera-Ortega., 1997).
The first report of the diagnosis of a specific agent involved in respiratory illness in backyard chickens in Yucatan was a serological survey for IBV and NDV. The isolation and characterization of IBV virus was also carried out demonstrating the active role of this virus in the communities of the state (Gutiérrez-Ruiz *et al*., 1998; 2000).
A survey carried out in 30 communities of Yucatan reported a prevalence of 2.2% of antibodies against the NDV virus in backyard chickens, matching with the free status given to the state (Gutierrez-Ruiz *et al*., 2000). Only a lentogenic virus (Ulster 2C type) was isolated in backyard chickens during the same survey (Gutiérrez-Ruiz, 2004).
The information on the diseases affecting the backyard chickens is poor. Most of the studies carried out are based on static collection of information of the system with a minimum of visits to the communities (Honhold *et al*., 1993; Rivera-Ortega, 1997).
Respiratory illness is mostly reported by the people of the rural communities. The respiratory problems observed are most likely of diverse etiology including viruses, mycoplasmas, bacterias, immunodepresive agents as well as management and unfavorable environmental conditions (Kleven *et al*., 1995).
Reports of interactions of IBV with other respiratory agents are common. Co-infection of different strains of the virus (Massachussets and variants) with avian metapneumovirus and/or NDV have been reported (Jones, 1997). NDV has the potential to cause important economic losses in the poultry industry because virulent variants of this disease cause mortalities as high as 100% (Jordan, 1990). Yucatan is free of NDV in its velogenic form and from Avian Infuenza, status that has been maintained for over 10 years (SENASICA, 2004; SAGARPA, 2006).
Other agents commonly involved are mycoplasmas, with Mycoplasma gallisepticum (MG) and Mycoplasma synoviae (MS) reported as the most important in avian production (Jones, 1997). The mycoplasma species than are reported to infect chickens in México are MG of which several strains are distinguished; A5969, S, F and R and MS from which only one strain is known 1853WVU (Etcharren *et al*., 1992).
The situation in the backyard system, where most of the time the animals are free and there is a close contact between birds of different houses with local wildlife as well as with migratory birds, creates the possibility of the introduction to the state of Yucatan of diseases considered exotic like velogenic NDV.
In different countries the high risk that exists for the introduction of disease agents

from wild birds to domestic poultry has been reported (Rosales, 1996; Kommers *et al*., Terregino *et al*., 2003). This is more likely to occur with free range domestic animals like poultry kept in the backyards of Yucatan (Acosta-Casanova, 2004).
Migratory birds have proved to be carriers of ND and IA viruses (Maldonado *et al*., 1994; Takakuwa *et al*., 1998; Schelling *et al*., 1999; Pfitzer *et al*., 2000). The coastal regions of Yucatan are in some of the migratory routes followed by birds traveling from North America to Central and South America and back, the marshes are used as resting and feeding areas for these birds.
Recent outbreaks of NDV and Avian influenza have occurred in the centre of Mexico as well as in the United States of North America, Canada, and some European countries (Nolen, 2003; OIE, 2003).
It is considered possible that resident wild birds as well as migratory birds pose a risk for the poultry production in Yucatan, especially for the backyard chickens due to the fact that in this system vaccination and biosecurity are non existent. Once an infection is established in the backyard poultry it can be easily introduced to the commercial farms.
Every day the need for monitoring health problems affecting backyard poultry is more evident, not only to know diseases causing economical losses but also for the early detection of exotic diseases that can affect both animals and people.
Considering all the information available it was decided to conduct an study to determine the true incidence rates of IBV, NDV and mycoplasmosis in backyard chickens from eight rural communities in the state of Yucatan, Mexico.

MATERIALS AND METHODS

Communities included in the study

The study was carried out in 8 rural communities selected by convenience. The communities included were five located on the sea coast; Dzilam de Bravo, Telchac Puerto, Celestún, Sisal and Río Lagartos, and three from the central zone where most of commercial flocks are located; Oxholón, Chablekal and Subincancab.

Origin of animals used in the study and their distribution in the communities

Fertile eggs from the Creole chicken flock of the Facultad de Medicina Veterinaria y Zootecnia de la Universidad Autónoma de Yucatán (FMVZ-UADY) were incubated. This type of bird, were selected because of its slow growth and rusticity features considered desirable for this study. Whithin 24 hours after hatching the birds were taken to a secluded room where they were maintained until they reached six weeks of age When the maternal antibodies should have waned and the birds were less susceptible to environmental conditions such as cold. At this age birds were proved clinically healthy as well as free from antibodies against the diseases included in the study.

Birds delivered to the houses

Packages of 4 animals were made. Each of The birds was identified with a different colored plastic strip on the leg which was replaced when considered necessary during the following visits. A phenotype and sex description of the animals was also recorded. Both procedures were done in order to maintain individual track of the birds.

On each of eight communities, six houses were selected and a package consisting of four chickens was delivered to each one. During the following visits more birds were delivered in order to replace dead or disappeared animals. In a few occasions houses were replaced for different reasons.
Initially, 192 Creole chickens were delivered and at the end of the study a total of 331 were used due to the replacements. 48 houses were selected at the beginning but 10 additional were required to replace some that could not continue for different reasons.
The houses were selected depending on the shape of the community making an imaginary division in the attempt to make an evenly distribution of the packages in 6 houses in each of the eight communities included in the study.

Collection, preservation and processing of samples
The samples were collected to all the birds prior to their delivery to the communities. Three consecutive visits were carried out with an interval of two months between them, making a total of four visits to each community. These visits were carried out from September 2004 to April 2005.
For serology at least 2 ml of blood from the brachial vein was obtained with 21G X 32mm syringes. The blood obtained was placed in 7 ml capacity sterile assay tubes. These were left at room temperature for approximately two hours to favour the formation of the cloth. The serum was obtained by centrifugation at 2500 G for 10 minutes. The serum was placed in 0.5 ml Eppendorf tubes and stored at -20°C until the serologic tests were performed, except in the case of the slide agglutination test for *Ms* and *Mg* were fresh diluted serum was used as indicated for this test.

Diagnostic tests
For IBV the haemagglutination inhibition (HI) technique described by Alexander *et al*., (1983) was performed using antigens from Massachussets 41 (M41) and SIN/YUC/MEX/1996 (SIN6) IBV serotypes.
For diagnosis of NDV, The HI test was performed using the Ulster strain with the methodology described in the Council directive 92/66/EEC (1997).
In order to accept the results the red blood cells control must had to show no sign of agglutination. The titres were read at 100% of inhibition, considered as so when the sliding of the red blood cells was equal to that of the red blood cells control by tilting the plates at 45º. The negative controls (SPF) had to show a titre of 3 $\log_2$ or less, and the titre of the positive control serum should have been the expected with no more than a double dilution difference (8 $\log_2$). The antigen had to contain 4 haemagglutination units. The titres of the serum samples were expressed in terms of $\log_2$, considering as a positive those samples with at least a 4 $\log_2$ titre (1/16) for both viruses.
For the diagnosis of Mycoplasma infections, the slide agglutination test was carried out using the commercial kits from Intervet™. For *Mg* the kit contained the Adler S6 strain (USA) and the WVU-1853 (A.T.C.C.) strain for *Ms*. The sera was diluted 1/10 in phosphate buffered saline to reduce false positive reactions as described in the methodology (Kleven,1998).
All the serology tests were carried out in the virology department of the diagnostic unit in the FVMZ-UADY

True incidence rate determination (TIR)
The TIR that measures the speed of infection was determined with the formula described by Martin *et al.,* (1987) as follows:

$$TIR = \frac{\text{Number of new cases that seroconvert in a period of time}}{\Sigma \text{ of the periods of time under risk of all the individuals in the population}}$$

Time animal at risk= Initial number at risk + ½ inputs – ½ outputs – ½ cases X period of observation.

RESULTS

True incidence rates for IBV was 0.54 bird-two month at risk when M41 antigen was used, 0.57 when SIN6/YUC/MEX/96 antigen was used and 0.65 when the results of both tests were used. For NDV the TIR was 0.002 bird-two month risk and for MG and MS 0.097 and 0.046 TIR were obtained.
TIR for individual communities included in the study are presented in table 1.

DISCUSSION

The true incidence rate for IBV was similar for both antigens used, but when a sample was considered positive when seroconversion occurred with either one of the antigens it led to a higher TIR (from 0.54 and 0.57 to 0.65 bird-two month risk). This was expected due to the fact that primary IBV infection produces a rather specific antibody response which is detected by the HI test (Brown and Bracewell, 1985; De Wit, 2000).
The use of two different antigens in the Hi test suggest that virus SIN6/YUC/MEX/96 is predominant in some communities of the state as indicated by a higher TIR in six of the eight communities included in the study. The higher TIR obtained with M41 antigens, observed in the remaining communities, could be due to the presence of this type of virus or more than one IBV type.
It was noticed that mortality of sentinel birds had an effect on TIR due to an inaccuracy in the formula for calculating the TIR, this is because dead birds are included in the calculation of animal time at risk although it is not possible to know if the animals were or not infected by IBV but still contribute to add some days to the total of days at risk for the population. This occurred in Rio Lagartos were the lowest incidence is reported but had the highest mortality which could have underestimated the incidence.
In respect to *M. gallisepticum* and *M. synoviae* infections the results indicate that for the former 10 of every 100 birds seroconverted and for the latter 5 of every 100 seroconverted in the two month period. The test used is highly sensitive but lacks in specificity which was minimized by trying to control some of the factor causing false positive reactions. However, it is possible that in three communities (Telchac, Oxholom and Subincancab), where few positive results were found and only in one household, these could be false positives. On the other hand we can be quite

confident that MG and MS are present in the other five communities included in the study, this being the first evidence of avian mycoplasmosis in the backyard system in Yucatan and as far as we know in Mexico.
For NDV, only one sample tested positive and with a low titre therefore the TIR was very low. The possibility of this positive serum of being a true positive of NDV was further investigated showing no signs of clinical illness and with no success in isolating NDV from this backyard. Taking in consideration the highly infectious character of this disease it would have not infected only one bird, supporting the hypothesis that this was a false positive.
This is the first report of the velocity of diseases in the backyard system in the state of Yucatan and Mexico. This study confirmed that IBV is present and widely distributed and infects most chicken in the backyards in a short time. The presence and distribution of mycoplasmosis in the backyard of most rural communities suggests that the combination of these agents with IBV could be responsible for the respiratory disease syndrome observed in backyard chickens. NDV seems not to be a problem for poultry in the communities studied.
More studies are needed to determine the role of other infectious agents in this respiratory syndrome.

REFERENCES

Acosta-Casanova, M.I. (2004). Caracterización del sub-sistema de ganadería de traspatio en 33 comunidades rurales del estado de Yucatán. Tesis de licenciatura. Facultad de Medicina Veterinaria y Zootecnia. Universidad Autónoma de Yucatán. Mérida, México.

Alexander DJ; Allan WH; Biggs PM; Bracewell CD; Darbyshire JH; Dawson PS; Harris AH; Jordan FTW; Macpherson I; McFerran JB; Randal CJ; Swarbrick O & Wilding GP (1983). A Standard technique for haemagglutination inhibition tests for antibodies to avian infectious bronchitis virus. The Veterinary Record, 113:64.

Brown AJ & Bracewell CD. (1985). Application of the haemagglutination inhibition test to typing of infectious bronchitis virus. Veterinary Record, 116:47-48.

Council directive 92/66/EEC (1997). Control of Newcastle disease. Official Journal of the European Communities, No. L 260:1-20.

De Witt JJ: (2000). Detection of infectious bronchitis virus. Avian Pathology, 29:71-93.

Etcharren, L.A.; Rodríguez, L.M.; Ceniceros, M.A., (1992). Aislamiento de *Micoplasma synoviae* y *micoplasma gallisepticum* de aves comerciales en México, identificados mediante inmunofluorescencia directa. En: XVII convensión nacional de ANECA. Puerto Vallarta, Jalisco.12: 85-86

Franco, C.C.; Santamaría, B.F. (1994). El traspatio o solar, una experiencia en la zona henequenera de Yucatán. En taller sobre: “La producción de traspatio en comunidades rurales: Los papeles de las instituciones gubernamentales, ONGs y Universidades y la posible Colaboración Inter.-institucional”, 29 de Julio de 1994. Mérida, Yucatán, México. pp. 2-12.

Gutiérrez-Ruiz, E.J. (2004). A survey for infectious bronchitis and other respiratory viruses from backyard chickens from Yucatán, México. PhD. Thesis, Royal Veterinary College, London. Pp. 2-139.

Gutiérrez-Ruiz, E.J.; Gough, R.E.; Zapata-Villalobos, D.M. (1998). Caracterización antigénica de un virus de la bronquitis infecciosa, aislado en pollos de traspatio en Yucatán, México. Veterinaria México, 29:351-357.

Gutiérrez-Ruiz, E.J.; Ramírez-Cruz, G.T.; Cámara-Gamboa, E.I.; Alexander, D.; Gough, E. (2000). A serological survey for avian infectious disease virus antibodies in backyard (free-range) village chickens in Mexico. Tropical Animal Health and Production, 32:381-390.

Jones, R.C. (1997). Interacciones entre el virus de la bronquitis infecciosa y problemas respiratorios emergentes. En: VIII Curso de Actualización AVI-MEX. El 26 de julio de 1997. Ciudad de México. Laboratorio Avimex. Pp. 22-29.

Jordan, F.T.W. (1990). Poultry diseases. University Press, Cambridge., London. pp. 125, 129, 141.

Kleven S.H. (1995). Micoplasmosis. En: Calnek (1995). Enfermedades de las aves. Manual moderno. Segunda edición. México DF. Pp 195- 207, 224- 230.

Kleven S.H. (1998). Micoplasmosis. En: D.E Swayne, J.R. Glisson, M.W. Jackwood, J. E. Pearson, W.M. Reed. Editores. A laboratory Manual for the Isolation and Identification of avian pathogens. 4ª edición. Florida, EEUU. Pp 74- 80.

Kommers G. D.; King D.J.; Seal B.S. y Brown C.C. (2003). Pathogenesis of chicken passaged Newcastle Disease viruses isolated from chickens and wild birds. Avian Diseases. 47:319-329.

Honhold, N.; Ferraez, N.; Allaway, C.; Wassink, G.; Rivera, T.; Carter, W.; Gutiérrez, M. (1993). Health and productivity of traspatio animals in Sinanche, Yucatán. Universidad Autónoma de Yucatán. Facultad de Medicina Veterinaria y Zootécnia. Mérida, México. pp 1-34.

Martin, S.W.; Meek, A.H., Willieberg L. (1987). Veterinary Epidemiology: Principles and methods. Iowa State University Press, Ames, Iowa, USA. 22-47.

OIE (2003). OIE. En: http://www.oie.int/eng/info/en_infoan.htm

Pfitzer, S.; Verwoerd, D. J.; Gerdes, G.H.; Labuschajne, A. E- Erasmus, A.; Manuell, R.J.; Grund, C.L. (2000). ND and Avian Influenza A virus in wild waterfowl in South Africa. Avian Diseases. 44:655-660.

Rejón-Avila, M.; Dajer-Abimerhi, A.; Honhold, N.; Lara y Lara, J. (1991). Diagnóstico comparativo de los aspectos socioeconómicos, de manejo y salud animal de la ganadería de traspatio en dos localidades del estado de Yucatán; San Pedro Chimay y Tekik de Regil de los municipios de Mérida y Timucuy. Universidad Autónoma de Yucatán, Facultad de Medicina Veterinaria y Zootécnia. Mérida, México. pp 1-36.

Rivera-Ortega, J.T. (1997). Cuantificación de la mortalidad y evaluación de algunos factores de riesgo que afectan a las aves de traspatio en Sinanché, Yucatán. MSc. Thesis, Facultad de Medicina Veterinaria y Zootécnia. Universidad Autónoma de Yucatán. Mérida, Yucatán, México. pp 1-19.

Rodríguez, B.J.C.; Beran, G. (1988). Estudio descriptivo del sistema de procucción de aves y cerdos de traspatio en la comunidad de Dzununcán, Estado de Yucatán. Facultad de Medicina Veterinaria y Zootécnia. Universidad Autónoma de Yucatán. p 15.

Rodríguez, J.; Allaway, C.; Wassink, G.; Segura, J.; Rivera, T. (1996). Estudio de la avicultura de traspatio en el municipio de Dzununcán, Yucatán. Veterinaria México 27(3):215.

Rosales, G. (1996). Algunas consideraciones sobre la influenza aviar. Memorias de la XXI Convención Anual ANECA. Cancún, México. pp. 4-11.

Schelling, E. Thür, B.; Griot, C.; Audigë, L (1999). Epidemiological study of Newcastle disease in backyard poultry and wild bird populations in Switzerland. Avian Pathology 28:263-272.

Secretaria de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (2006). SAGARPA. Campaña nacional contra la enfermedad de Newcastle presentación velogénica. Situación actual en México. Lunes 3 de abril, 2006. En: http://www.tml.sagarpa.gob.mx/ganaderia/campa%F1as/newcastle.htm

SENASICA (2004a). SENASICA. Situación actual de la influenza aviar en México. En: http://web2.senasica.sagarpa.gob.mx/xportal/dgsa/czoo/Doc463/

Takakuwa H. Ito, T. Tadaka, A. Okazi, K. Kida, H. (1998). Potentially virulentNewcastle disease viruses are maintained in migratory waterfowl populations. Journal Veterinary Research. 45:207-215.

Terregino C.; Cattoli G.; Crossele B.; Bertoli E. y Capua I. (2003). Caracterization of Newcastle disease virus isolates obtained from Euroasian collored doves (*Streptopelia de caocto*) in Italy. Avian Pathology. 32:63-68.

Table 1. True incidence rate (Two-month at risk interval) for Infectious Bronchitis, Newcastle disease virus, *M. synoviae* and *M. gallisepticum* in sentinel chickens from the backyard of 8 rural communities from the state of Yucatan, México.

community	BI-M41	BI-SIN6	BI-M41 + BI-SIN6	NDV	*Mg*	*Ms*
Dzilam de Bravo	0.63	0.54	0.77	0	0.09	0.060
Telchac Puerto	0.45	0.64	0.69	0	0.00	0.040
Celestun	0.53	0.60	0.60	0.016	0.265	0.089
Sisal	0.55	0.63	0.64	0	0.102	0.047
Oxholom	0.60	0.60	0.63	0	0.055	0.018
Subincancab	0.53	0.57	0.65	0	0.035	0.00
Chablekal	0.75	0.67	0.85	0	0.153	0.055
Rio Lagartos	0.32	0.38	0.44	0	0.128	0.067
Total (8 communities)	0.54	0.57	0.65	0.002	0.097	0.046

PATHOGENICITY AND VIRULENCE OF MEXICAN INFECTIOUS BRONCHITIS VIRUS, SIN6/YUC/MEX/96, FOR COMMERCIAL TYPE CHICKENS.

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SUMMARY

The control of Infectious Bronchitis virus (IBV) is sometimes complicated due to the emergence of variant viruses to which existing vaccines are ineffective.
A variant virus (SIN6/YUC/MEX/96) from commercial broilers as well as from backyard chickens from Yucatan, Mexico was isolated and characterized in 1996 (Gutiérrez-Ruiz *et a*l., 1998).
The objective of this study was to determine if IBV SIN6/YUC/MEX/96 was pathogenic for commercial chickens and if so what was its virulence for three-week-old layers (experiment 1) and for one-day-old broilers (experiment 2).
Two experiments were carried out, on each of them four groups, of ten birds each, were inoculated with one of the following treatments: 1) IBV SIN6/YUC/MEX/96, 2) *Escherichia coli (E. coli)*, 3) IBV SIN6/YUC/MEX/96 and *E. coli* and 4) sterile distilled water.
In the pathogenicity determination, signs and lesions characteristic of IBV were observed in both experiments in treatments one and three with a more prolonged and more severe presentation in the latter. No signs were observed for treatments two and four. The virulence index for broilers was 1 and for layers was 2, indicating that the virus was of low and medium virulence respectively.

INTRODUCTION

The infectious bronchitis virus (IBV) can be an important cause of losses to poultry production, increasing mortality and reducing growth rate and the quality and quantity of eggs laid.
The fact that neither the avian influenza virus nor Newcastle disease virus are present in the state of Yucatan, Mexico (Estudillo and Retana, 1998) makes the presence of IBV a relevant finding and probably one of the main causes of respiratory disease reported in chickens in Yucatan.
In the state of Yucatan, Mexico, a variant virus different to reference strains was

isolated in 1996 and subsequently characterized (Gutiérrez-Ruiz, 1998). This was the first report of a variant IBV in México.
The SIN6/YUC/MEX/96 virus was isolated from commercial broilers as well as from backyard creole chickens both presenting respiratory signs like sneezing, tracheal rales, malaise and increased mortality (Gutiérrez-Ruiz *et al*., 1998).
In a serological survey using SIN6/YUC/MEX/96 a prevalence of 75% was obtained compared with 56% with the M41 antigen using the same sera in the haemagglutination inhibition test, indicating that probably the variant virus is widely distributed in the chicken population of Yucatán (Gutiérrez-Ruiz *et al*., 2000, 2002).
Some IBV variants do not show a cross-protective response to type3s included in vaccine preparations (Collison *et al*., 1996).
Before performing cross-protection studies and determining if existing vaccines can protect against variant virus it is important to determine if these viruses are pathogenic for chickens and in that case how virulent they are.
The use of a model to classify the virulence of field isolates of IBV combining IBV and E. coli has proved useful because the virus alone usually causes mild disease (Nakamura *et al.*, 1992; Avellaneda *et al*., 1994).

MATERIALS AND METHODS

Two experiments were carried out, each experiment included four treatments, for each treatment 10 birds were used.
In treatment one, chickens were inoculated with 0.1 ml of allantoic fluid containing $10^{5.2}$ ID_{50}/ml of IBV SIN6/YUC/MEX/96. Treatment two comprised the intranasal inoculation of Phisiologic solution containing 2 X 109 colony forming units of a field isolate of *E. coli.* In treatment three chickens were inoculated with a both SIN6/YUC/MEC/96 and *E. coli* as described previously. Treatment four was the intranasal inoculation of 0.1 ml sterile distilled water.
Experiment one was carried out using three-week-old commercial type layers from the Isa-Babcock line, obtained from the flock of the Faculty of Veterinary Medicine, Universidad Autonoma de Yucatán. The hens of this flock were maintained free of antibodies to Infectious Bronchitis viruses. The experimental chickens were raised up to three weeks of age in an isolated room.
For experiment two, 40 one-day-old broilers chicks from the Ross line were obtained from a commercial incubator. These chicks were negative to antibodies against SIN6/YUC/MEX/96 and had low levels of antibodies to M41.
Chickens for experiment one (layers) were placed in 4 wire cages and taken to one of 4 different buildings not used for chicken accommodation before. At the entrance of each building a foot bath was available as well as protective footware and clothing. The same procedure and accommodation was used for experiment 2 with a difference of 30 days and after the building was cleaned and disinfected. Additionally birds from experiment 2 were provided with a source of heat in the form of incandescent light bulbs.
IBV SIN6/YUC/MEX/96 was propagated and the average infectious dose (ID_{50}) determined using SPF eggs from IASA™ Mexico. The ID_{50} was calculated using the method by Reed and Muench, (1938).
The *E. coli* strain used in both experiments was obtained from the bacteriology department of the FMVZ-UADY. The bacteria was isolated from an outbreak of

respiratory disease but was not characterized. The dose challenge was the same useded by Smith *et al*., (1985).
In order to determine the serological status of the birds used in both experiments the HI test using M41 and SIN6/YUC/MEX/96 antigens was carried out prior the challenge of the birds and on days 3, 7, 12, 17 and 21 post-inoculation.
Pathogenicity was determined using clinical and pathological observation. For the identification of clinical signs the birds were observed every day for 21 days.
On each experiment two birds were humanly sacrificed on days 3, 7, 12, 17 and 21 post-inoculation, immediately after the sacrifice the trachea, lungs, air sacs and intestine were examined. Samples from trachea, kidneys and intestine were collected and placed in buffered formaline. The method by Lesson and Lesson (1990) was used by the histopathology.
For the determination of the virulence a modification of the method described by Avellaneda *et al* (1994) was used. In the present study only clinical signs and mortality were considered.
Birds were examined daily for ten days. The virulence index is the average value per bird per day in ten days. Birds were described as: 1) Light disease: Birds showing only sneezing and coughing (low virulence); 2) Moderate disease: Birds showing sneezing, coughing, tracheal rales, ruffled feathers and malaise (moderate virulence); 3) Severe disease: Beside the previous signs also death (high virulence).

RESULTS

Serology

In experiment one (layers) all birds had negative results in the HI test with both antigens.
Birds inoculated with IBV or IBV and *E. coli* showed positive titres by day 12 post-inoculation. Birds inoculated with *E. coli* or distilled water remained seronegative throughout the experiment.
In the second experiment (broilers), all birds were positive to antibodies against M41 (log_2 6-7) becoming negative by day 17 post-inoculation. In comparison all birds were negaive to antibodies against SIN6/YUC/MEX/96 on reception.
Birds inoculated with IBV or IBV and *E. coli* showed positive titres by day 12 post-inoculation. Birds inoculated with *E. coli* or distilled water remained seronegative throughout the experiment.

Pathogenicity

Clinical Signs

In experiment one birds inoculated with IBV presented sneezing from day 3 to 16 no mortality was observed.
Chickens inoculated with IBV and *E. coli* showed sneezing on day 9 and tracheal rales the next day. By day 11 they did not have any signs of disease.
Birds inoculated with E. coli had no signs of respiratory disease presenting only mild diarrhea from day five to ten.
Chickens inoculated with distilled water did not show any sign of disease throughout the experiment.

Chickens in experiment two (broilers) and treatment one (IBV) presented sneezing on day one post-inoculation, increasing on intensity until day five, when tracheal rales were also present, signs did not disappear throughout the experiment.
Chickens inoculated with IBV and *E. coli* presented strong sneezing, malaise, breathing difficulty and tracheal rales by day four post-inoculation lasting to the end of the experiment. On day three yellow and soft faeces were observed.
Chickens inoculated only with *E. coli* did not present respiratory signs of disease but had yellow and soft faeces at day three.
Inoculation of distilled water did not cause any sign of disease.
Mortality was not observed on any treatment in both experiments.

Gross Pathology
Main findings for commercial type layers inoculated with IBV were air sack thickening, haemorrages and presence of exudates in the trachea, enlarged and haemorrhagic thymus and pale kidneys.
The same lesions were observed on birds inoculated with IBV and *E. coli* with the addition of pale lungs and the presence of haemorrhagic exudates and liquid contents in the gut.
Chickens only inoculated only with *E. coli* had the same finding in the intestine.
The birds inoculated with distilled water did not present any apparent lesion.
In the broilers the main gross pathological changes were on birds inoculated with IBV was the enlargement or the thymus, thickening of air sacs, haemorrhagic lungs, and exudates with IBV and *E. coli* but lesions were observed earlier in birds inoculated only with *E. coli* there were haemorrhages and presence of liquid contents in the intestine.
Chicks inoculated with distilled water did not present any lesion.

Histopathology
Trachea.- In commercial layers inoculated with IBV the hyperplasia of the caliciform cells in the epithelium and moderate submucosal oedema was observed. In birds inoculated with IBV and *E. coli* besides the lesions reported for treatment one, there was a moderate infiltration of lymphocytes.
In birds inoculated only with *E. coli* there was a lymphocyte infiltration, moderate oedema and hyperplasia of calciform cells.
Birds inoculated with distilled water presented a moderate oedema.
Intestine**.-** In birds inoculated with IBV there was an absence of epithelium, light lymphocyte inflilltration, severe oedema, hyperplasia of calciform cells and cystic dilatation of glands. The same lesions were observed for treatment 3 (IBV and *E. coli*).
In chicks inoculated with only *E. coli* there was a moderate lymphocyte infiltration and light oedema.
In the control chicks (inoculated with distilled water) there was only the presence of moderate oedema.
Kidneys.- Birds in treatment one (IBV) presented congestion, multifocal intersticial lymphocitic infiltration, nephrosclerosis and cystic dilatation.
Chicks inoculated with IBV and *E. coli* presented multifocal intersticial nephritis and congestion.
On birds in treatment 2 (*E. coli*) and 4 (distilled water) there was only congestion.

In experiment 2 (broilers) the following was observed:

Trachea.- In birds inoculated with IBV there was a severe focal absence of cilia, epithelial hyperplasia of caliciform cells and moderate oedema. In chicks inoculated with IBV and *E. coli* the same lesions were observed with the addition of a discrete lymphocyte infiltration which was also observed in birds inoculated only with *E. coli.*
Control chicks (inoculated with distilled water) only presented moderate oedema.
Intestine.- IBV inoculated chicks presented discrete lymphocitic infiltration, hyperplasia of caliciform cells and oedema. The IBV and *E. coli* group had also mucosal associated lymphoid hyperplasia.
Birds inoculated only with *E. coli* presented oedema and discrete lymphocytic infiltration.
Kidney.- There was moderate multifocal intersticial nephritis in chicks inoculated with IBV. In birds inoculated with IBV and *E. coli*, there was focal nephritis followed by moderate multifocal intesticial nephritis.
Birds in treatment 2 and 4 only presented moderate congestion.

Virulence
Experiment 1 (layers): In treatments 2 (*E. coli*) and 4 (distilled water) no clinical signs were observed.
In treatments 1 (IBV) three birds presented clinical signs while four presented signs in the group inoculated with IBV and *E. coli*.
The virulence was determined to be 1 (low virulence)

Experiment 2 (broilers): In treatments 2 and 4 no clinical signs were observed.
In IBV inoculated chicks there was sneezing, coughing and subsecuently tracheal rales. The same observations were recorded for treatment 3 (IBV and *E. coli*) but earlier. The virulence index was reported as 2.

DISCUSSION

In the experiment 1, chickens did not have antibodies to IBV as they were produced from seronegative hens. The titres to the challenge virus increased by day 12 indicating that there was infection.
In Experiment 2, the chicks had antibodies to M41 most likely from maternal origin as they disappeared by the end of the experiment. These chicks had no previous contact with IBV SIN6/YUC/MEX/96 as shown by the lack of antibodies against this virus at the beginning followed by seroconversion of the challenged groups.
The clinical signs observed in both experiments are characteristic of IBV infections (McMartin, 1993; Dhinakar and Jones, 1996; Gelb Jr, 1998) being more severe for one-day-old chicks.
The association of IBV with bacteria like *E. coli* usually produces more severe infection than the infection with IBV alone as observed in this study (Jordan, 1990; Butcher *et al*., 1998).
In both experiments chickens inoculated with IBV or IBV and *E. coli* presented the same macroscopic lesions, the casseous material in the trachea and haemorrrhages in the lungs observed in this study are lesions associated with IBV (Butcher *et al* 1998, Capua *et al*, 1994; Dhinaker and Jones, 1996).

Birds inoculated with IBV had pale but not enlarged kidneys as reported for IBV infections due to the urate accumulation (Butcher *et al*., 1998).
In chickens of both experiments inoculated with IBV the thymus was enlarged and haemorrhagic, this finding could not be found on the revised literature, but the thymus is a gland whose function is to develop cell mediate immune responses and has been reported to increase its size during infectious diseases (Gurtler *et al*., 1976; Tizard, 1998).
The main histopathological changes observed in the chickens, of both experiments, inoculated with IBV were hyperplasia of the calciform cells of the epithelium, severe focal absence of cilia in agreement with previous reports (Dutta, 1975, MacDonald and McMartin, 1976).
Birds inoculated with IBV and E. coli presented, as well as the lesions reported for IBV alone, a discrete lymphocitic infiltration in agreement with Ambali and Jones (1990) who stated that the regeneration process starts from 6 to 10 days after infection, with tissues coming back to normality after two to three weeks remaining only a discrete lymphocitic infiltration.
It has been reported that IBV replicates in the digestive tract and occasionally causes enteritis (Ambali and Jones, 1990). Lesions observed in both experiments were more severe for treatment 3 than for treatment one due to the complication caused by the bacteria.
SIN6/YUC/MEX/96 also affected the kidneys in both experiments it was more aggressive in combination with *E. coli* than alone as reported for other IBV (Smith *et al*, 1985, Chen *et al*, 1996).
SIN6/YUC/MEX/96 showed low virulence for three-week-old Isa Babcock layers and medium virulence for one-day-old Ross broilers. The virus in association with *E. coli* produced more severe signs and lesions in both chicken lines. The virus also had predilection for the respiratory tract and affects the kidneys mildly. Under field conditions the outcome of infection could be different due to the occurrence of many factors like concurring agents, stress and wrong management practices (Marquez *et al*, 1997).

REFERENCES

Ambali A.G., Jones R.C. (1990). Early pathogenesis in chicks of infection with an enterotropic strain of infectious bronchitis virus. Avian Diseases, 34:809-817.

Avellaneda G.E., Villegas P., Jackwood M.W., King D.J. (1994). *In vivo* pathogenicity of field isolates of infectious bronchitis virus. Avian Diseases, 38:389-397.

Butcher G.D., Miles R.D. (1998). Vaccine failure in poultry: Factors to consider. Available at http://edis.ifas.ufl.edu

Capua I., Gough R.E., Manzini M., Casaccia C., Weiss C. (1994). A “novel” infectious bronchitis strain infecting broiler chickens in Italy. Journal of Veterinary Medicine, 41:83-89.

Chen B.Y., Hosi S., Nunoya T., Itakura C. (1996). Histopathology and immunohistochemestry of renal lesions due to infectious bronchitis virus in chicks. Avian Pathology, 25:269-283.

Collison E., Xu Y., Junker D., Hock L., Wang L. (1996). La evolución del virus de la bronquitis infecciosa depende del intercambio de material genetico entre virus existents. Proceedings XXI Convencion anual de la Asociación Nacional de Especialistas en Ciencias Avícolas. May 1-5 1996, Can Cun, Quintana Roo, Mexico, 64-65.

Dhinakar Raj G., Jones R.C. (1996). Immunopathogenesis of infection in SPF chicks and commercial broiler chickens of a variant infectious bronchitis virus of economic importance. Avian Pathology, 25:481-501.

Dutta S.K. (1975). Morphological changes of chicken tracheas and tracheal organ cultures infected with avian infectious bronchitis virus studied in scanning electron microscopy. Avian Diseases, 19:429-436.

Estudillo L.J., Retana R.A. (1998). Influenza aviar en Mexico, interrelacion aves silvestres-virus de influenza; realidades y mitos. Proceedings XXIII Convencion anual de la Asociación Nacional de Especialistas en Ciencias Avícolas. May 6-9 1998, Puerto Vallarta, Jalisco, Mexico, 43-51.

Gelb Jr. J. (1989). Infectious Bronchitis. In: A laboratory manual for the isolation and identification of avian pathogens. American Association of Avian Pathologists. (Ed. H. Graham Purchase; Lawrence H. Arp; Charles H. Domermuth & James E. Pearson). Kendall/Hunt Publishing Company, Pennsylvania. 124-127.

Gurtler H., Ketz H.A., Schroder L., Seidel H. (1976). Fisiologia de los liquidos corporales. Fisiologia Veterinaria 2nd. Ed. Acribia, Spain, 475.

Gutierrez-Ruiz E, Gough R, Zapata-Villalobos D. (1998). Caracterización antigénica de un virus de la bronquitis infecciosa, aislado en pollos de traspatio en Yucatán, México. Veterinaria México 29 (4):351-358.

Gutierrez-Ruiz E, Ramirez-Cruz G, Câmara-Gamboa E, Alexander D, Gough R. (2000). A serological survey for avian infectious bronchitis virus and Newcastle disease virus antibodies in back yard (free range) village chickens in Mexico. Tropical Animal Health and Production, 32:381-390.

Gutierrez-Ruiz E, Ramirez-Cruz G, Câmara-Gamboa E. (2002). Estudio serológico de la bronquitis infecciosa con el virus SIN6/YUC/MEX/96 en aves de traspatio en 30 comunidades del estado de Yucatán, México. Veterinaria México, 33 (2):101-108.

Jordan F.T.W. (1990). Infectious Bronchitis. Poultry Diseases. Balliere-Tindall, Cambridge, UK. 159-166.

Lesson T.S., Lesson C.R. (1990). Atlas de Histologia. Interamericana-McGrawHill. Mexico. 255.

McDonald J.W., McMartin D.A. (1976). Observations on the effects of the H52 and H120 vaccine strains of infectious bronchitis virus in the domestic fowl. Avian Pathology, 5:157-173.

Marquez V.M.A., Lucio E.D., Rios C.J.F. (1997). Evaluacion de la sensibilidad in vitro de E. coli aisladas de casos clinicos durante septiembre de 1995 a febrero de 1997frente a diferentes quimioterapeuticos. . Proceedings XXII Convencion anual de la Asociación Nacional de Especialistas en Ciencias Avícolas. May 7-11, 1997, Ixtapa Zihuatanejo, Guerrero, Mexico, 127-140.

McMartin D.A.(1993). Infectious bronchitis. In: Virus infections of birds. (Ed. McFerran JB & McNulty MS). Elsevier, London UK, 249-275.

Nakamura K, Cook JK.A., Frazier J.A., Narita M. (1992). *Escherichia coli* multiplication and lesions in the respiratory tract of chickens inoculated with infectious bronchitis virus and/or *E. coli*. Avian Diseases, 36:881-890.

Reed L.J., Muench H. (1938). A simple method of estimating fifty per cent endpoints. The American Journal of Hygiene, 27:493-497.

Smith W.H., Cook J.K.A., Parsell Z.E. (1985). The experimental infection of chickens with mixtures of infectious bronchitis virus and *Escherichia coli*. Journal of General Virology, 66:777-786.

Tizard I.R. (1990). Organos del Sistema Inmunitario. Inmunología Veterinaria. McGrawHill-Interamericana, Mexico. 84-85.

GENETIC DIVERSITY ON S1 GLYCOPROTEIN OF AVIAN INFECTIOUS BRONCHITIS VIRUS STRAINS ISOLATED IN BRAZIL BETWEEN 1988-2000

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SUMMARY

Twelve Brazilian field isolates of infectious bronchitis virus (IBV); were recovered, between 1988 and 2000, from commercial broiler or layer flocks in three different States, which are located in South and Southeast Brazilian regions. Among them, there are flocks that had been vaccinated with live Mass viral strain (H120), along with others that had not. Two oligonucleotide sets were used after propagation in embryonated chicken eggs to amplify the S1 genes of the spike glycoprotein. The restriction maps generated by *Hae*III, *Xcm*I and *Bsty*I showed, respectively, 3, 4 and 1 different patterns from common pattern of strains classified as Mass serotype (H120/M41 strains). Further molecular analysis of IBV isolates, previously detected as different genotypes, revealed point mutations and a nucleotide insertion in hypervariables region one and two (HVR-1 and HVR-2) of S1 gene. Major amino acids changes in these six isolates involved insertion of two stretches (aa118-119) and (aa-141-145) located at N-terminal and C-terminal regions of HVR-2. The results led to conclude that different genetic variant strains of IBV are circulating in vaccinated and non-vaccinated flocks in Brazil.

INTRODUCTION

Infectious bronchitis virus (IBV) is the etiological agent of infectious bronchitis (IB), which is an acute and highly contagious disease of chickens, resulting in significant economic losses in commercial broiler, layer and breeder chickens. Viral damage to the respiratory tract is a dominant pathological find in this disease, but there are some strains of IBV able to replicate in the kidney and oviduct, causing nephritis and reduced egg production, respectively (Cavanagh & Naqi, 2003).
IBV is the prototype virus of the *Coronaviridae* Family (Cavanagh, 1997) and its genome consists of the single stranded positive-sense RNA, varying from 27 to 32 kb (Lai & Cavanagh, 1997). The virion has three major virus-encoded structural

proteins: the spike (S) glycoprotein, the membrane (M) glycoprotein and the nucleocapsid (N) protein. The S glycoprotein is processed post-translationally into N-terminal S1 and C-terminal S2 subunits (Kusters et al., 1987). S1 forms the bulb part of the peplomer and is responsible for attachment and virus-neutralizing antibody responses, while S-2 anchors S-1 to the viral envelope (Cavanagh, 1983).
IB has occurred frequently in vaccinated and non-vaccinated flocks, because there are multiple IBV variants and the disease is highly transmissible. Though the vaccination has been used, several IBV serotypes and variant strains have continuously been isolated worldwide (Gelb et al., 1991). Different variants of IBV are thought to be generated by amino acid changes resulting from nucleotide insertions, deletions or point mutations, specially in the S1 subunit, as the result of a lack of proofreading enzymes acting on the viral polymerase, as well as the high rate of replication of this virus. Another mechanism for variability in the S1 subunit may be RNA recombination (Cavanagh et al., 1992, Holland et al., 1992, Wang et al., 1993).

Brazil is currently one of the major poultry-producing countries in the world and in the last few years, IB has become a serious problem in this country (Di Fabio et al., 2000). The first isolation of IBV was recorded in Brazil in 1957 (Hipólito, 1957) and despite the official introduction of IBV vaccination in 1979, using the strains H52 and/or H120, the outbreaks have been ongoing (Ito, 2006). Apparently, the majority of Brazilian IBV field isolates recovered until 1989 were classified by cross-neutralization test as Mass serotype (Ito, 2006). Di Fabio et al. (2000) investigated by cross-neutralization tests in tracheal organ cultures a group of fifteen IBV field isolates collected from commercial flocks in Brazil, which had experienced a variety of IB-like conditions, including respiratory disease, digestive and kidney problems, and/or drops in egg production. One of the isolates was associated to Massachusetts (Mass) serotype and the fourteen remainder viruses were classified in at least four antigenic groups, all distinct from ones described previously in other countries.
More recently, the presence of a significant number of Brazilian IBV field isolates, differing in phylogenetic analysis from the genetic patterns for S1, N or non-structural protein code genes of American, European, Australian or even Asiatic strains, have been reported to be circulating in the commercial poultries (ABREU et al., 2006, BRENTANO et al., 2006, VILLARREAL et al., 2006[a/b]).
However, antigenic, genetic, pathological and epidemiological analysis of IBV isolates in Brazil has not been systematically performed. The genetically relationships between Brazilian isolates and foreign IBV strains have also remained barely known. Thus, there is particular interest to know whether the current IBV isolates in Brazil were newly introduced from other countries or whether they arose by mutation or recombination of circulating local IBV strains, including the vaccine strain.
The objective of the present study was to characterize genetically a fragment encompassing the 5'-part of the S1 glycoprotein gene of twelve IBV strains isolated from Southern and South-eastern in Brazil, between 1988 to 2000.

MATERIALS AND METHODS

Viruses
The IBV isolates used in this study are listed in Table 1. Most of the IBV isolates were obtained from regional Laboratories in Brazil and were isolated after two to three passages using embryonated specific pathogen-free (SPF) eggs.

RNA Extraction
Isolates of IBV were inoculated into the allantoic cavities of 10-day-old chicken embryos and incubated for 40 h at 37ºC. The eggs were kept at 4ºC for 4-6 h before the allantoic fluid was harvested. Viral RNA was extracted using TRIZOL (Invitrogen, Carlsbad, CA) and following the instructions of manufacturer.

RT-PCR
A reverse-transcriptase (RT) reaction was carried out with SuperScript II (Invitrogen, Carlsbad, CA) using random hexamers. Two sets of primers were used for the amplification of S1 gene. Primers S1OLIGO-5' and S1OLIGO-3' were used to amplify the whole S1 coding sequence of IBV using the cycle conditions described by Kwon et al. (1993). Primers SYU+ and SYU- were used to amplify the 5'-part of S1 gene following the cycle conditions reported by Yu et al. (2001).

RFLP
The products with the expected size (approximately 1720 kb) obtained from PCR using the S1OLIGO-5' and S1OLIGO-3' primers were excised from an agarose gel and purified using GFX kit (Amersham Biosciences). The restrictions enzymes *Hae*III, *BstY*I and *Xcm*I (Biolabs, New England) were used to digest the PCR products of S1 gene as described Kwon et al. (1993). The RFLP patterns were observed after electrophoresis on 2% agarose gel at a constant voltage of 100V.

DNA Sequencing
The products with the expected size (approximately 452 pb) obtained from PCR with the SYU+ and SYU- primers were purified by GFX kit (Amersham Biosciences) and used as a template for sequencing from both directions on Applied Biosystems 373 automated DNA sequencer, using dye terminator cycle sequencing chemistry (Perkin-Elmer/Applied Biosystems, Foster City, CA, USA).
Sequecing data were edited using BIOEDIT software (Hall, 1999). A consensus sequence was obtained for each isolate using data from both sequencing directions. Nucleotide sequences were analyzed through GenBank searches and submitted to multiple alignment, using Clustal W version 1.7 (Thompson et al., 1997) and the trees were constructed by neighbour-joining method in MEGA 3.1 version (Kumar et al., 2004). The matrices of identity and similarity between amino acid sequences were determined by MatGAT 2.0 version software (Campanella et al., 2003). The reference S1-gene sequence data from GenBank used in these analysis were identified by the following characteristics and accession numbers: Holland (H120) (M21970), Massachusetts 41 (Mass 41) (X04722), Connecticut (Conn) (L18990), Ark DPI (AF006624), Florida 18288 (AF0275120, Chinese isolate Jilin (AY839144), Swedish isolate 1096/97 (AF420330) and four Brazilian field isolates (DQ355995; DQ448277; DQ448275 and DQ448276).

RESULTS

Reverse transcriptase and polymerase chain reaction in conjunction with RFLP analysis were initially performed to genotype the Brazilian field IBV isolates and to compare them with the digestion patterns of the S1 gene amplified from H120 vaccine strain of IBV. The digestion of the S1 gene PCR product (1.7 kb) with the restriction enzymes *HaeIII* generated four different RFLP patterns for these twelve viruses. A RFLP profile corresponding to the digestion of S1 gene of H120 strain (Mass genotype) with this enzyme was observed for isolates IBVPR03, IBVSP01, IBVPR07, IBVSC01 and IBVSC03. Two other isolates (IBVPR04 and IBVSP02) showed identical RFLP patterns to one another (Variant A), but they were distinct from the Mass pattern, as well as from other two patterns observed for the isolates IBVPR01, IBVPR02 (Variant B), or for the isolates IBVPR05 and IBVPR06 (Variant C). Finally, only one RFLP pattern generated by the enzyme *XcmI* was able to distinguish the isolate IBVSC02 (Variant D) from that recorded for the H120 vaccine strain of IBV and from the other field isolates analyzed here. The enzyme *BstyI*, confirmed the distinction of the isolates IBVPR04 and IBVSP02, because it generated a singular RFLP pattern, distinguishing these viruses from Mass genotype as well from those patterns recorded for the others Brazilian IBV isolates (Fig. 1).

The nucleotide sequences of 399 bp each of 5'-part of S1 gene, including the middle of HVR-1 and the HVR-2 in the S1 subunit gene, which were obtained from direct sequencing of twelve Brazilian IBV field isolates, were aligned and firstly analyzed in comparison to the H120 vaccine strain genetic data. S1 phylogenetic analysis using, either the nucleotide or the partial amino acid deduced sequences of these isolates, supported mostly of the RFLP findings (Fig.1). The identity values for S1 sequence of the isolates classified by RFLP as Mass genotype along with the H120 strain, were high and ranged from 96.4 to 100%, generating an individualized cluster of these viruses (clade I). Isolate IBVSC02, which had a unique *XcmI*-RFLP profile, displayed a less intense relationship with H120 strain (88.4% of identity) and represented a distinct genotype, which was classified into a separate branch (clade II). The other six field isolates, identified by *HaeIII*-RFLP as three different genotypes, were less related to Mass genotype, exhibiting lower identity values to H120 strain, which ranged from 64.6 to 67.6%. Close relationships were distinguished for each one of three groups containing two of these isolates each, leading to separate them by phylogenetic analysis into clade III, clade IV and clade V. The first clade contained the isolates IBVPR04 and IBVSP02, which displayed a high identity between their amino acids sequences (97%), while clade IV clustered the isolates IBVPR01, IBVPR02, and revealed also a high identity degree (99.2%) and clade V grouped IBVPR05 and IBVPR06 isolates characterized by a sequence identity of 92.4%. Each pair of viruses differed from other by the range of 13 to 33.3%.

The isolates classified into the clades III, IV and V had another characteristic in common, that is major changes at nucleotide level, which resulted in the insertions of two short amino acids stretches, corresponding to the residues 119-120 and to the residues 141-145. Many point mutations were also detected along this 5'-part of S1 gene from these viruses. Additionally, two of these isolates (IBVPR01 and IBVPR02), belonging to clade IV, showed a deletion of three amino acids between the residues 56 to 60, which was similar to the deletion observed for the isolate IBVSC02, except

it encompassed four residues and it was localized between the amino acids 57 to 62 (Fig. 2).
A similar topology of branches were obtained, when another phylogenetic tree (Fig.) was constructed for grouping the amino acid sequences of the N-terminus of S1 gene from the twelve Brazilian IBV isolates along with the amino acid sequences derived from eleven other IBV strains or isolates which were searched in GenBank, which were selected baed on their higher degrees of identity with these Brazilian field viruses. The isolates IBVPR03, IBVSP01, IBVPR07, IBVSC01 and IBVSC03 confirmed their high relatedness (91.2 to 100% of identity) to the Mass reference trains (H120 and M41), and to the Swedish isolate 1096/97 (Farsang et al. 2002), defining the clade I. The isolate IBVSC02, which was previously identified as a distinct genotype, composed with the strains Flor-18288 and Connecticut the clade II, and their identity values with these reference strains ranged from 93.4 to 99.2%. The isolates IBVPR04 and IBVSP02 were close related one to another, as demonstrated the first dendogram and in this second phylogenetic analysis, they were localized in the same branch of IBV strains ARK DPI and Jilin, constituting the clade III. The former clades IV and V observed in the first phylogentic tree were more defined in two separated clusters (clades IV and V), when four other amino acid sequences encompassing the first half of S1 glycoprotein from IBV variants isolated between 2004 and 2005 in Brazil from chickens with enteric diseases or reproductive problems (Villarreal et al., 2006), were added to this phylogenetic analysis. The clade IV was formed by only two isolates (IBVPR01 and IBVPR02), while the clade V group or sub-grouped the isolates Br-USP-01, Br-USP-12, IBVPR06, Br-USP-10, Br-USP-03 and IBVPR06 (Fig. 3).

DISCUSSION

RFLP analysis and S1 partial sequencing were used to determine the relationship of twelve Brazilian field isolates of infectious bronchitis virus (IBV), which were recovered, between 1988 and 2000, from commercial broiler or layer flocks located in South and Southeast regions of Brazil. The criteria used by Gelb et al. (2005) to cluster US and Israeli IBV isolates in categories was adopted in this study. Thus, there were isolates highly related to the vaccine strain used in Brazil (clade I / Mass / H120 strain); isolates closer related to classical reference strains, such as Connecticut (clade II), or ARK DPI (clade III), or isolates related to, yet distinct from vaccine or reference strain, or even isolates behaving as variants, because they were unrelated to vaccine or classical reference strains, but showed a high degree of identity to new isolates from this country of which the nucleotide sequences of the first half of S1-gene were very recently deposited in GenBank (clades IV and V).
Five of twelve isolates in this study (IBVPR03, IBVSC01, IBVSC03, IBVSP01 and IBVPR07) were highly related to H120 vaccine strain, since their identity values with this reference strain ranged from 94.4 to 100%. IBVPR03, IBVSC01 and IBVSP01 were isolated from birds with renal problems, whereas the isolates IBVSC03 and IBVPR07 were recovered from chickens with respiratory lesions. All the flocks where these viruses were isolated were vaccinating their birds against IB. H120 vaccine strain may have persisted in the birds and could be accidentally recovered from the flock, but it was not the cause of the renal or respiratory diseases. An investigation is recommended here to define the effective role of these viruses in the IB

pathogenesis. Similar results were reported by Farsang et al. (2002) and Gelb et al. (2005) on the characterization of field IBV isolates from Swedish and from US or Israel, respectively.

Isolate IBVSC02 exhibited a very close relationship with Connecticut strain (99.2%) and a lower identity with Florida-18288 (93.4%). There is no consistent explanation for this intriguing result. This virus was isolated in 1999 from a flock located in Santa Catarina State that was experiencing respiratory problems and had been administering a H120 vaccination to the birds. In spite of this, a similar finding was reported recently by Alvarado et al. (2005) in an investigation, where two Colombian field isolates were identified by phylogenetic analysis of 5'-part of S1 gene as close related to Connecticut strain, leading them to speculated that the possible reason for the presence of these viruses could be associated to the circulation of a Connecticut vaccine strain, which was once available and authorized to be used in that country. However, this hypothesis does not seem to be applicable to our situation, because only H52 and H120 IBV strains were officially licensed for using in Brazil, since the beginning of IBV vaccination in our country.

Another unexpected and interesting relationship was detected for the isolates IBVPR04 and IBVSP02, that besides displaying a high identity one to another (97%), they were highly (97%) or totally (100%) related to Chinese isolate Jilin and to the reference strain ARK DPI, respectively. As stated before and considering that vaccines which are formulated with ARK IBV strains are not authorized in Brazil, these field isolates may have derived from a naturally occurring virus or may have introduced from a foreign country.

The isolates IBVPR01, IBVPR02, IBVPR05 and IBVPR06 seem to be indigenous, because they demonstrated a low genetic relation with the majority of the reference strains obtained from GenBank and representing IBV isolates from North America, Europe and Asia, but they were moderately to highly related one to another (86.4 to 99.2% of identity) and exhibited an intermediate to high relationship with the new Brazilian isolates of which the nucleotide sequences were recently deposited. It is noteworthy that the viruses isolated earlier, in 1988, were placed, after the phylogenetic analysis, in a branch more distant from those branches clustering the viruses, which were isolated more recently in the years of 2000 (IBVPR05 and IBVPR06) or 2004 and 2005 (Br-USP-01, Br-USP-12, IBVPR06, Br-USP-10, Br-USP-03 and IBVPR06). Additionally, the four isolates analyzed in this study derived from birds affected by respiratory disease, whereas the other Brazilian viruses were recovered from chickens with enteric (Br-USP-12, IBVPR06, Br-USP-10, Br-USP-03 and IBVPR06) or male fertility (Br-USP-01) problems.

The main differences observed in the deduced amino acid sequences of N-terminus of S1 glycoprotein were distributed along with this segment and concentrated between the residues 52 to 96 and between the residues 117 to 147. These regions were similar to HVR I (residues 56-69) and HVR II (residues 117-131) of Mass and European strains and they were also nearly coincident with the antigenic sites I (residues 38-67) and II (residues 97-141), which were associated with two separate virus-neutralizing and conformational-dependent epitopes (Kant et al., 1992). In addition, three regions of insertions and deletions were identified between the residues 57-62, 118-121 and 140-146 of S1 protein, respectively in the HVR I and HVR II regions.

According to known concept that many different antigenic variants and serotypes of IBV exist worldwide, the detection of IBV variants in this study it is not unexpected. Thus, the presence of other antigenic types of IBV in Brazil was reported before, including the occurrence of an Arkansas-like IBV in 1980's (Branden et al, 1986). Moreover, the identification of at least four antigenic groups among fourteen viruses isolated during 1995 in this country; all distinct from ones found previously in other countries, was also described, though there was no of these isolates that could be associated with Arkansas or Connecticut serotypes. The Mass serotype of IBV was first reported in Brazil in 1950's (Hipólito, 1957), but this virus is evolving continuously and it can be speculated that after the introduction of vaccination with H52 and H120 at the end of 1970's, the Brazilian IB viruses experienced a steady and higher increase in the variability of its most important genes; particularly that coding for S1 glycoprotein.

In conclusion, the main results of this study, though restricted in the number of virus samples and lacking an appropriate epidemiological design, pointed out the presence of significant genetic variability among IBV field isolates in Brazil and served as a partial retrospective analysis about the IBV evolving process, since isolates from 1980's, 1990' and 2000 were investigate herein.

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REFERENCES

Alvarado, R., Villegas, P., Mossos, W., Jackwood, M.W. Molecular characterization of avian infectious bronchitis vírus strains isolated in Colômbia during 2003. Avian Dis., 45, 201-209, 2005.

Abreu, J.T., Resende, J.S., Flatschart, A.V.F., Mendes,A.C.R., Martins, N.R.S., Silva, C.B.A., Ferreira, M.C., Machado, A.L.C. Genotipificação de isolados do vírus da bronquite infecciosa das galinhas (VBIG) pelo sequenciamento direto de parte de S2 e do segmento S1. Revista Brasileira de Ciência Avícola, 2006, Suplemento 8, p.210.

Branden, R.C., Silva, E.N. Ocurrencia de nuevos serotipos de bronquite infecciosa em Brasil. In: Proc. VI Seminário International de Patologia Aviar. Athens, Geórgia. P. Villegas, ed., 1986.

Brentano, L., Esteves, P.A., Trevisol, I.M., Hayashi, M.M., Luciano, R.L., Castro, A.G.M., Klein, T.A.P., Molinari, M. Sequenciamento do gene S1 de vírus da bronquite infecciosa de surtos da doença associada a lesões de miopatia peitoral. Revista Brasileira de Ciência Avícola, 2006, Suplemento 8, p.241.

Campanella, J.J., Bitincka, L., Smaley, J. MatGAT: an application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics, v.4., p.29, 2003.

Cavanagh, D. 1983. Coronavirus IBV: further evidence that the surface projections are associated with two glicopolypeptides. J. Gen. Virol. **64**, 1787-1791.

Cavanagh, D., Davis, P.J., Cook, J., Infectious bronchitis virus: evidence for recombination within the Massachussets serotype. Avian Path., 21, 401-408, 1992.

Cavanagh D, Mawditt K, Shaw K, Britton P, Naylor C. Towards the routine application of nucleic acid technology for avian disease diagnosis. Acta Veterinaria Hungarica 1997; 45: 281-298.

Cavanagh D, Naqi S. Infectious Bronchitis. In: Calnek BW, Barnes HJ, Beard CW. Diseases of poultry; 2003. 11 ed. Ames: Iowa State University Press; p 101-119.

Di Fabio, J., Rossini, L.I., Orbell, S.J., Paul, G., Huggins, M.B., Malo, A., silva, B.G.M, Cook, J.K.A. Characterization of infectious bronchitis viruses isolated from outbreaks of disease in commercial flocks in Brazil. Avian Dis., 44, 582-589, 2000.

Farsang, A., Ros, C., Renströn, L.H.M., Baule, C., Soós, T., Belák, S. Molecular epizootiology of infectious bronchitis vírus in Sweden indicating the involvement of a vaccine strain. Avian Path., 31, 194-203, 2002.

Gelb Jr J, Wolff JB, Moran CA. Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Diseases 1991; 35: 82-87.

Gelb, J. Jr., Weisman, Y., Ladman, B.S., Meir, R. S1 gene characterisitics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Path., 34, 194-203, 2005.

Hall, T.A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser., v.41, p.95-98, 1999.

Hipólito, O. Isolamento e identificação do vírus da bronquite infecciosa das galinhas no Brasil. Arq. Esc. Vet. Univ. Minas Gerais, 10, 131-151, 1957.

Holland, J., Spindler, K.,Horodyski, F., Grabau, E., Nichol, S., Van Pol, S. Rapid evolutionof RNA genomes. Science, 215, 1577-1580, 1982.

Ito, N.K., Miyaji, C., Okabayashi, S. Controle de variantes do vírus da bronquite infecciosa das galinhas. In: Anais da Conferência APINCO – 2006, Santos – SP, Brasil, 2006, p.75-99.

Kant, A.G., Koch, D.J., van Rozelaar, J.G.Kusters, F.A., Poelwijk, van der Zeijst, B.A. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J. Ge. Virol., 73, 591-596, 1992.

Kwon HM, Jackwood MW, Gelb Jr JF. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Diseases, 37: 194-202, 1993.

Kumar, S., Tamura, K., Nei, M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163, 2004.

Kusters, J.G., Niesters, H.G. M., Bleumink-Pluym, N.M.C. Molecular epidemiology of infectious bronchitis virus inn The Netherlands. J. Gen. Virol., 68, 343-352, 1987.

Lai MMC, Cavanagh D. The molecular biology of coronaviruses. Advances in Virus Research 1997; 48: 71-77.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tool. Nucleic Acids Research, v. 25, p.4876-4882, 1994.

Villarreal, L.B.Y., Brandão, P.E., Assaayag, M.S., Saindberg, A.B.S., Jerez, J.A., Ferreira, A.P.F. Propriedades Moleculares e biológicas de um coronavírus aviário relacionado aos coronavírus do grupo II. Revista Brasileira de Ciência Avícola, Suplemento 8, p.211, 2006.

Villarreal, L.Y.B., Brandão, P.E., Chacón, J.L.V., Raffi, P., Sestak, L., Ferreira, A.P.F. Vírus da bronquite infecciosa das galinhas e pneumovírus aviário associados com problemas de fertilidade em galos. Revista Brasileira de Ciência Avícola, Suplemento 8, p.212, 2006.

Wang, L., Junker, D., Collison, E.W., Evidence of natural recombination within S1 gene of infectious bronchitis vírus. Virology, 192, 710-716, 1993.

Table 01 – Infectious bronchitis virus isolates from field cases in chickens in Brazil analysed in this study

IBV isolates	Production Type	IBV Vaccination	Form of the disease	Year of Isolation	Location
IBVPR-01	Broiler	Unknown	Respiratory	1988	Paraná
IBVPR-02	Broiler	Unknown	Respiratory	1988	Paraná
IBVPR-03	Layer	+	Renal	2000	Paraná
IBVPR-04	Broiler	-	Respiratory	2000	Paraná
IBVPR-05	Broiler	-	Respiratory	2000	Paraná
IBVPR-06	Broiler	-	Respiratory	2000	Paraná
IBVPR-07	Broiler	+	Respiratory	1999	Paraná
IBVSP-01	Broiler	+	Renal	1998	São Paulo
IBVSP-02	Broiler	+	Renal	1999	São Paulo
IBVSC-01	Broiler	+	Renal	1999	Sta. Catarina
IBVSC-02	Broiler	+	Respiratory	1999	Sta. Catarina
IBVSC-03	Layer	+	Respiratory	1999	Sta. Catarina

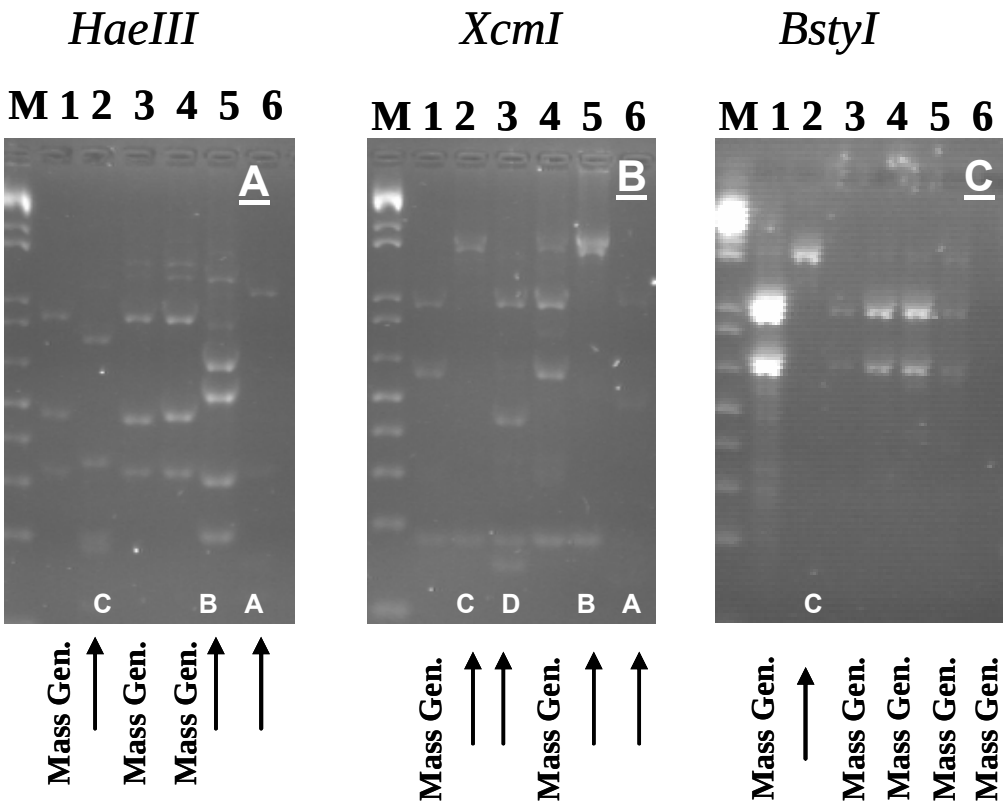

Fig. 1 - Electrophoretic profile of S1 gene PCR product of Brazilian field isolates, amplified with the oligonucleotides S1-OLIGO-5' and S1-OLIGO-3' and diggested with endonucleases *HaeIII* (A), *XcmI* (B) and *BstyI* (C). Lane M – 1kb plus DNA marker, Lane 1 – H120 strain, Lane 2 – IBVPR01, Lane 3 – IBVPR03, Lane 4 – IBVSC01, Lane 5 – IBVPR06 and Lane 6 – IBVSP02 (Besides Mass Genotype, four different genotypes were detected and they were identified by the capital letters A, B, C and D).

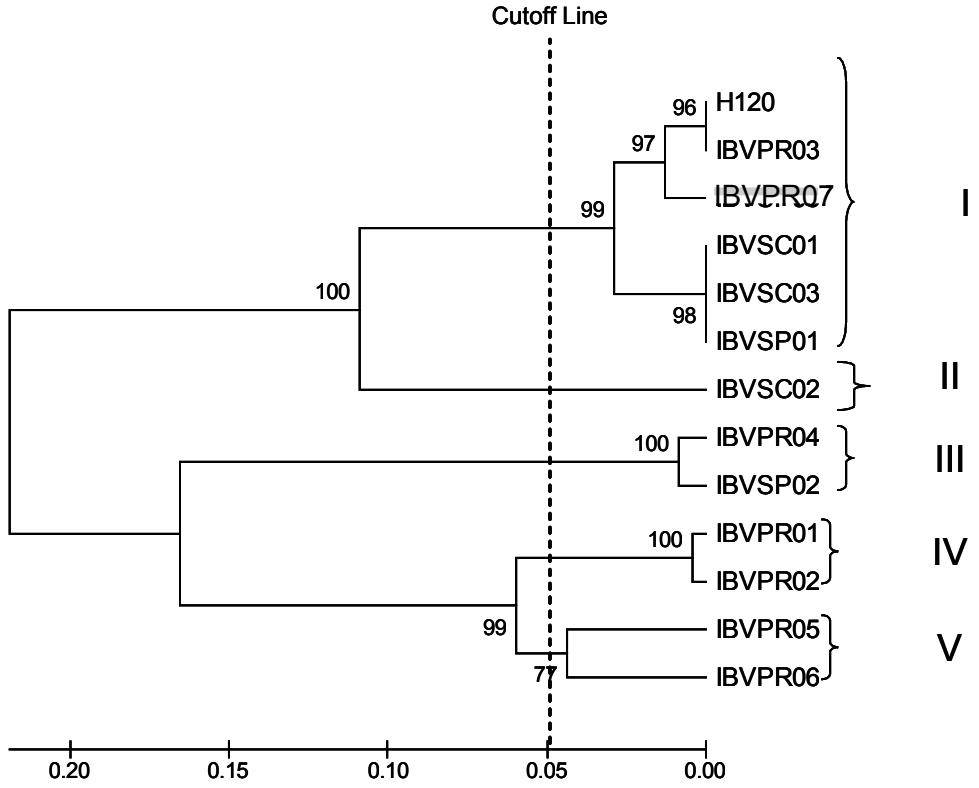

Fig. 2. Dendrogram of deduced amino acid sequences of S1 glycoprotein of Brazilian field isolates of IBV. The partial sequence of amino acids 48 to 184 of the H120 strain were used for phylogenetic analysis at the amino acid level. Horizontal distances were proportional to the minimum number of amino acid differences required to join the nodes and sequences

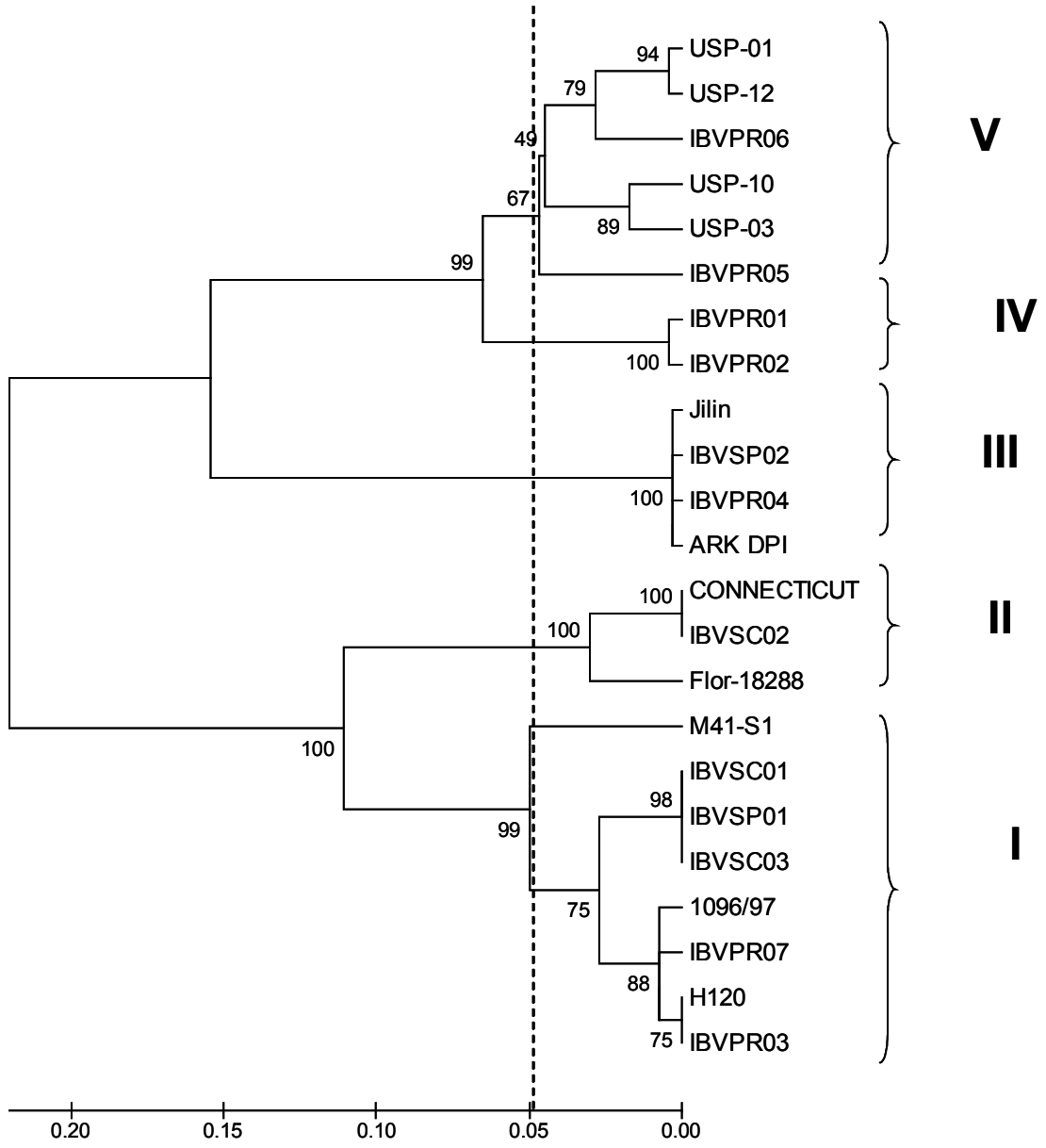

Fig. 3. Dendrogram of the phylogenetic relationships of the twelve Brazilian IBV viruses based no the deduced amino acid sequences of N-terminus of S1 glycoprotein (Amino acids 48 to 184) compared with those of the databanks. Horizontal distances were proportional to the minimum number of amino acid differences required to join the nodes and sequences.

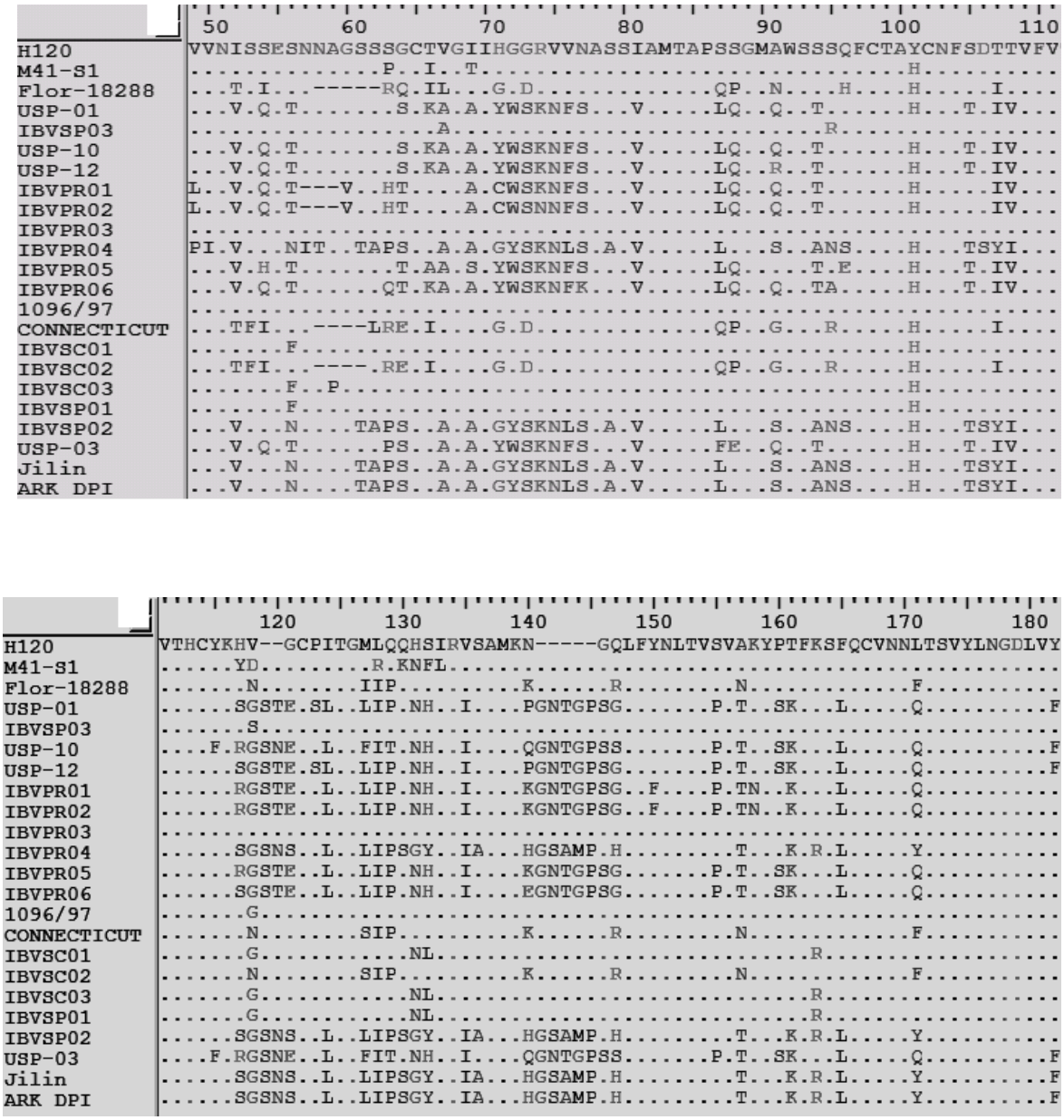

Fig. 4. Multiple alignment of deduced amino acids of representative isolates of the twelve Brazilian IBV isolates (IBVPR01, IBVPR02, IBVPR03, IBVPR04, IBVPR05, IBVPR06, IBVPR07, IBVSC01, IBVCS02, IBVSC03, IBVSP01 and IBVSP02) and 11 published IBV sequences obtained from GenBank. Identical amino acids are displayed by dots (.) and the positions of deletions by dashes (-).

ENHANCED LABORATORY SURVEILLANCE OF GROUP 3 CORONAVIRUSES IN LIFE POULTRY MARKETS IN GUANGDONG PROVINCE, CHINA, AFTER THE SARS OUTBREAK

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SUMMARY

One hundred and seven chickens or silky chickens were tested for infectious bronchitis virus between August 2003 and December 2005 in Guangdong and Hunan Chinese provinces, from which SARS coronavirus has initially emerged. IBV was detected in 82 birds (prevalence: 77%). Limiting the IBV detection PCR to tracheal or cloacal swabs would have led to a considerable underestimation of virus prevalence of 50 to 66% only. 15 sequences of 362 bp of the spike 1 gene (S1) were obtained. 13 strains clustered with Chinese genotype IV strains, which were recently reported in South China too. Genotype IV also showed the larger evolutionary distances in comparison to other Chinese genotypes. IBV/CK(T)/GD.CH/05-04/3587 strain clustered with genotype III virus, showing that genotype III continues to circulate in Guangdong province at least. A vaccine strain was probably detected in a bird as IBV/CK(C)/HN.CH/05-06/2904 was identical to H120 and H52 vaccines which are commonly used in Chinese poultry farms. It is nevertheless not likely that the IBV strains which could not be sequenced were vaccine strains: since both detection and sequencing PCRs were equally sensitive for the vaccine strain, sequencing would rather overestimate vaccine strains than wild-type variants. Our results suggest that at life-bird markets almost all birds carry wild-type IBV and that these markets may be an important and so far underestimated source of infection for IBV.

INTRODUCTION

The recent emergence of a coronavirus variant causing severe acute respiratory syndrome (SARS) in humans has renewed interest in the *Coronaviridae* family of viruses. This enveloped virus was first found in the United States in 1930 and since

has been reported from most countries throughout four continents: America, (Johnson and Marquardt, 1975), Europe, (Capua et al., 1994; Cavanagh and Davis, 1993; Gough et al., 1992), Asia, (Wang et al., 1997) and Australia, (Ignjatovic and McWaters, 1991; Lohr, 1976). Coronaviridae form 3 genetic groups, two of which (group 1 and 2) are pathogenic in humans. Group 1 and 2 viruses cause upper respiratory infections that vary in frequency and severity in different outbreaks (Holmes, 2001). SARS CoV was classified with group 2 viruses but was highly lethal in humans. Group 1 and 2 viruses also infect canines, rodents, felines, porcines and bovines (Lai, 2001). Turkeys can be infected by group 2 as well as group 3 turkey coronaviruses (Lai, 2001). Group 3 viruses such as Infectious Bronchitis Virus (IBV) (Cavanagh, 2000; Enjuanes, 2000), occur only in birds. So far group 3 viruses have not been found in human but phylogenetic analysis of SARS-CoV showed that its genome contained sequences that seem to be of group 3 origin (Stavrinides and Guttman, 2004).

SARS coronavirus has initially emerged from the province of Guangdong in Southeast China. In an effort to understand the natural history of this virus, laboratory surveillance of different host species has been intensified. The Chinese horseshoe bat has been suggested as possible natural reservoir of SARS-like coronavirus (Lau et al., 2005). In a recent study, we showed that bat coronaviruses cluster in several distinct groups, emphasizing the complexity of this viral family and the special role of bats in its ecology and evolution (Tang, 2006).

In China, IBV strains have first been isolated in 1982 (Liu et al., 2006). Outbreaks have occurred frequently and they continue to damage the poultry industry, although vaccines such as H120 and H52 are in used since many years (Liu et al., 2006). IBV infects primarily the respiratory tract, kidney and oviduct (Cavanagh, 2003). Recent reports suggest a shift in tissue tropism (Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001; Zhou et al., 2004) and an extended host range into new bird species reared close to domestic fowl. For instance, IBV was recently detected in Chinese peafowl (*Pavo*), guinea fowl (*Numida meleagris*), partridge (*Alectoris*) and teal (*Anas*) (Cavanagh, 2005).

IBV is an enveloped and positive stranded RNA virus with a genome of about 27 kb. While the nucleoprotein is the most conserved gene of IBV, the spike 1 (S1) subunit of the spike protein gene is the most variable (Cavanagh, 2003). This subunit is responsible for inducing neutralizing and serotype-specific antibodies. Mutations within this genome region may therefore result in emergence of new variants against which vaccines do no longer protect (Moore et al., 1998). Here we present a surveillance study of group 3 coronavirus IBV in life poultry markets mainly in Guangdong province in Southeast China.

MATERIALS and METHODS

Field samples

Since July 2000, systematic influenza virus surveillance was carried out in Southeastern Chinese live birds markets: cloacal, tracheal and fecal swab samples were collected once every 7-10 days in each market from apparently healthy birds. For the present study, 107 chicken or silky chicken cloacal and tracheal swab samples collected between August 2003 and December 2005 in Guangdong and Hunan provinces were tested. Market-vendors buy their birds from a wholesale

market where poultry from both large and family farms are mixed. Meat chickens from large industrial farms are between 50 and 60 days of age while family farm chickens are usually older than 4 months of age. Old layers and breeders are also sold on these markets. The samples were stored at –80°C in virus transport medium (PBS + 6 antibiotics) and had either never been thawed or thawed just once (for AIV isolation attempt) before being processed within the framework of the present study.

RNA isolation and Reverse Transcription - Polymerase Chain Reaction (RT-PCR)
RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), following the instructions of the manufacturer. RNA was eluted in 50 µl elution buffer and processed immediately without intermediate freezing-thawing step. The extracted RNA was first reverse transcribed with random primers and SuperScript III (Invitrogen, Merelbeke, Belgium), following the instructions of the manufacturer. Complementary DNA was first screened for IBV genome using a highly sensitive nested detection PCR of a constant region of the nucleoprotein gene (Akin et al., 2001). The S1 gene was amplified from IBV-positive samples in a semi-nested format (Adzhar, 1997) for sequencing purposes. All PCRs were performed in 25 µl with 0.5 U Platinum® Taq DNA Polymerase (Invitrogen, Merelbeke, Belgium) per reaction for the 1st round PCR and 1 U of Platinum® Taq per reaction for the nested PCR. The equivalent of 0.5 µl of the reaction mix of the RT or of the first PCR was transferred to a new tube for the 1st round or the nested reactions, respectively. Primer concentrations were 0.5 µM and 1 µM for detection and sequencing PCRs, respectively. $MgCl_2$ concentrations were optimized and ranged between 1.5 and 5 µM. All PCRs counted 35 cycles, annealing temperatures were 55°C, 58°C, 53°C and 55°C for detection 1st round, detection nested, sequencing 1st round and sequencing semi-nested PCRs respectively. All programmed cycling was performed in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). PCR amplicons were analyzed in a 1.5% agarose gel (Ultrapure, Invitrogen, Merelbeke, Belgium), using 1X TAE as electrophoresis running buffer and stained with ethidium bromide (15 µg in 100 ml of agarose gel). IBV vaccine strain H120 kindly provided by Dr. Palya and Penzes, Phylaxia Veterinary Biologicals Co. Ltd, Budapest, Hungary, was used as positive PCR control and to optimize the different PCRs.

Sequencing
PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. 10-100 ng DNA were used for sequencing in both directions with the Big Dye Terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster city, USA) on a 16 capillary sequencer (model 3130xl**,** Applied Biosystems, Foster city, USA) using the PCR primers (Eurogentech, Seraing, Belgium) as sequencing primers. South-Eastern Chinese S1 sequences were submitted to GeneBank under the accession numbers AM262512 to AM262521. Identical sequences were submitted only once. Strains were designated as follows: IBV/host code(sample type code)/location.WHO country code/year-month/sample number. Hosts were designed either with CK for chicken or with SCK for silky chicken. Sample types were either tracheal or cloacal swabs designed with T and C respectively.

Data Analysis

Sequences were analyzed using the Bioedit program (Hall, 1999). This program was also used to read the sequencing electropherograms and to exclude nucleotide ambiguity. Sequences were aligned with ClustalW (Chenna et al., 2003). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 (Kumar S, 2001). Phylogenic analysis of nucleic acid and deduced amino acid sequences was done with the Neighbor Joining method, Kimura 2 parameters, pairwise deletion. Amino acid sequences were also analyzed with the Neighbor Joining method, with the Poisson correction. Bootstrap values (1000 replications) were indicated on the tree. Win Episcope 2.0 (Thrusfield et al., 2001) was used for the kappa test (Cohen, 1960).

RESULTS

Detection of IBV

107 chickens or silky chickens were tested for IBV genome both in cloacal and tracheal swab samples. 53 (50%) tracheal and 66 (62%) cloacal swabs were positive in the IBV detection PCR. Only 36 birds (34%) were positive for both swabs (Table 1). Kappa coefficient of agreement was poor (Landis and Koch, 1977) 0.123 (confidence interval: 95%, Table 1). The prevalence of IBV was 77%. Although IBV was detected in 82 birds, only 15 sequences of 362 bp of the spike 1 gene (S1) were obtained, corresponding to 18% of the positive birds. Samples from 2003 (1/3), 2004 (18/22) as well as from 2005 (63/82) were detected positive for IBV, from Guangdong (75/99) as well as from Hunan (7/8) province.

Phylogenetic analysis of nucleotide sequences

12 strains clustered with genotype IV strains while IBV/CK(T)/GD.CH/05-04/3587 clustered with genotype III strains and IBV/CK(C)/HN.CH/05-06/2904 with Massachusetts genotype V strains (genotype numbering according to Liu et al., 2006, Fig. 1). Within the genotypes III and IV proposed by Liu et al. (2006), several sub-groups IIIa and IIIb, IVa to IVd became apparent: the genotype III strain IBV/CK(T)/GD.CH/05-04/3587 seems to belong to cluster IIIb but was genetically essentially as close to cluster IIIa strains as to cluster IIIb strains. Its average genetic distance to cluster IIIa strains was 7.25%±0.35%, while the distance to cluster IIIb was 6.70%±0.52%. IIIa and IIIb strains -available on GenBank and for which the location is known- originated from Guangdong (IBV/CH/DQ167133-CK/CH/LGD/03I) or Tianjin (IBV/CH/AF257075-TJ/96/02 and IBV/CH/DQ167151-CK/CH/LTJ/95I) provinces.

Our genotype IV strains belonged to 2 sub-clusters: IBV/CK(C)/GD.CH/05-01/518, IBV/CK(T)/GD.CH/05-01/215 and IBV/CK(C)/HN.CH/05-01/388 were closest to genotype IVa, the predominant cluster, while IBV/CK(T-C)/HN.CH/05-01/316-387-388-like strains grouped with IBV/CH/AY837465-TA03 to genotype IVd (Fig. 1). The new strains slightly increase the genotype IV maximum diversity (on the 362 nt) from 19.2% (between IBV/CH/DQ167128-CK/CH/LAH/03I and IBV/CH/DQ167135-CK/CH/LGD/04III) to 20.6% (between IBV/CH/DQ167128-CK/CH/LAH/03I and IBV/SCK(T)/GD.CH/05-04/3495).

Strains from our study showed specific nt in only 2 positions in comparison to all other Chinese IBV strains: ^{176}G for IBV/CK(C)/GD.CH/05-11/19466 and ^{279}G for most genotype IVd strains (nt numbers according to sequenced bases: nt1 to nt362). Our

genotype V strain IBV/CK(C)/HN.CH/05-06/2904 was identical with the H120 and H52 vaccines.

Amino acid sequence comparison
The maximal distance between aa sequences of our Chinese S1 gene fragments was 25.2% (between IBV/CH/AF352315-H52 and IBV/CH/DQ167137-CK/CH/LHB/96I, between IBV/CH/DQ167143-CK/CH/LHN/00I and IBV/CH/DQ075323-SH1, between IBV/CK(C)/HN.CH/05-06/2904 and IBV/CH/DQ167137-CK/CH/LHB/96I, between IBV/vaccine H120 and IBV/CH/DQ167137-CK/CH/LHB/96I). These strains did not increase the overall Chinese strain diversity at the aa level. There was only one unique aa found (^{60}R in IBV/CK(C)/GD.CH/05-11/19466, aa numbers: aa1 to aa121).

DISCUSSION

Limiting the IBV detection PCR to tracheal or cloacal swabs would have led to a considerable underestimation of virus prevalence of 50 to 66% only. Testing both tracheal and cloacal swabs brought the prevalence up to almost 80%. Recent studies reported a shift in tissue specificity (e.g. the proventricular, Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001) suggesting that in life-bird markets the prevalence of virus carriers may even be higher, if only enough tissues are sampled.
82 birds were positive in the nucleoprotein detection PCR, but only 15 sequences of S1 gene fragments were obtained, although both PCRs were equally sensitive for the H120 vaccine strain (10^{-3} $TCID_{50}$). The difficulties to amplify S1 gene fragments could be due to RNA degradation, although this would equally affect the nucleoprotein and the S1 PCR. Mutations at primer locations of the more variable spike protein gene used for detection (Cavanagh, 2003) seem to be a more likely explanation.
In China, the life-attenuated IBV vaccine (e.g. H120 or H52) is recommended for commercial meat chickens at 4, 14 and 40-45 days of age. IBV vaccination is common but not systematic in industrial farms in Southeastern China. Therefore, we cannot fully exclude that vaccine virus was detected in our tests. However, among 15 strains only one (7%) had a vaccine-like spike gene. Since both detection and sequencing PCRs were equally sensitive for the vaccine strain, sequencing would rather overestimate vaccine strains than wild-type variants. Moreover, as much as 50% of the birds tested may be from family farms which rarely vaccinate. Thus our results suggest that at life-bird markets almost all birds carry wild-type IBV and that life-bird markets may be an important and so far underestimated source of infection for IBV.
Our results further show that genotype III strains that have been found in China since 1995 continue to circulate at least in Guangdong province in 2005. Genotype IV strains seem to be of a more recent origin as genotype III strains as they have mostly been found after 2000 (Liu et al., 2006). Nevertheless this genotype showed a larger evolutionary diversity both in our as well as previous studies: distances within groups IVa and IVd were 5.59 and 4.91% respectively, while the mean distance between the 2 groups reached about 20% both at the nt and aa level. The detection of IBV genome is not necessarily proof of current or former disease, but our strains are closely related to nephropathogenic and proventricular strains found since 2000 (Liu and Kong, 2004; Liu et al., 2006; Yu et al., 2001; Zhou et al., 2004). The low

efficiency of the sequencing PCR may indicate that the diversity of IBV in Southeastern China may be greater than reflected by the sequences.
Our study shows that the prevalence of IBV is extremely high in life birds markets and the diversity is probably even higher than so far estimated. Stavrinides and Guttman (2004) showed that at least partial sequences of the matrix, nucleoprotein and spike genes of SARS-CoV may be of avian origin. It is therefore necessary further characterize the genes of these IBV strains as well as potential variants that may have gone undetected.

ACKNOLEDGMENTS

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REFERENCES

Adzhar, A., R.E. Gough, D. Haydon, K. Shaw, P. Britton, D. Cavanagh (1997). Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol* **26,** 625-640.

Akin, A., Lin, T. L., Wu, C. C., Bryan, T. A., Hooper, T., and Schrader, D. (2001). Nucleocapsid protein gene sequence analysis reveals close genomic relationship between turkey coronavirus and avian infectious bronchitis virus. *Acta Virol* **45**(1)**,** 31-8.

Capua, I., Gough, R. E., Mancini, M., Casaccia, C., and Weiss, C. (1994). A 'novel' infectious bronchitis strain infecting broiler chickens in Italy. *Zentralbl Veterinarmed B* **41**(2)**,** 83-9.

Cavanagh, D. (2000). Coronaviruses and toroviruses. *In* "Principles and practice of clinical virology" (a. J. R. P. J. E. B. A.Z. Zuckerman, Ed.), pp. 345-356. John Wiley and Sons, Chichester, United Kindom.

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathol* **34**(6)**,** 439-48.

Cavanagh, D., and S. A. Naqi (2003). Infectious Bronchitis. 11 ed. *In* "Diseases of Poultry" (H. J. B. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, and D. E. Swayne, Ed.), pp. 101-119. Iowa state University Press, Ames.

Cavanagh, D., and Davis, P. J. (1993). Sequence analysis of strains of avian infectious bronchitis coronavirus isolated during the 1960s in the U.K. *Arch Virol* **130**(3-4)**,** 471-6.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G., and Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**(13)**,** 3497-500.

Cohen, J. (1960). A coefficient of agreement for nominal scales. *Educ. Psychol. Meas.* **20,** 27-46.

Enjuanes, L., Brian, D., Cavanagh, D., Holmes, K., Lai, M. M. C., Laude, H., Masters, P., Rottier, P., Siddell, S., Spaan, W. J. M., et al. (2000). Coronaviridae. *In* "Virus Taxonomy" (C. M. F. F. A. Murphy, D. H. L. Biship, S. A. Ghabrial, A. W. Jarvis, G. P. Martelli, M. A. Mayo, and M. D. Summers, Ed.), pp. 835-849. Academic Press, New York.

Gough, R. E., Randall, C. J., Dagless, M., Alexander, D. J., Cox, W. J., and Pearson, D. (1992). A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. *Vet Rec* **130**(22)**,** 493-4.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41,** 95-98.

Holmes, K. V. (2001). Coronaviruses. 4 ed. *In* "Fields Virology" (D. M. Knipe, Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E., Ed.), Vol. 1, pp. 1187-1203. 2 vols. Lippincott Williams & Wilkins, Philadelphia.

Ignjatovic, J., and McWaters, P. G. (1991). Monoclonal antibodies to three structural proteins of avian infectious bronchitis virus: characterization of epitopes and antigenic differentiation of Australian strains. *J Gen Virol* **72 (Pt 12),** 2915-22.

Johnson, R. B., and Marquardt, W. W. (1975). The neutralizing characteristics of strains of infectious bronchitis virus as measured by the constant-virus variable-serum method in chicken tracheal cultures. *Avian Dis* **19**(1)**,** 82-90.

Kumar S, T. K., Jakobsen IB, Nei M (2001). MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* **17,** 1244-5.

Lai, M. M. C., Holmes, K.V (2001). Coronaviridae: the viruses and their replication. 4 ed. *In* "Fields Virology" (D. M. Knipe, Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E., Ed.), Vol. 1, pp. 1163-1185. 2 vols. Lippincott Williams & Wilkins, Philadelphia.

Landis, J. R., and Koch, G. G. (1977). The measurement of observer agreement for categorical data. *Biometrics* **33**(1)**,** 159-74.

Lau, S. K., Woo, P. C., Li, K. S., Huang, Y., Tsoi, H. W., Wong, B. H., Wong, S. S., Leung, S. Y., Chan, K. H., and Yuen, K. Y. (2005). Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* **102**(39)**,** 14040-5.

Liu, S., and Kong, X. (2004). A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathol* **33**(3)**,** 321-7.

Liu, S. W., Zhang, Q. X., Chen, J. D., Han, Z. X., Liu, X., Feng, L., Shao, Y. H., Rong, J. G., Kong, X. G., and Tong, G. Z. (2006). Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004. *Arch Virol*.

Lohr, J. E. (1976). Serologic differences between strains of infectious bronchitis virus from New Zealand, Australia, and the United States. *Avian Dis* **20**(3)**,** 478-82.

Moore, K. M., Bennett, J. D., Seal, B. S., and Jackwood, M. W. (1998). Sequence comparison of avian infectious bronchitis virus S1 glycoproteins of the Florida serotype and five variant isolates from Georgia and California. *Virus Genes* **17**(1)**,** 63-83.

Stavrinides, J., and Guttman, D. S. (2004). Mosaic evolution of the severe acute respiratory syndrome coronavirus. *J Virol* **78**(1)**,** 76-82.

Tang, X. C., Zhang, J.X., Zhang, S.Y., Wang, P., Fan, X.H., Li, L.F., Li, G., Dong, B.Q., Liu, W., Cheung, C.L., Xu, K.M., Song, W.J., Vijaykrishna, D., Poon, L.L.M., Peiris, J.S.M., Smith, G.J.D., Chen, H., Guan, Y. (2006). Prevalence and diversity of coronaviruses in bats from China. *Journal of Virology*.

Thrusfield, M., Ortega, C., de Blas, I., Noordhuizen, J. P., and Frankena, K. (2001). WIN EPISCOPE 2.0: improved epidemiological software for veterinary medicine. *Vet Rec* **148**(18)**,** 567-72.

Wang, H. N., Wu, Q. Z., Huang, Y., and Liu, P. (1997). Isolation and identification of infectious bronchitis virus from chickens in Sichuan, China. *Avian Dis* **41**(2)**,** 279-82.

Yu, L., Jiang, Y., Low, S., Wang, Z., Nam, S. J., Liu, W., and Kwangac, J. (2001). Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. *Avian Dis* **45**(2)**,** 416-24.

Zhou, J. Y., Zhang, D. Y., Ye, J. X., and Cheng, L. Q. (2004). Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. *J Vet Med B Infect Dis Vet Public Health* **51**(4)**,** 147-52.

Table 1. Detection of IBV tracheal and cloacal swabs of life-bird markets.

Kappa=0.123 (95% CI)	Tracheal swab positive	Tracheal swab negative	Total
Cloacal swab positive	36	30	66
Cloacal swab negative	17	24	41
Total	53	54	107

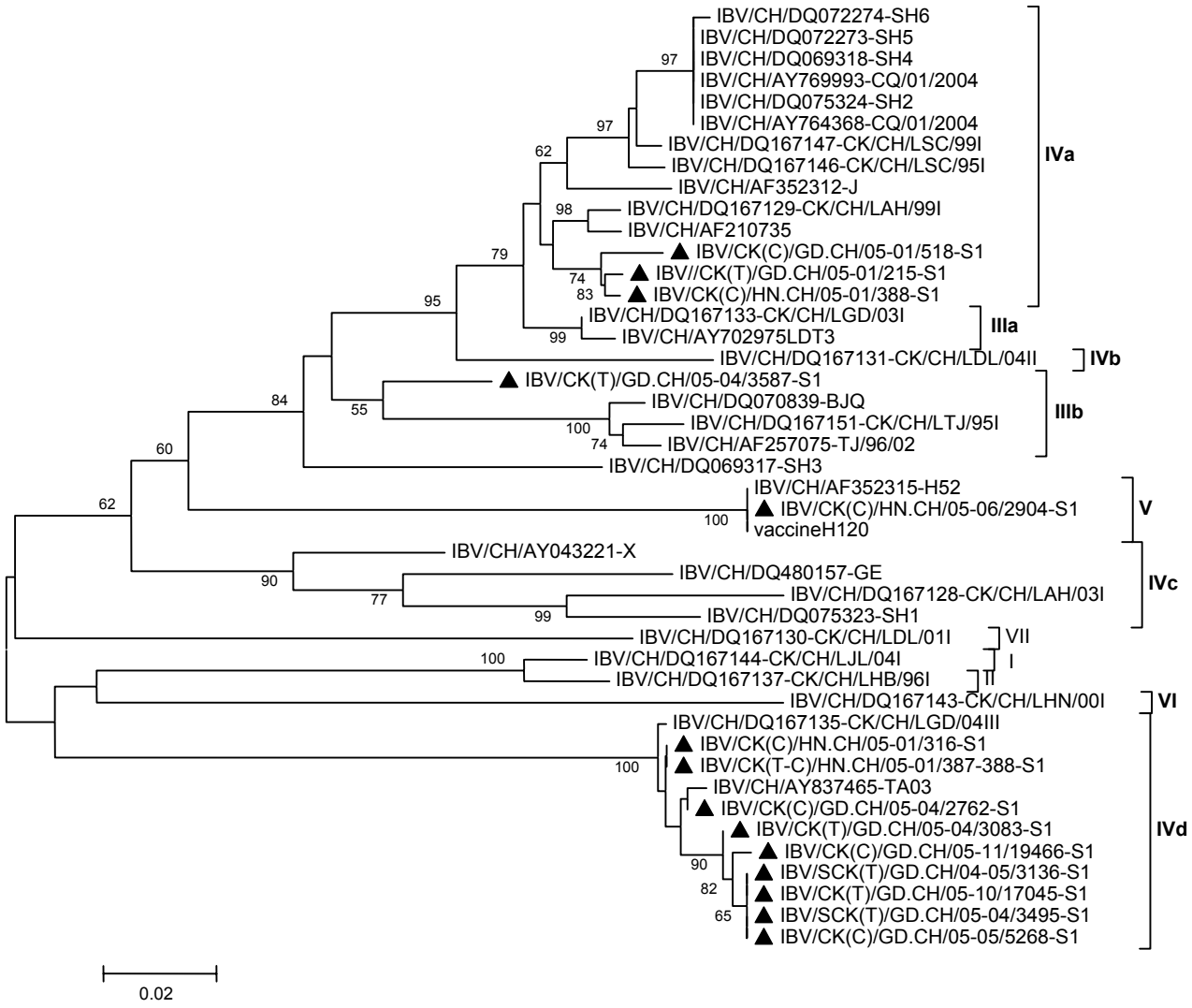

Fig. 1. Phylogenetic analysis of a 362 bp fragment of the spike 1 gene of IBV. Our 14 South-Eastern Chinese IBV strains are compared with 30 other Chinese strains whose sequences are available on GenBank. All Chinese genotypes III and IV strains were included while a single Chinese strain was randomly selected for genotypes I, II, VI and VII. Numbers at nodes correspond to bootstrap values >49. Closed symbols indicate strains reported in the present study. Genotypes were indicated on the right hand side with I, II, IIIa, IIIb, IVa, IVb, IVc, IVd, V, VI and VII.

IB IN SWEDEN 1994-1998
FIVE OUTBREAKS IN A NAÏVE POPULATION

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SUMMARY

Sweden, like Norway and Finland, was in the beginning of the 1990´s countries not vaccinating their poultry against infectious bronchitis (IB). Furthermore, IB was a notifiable disease and Sweden was in negotiations for membership in the European Union asking for additional guarantees for this disease, among others. In the period 1994 up to 1998 a number of outbreaks of IB in commercial poultry; layers, broiler breeders and broilers, drastically changed the conditions for a non vaccinating policy. Here a brief description of the outbreaks, management and possible origin is presented. The discussion in the farmer's organisations pro and against vaccination and economical considerations eventually leading to a vaccination policy is covered.

INTRODUCTION

Due to a favourable geographic location and a restricted policy concerning import of animals many infectious diseases endemic in other European countries are seldom or never diagnosed in Sweden. Furthermore eradication of infectious diseases has in many cases been successful. Bovine tuberculosis and brucellosis were eradicated in the 1950´s and Bovine virus diarrhea will be in the very near future. The same applies to poultry and Avian influenza has never been diagnosed in poultry. Newcastle disease was diagnosed in the 1950´s and after that not until 1995 but now 2006 additional seven times. Infectious bronchitis (IB) was noted in 1970´s when 25 farms were infected and stamping out was applied. A Health Control Programme in practice from 1992 on had shown only one IB positive layer farm in total. The same period broiler flocks and breeders had tested negative. But in 1994 another outbreak was diagnosed at the National Veterinary Institute (SVA).
In 1994 Sweden had two broiler breeding companies and three major layer breeding companies. These five companies imported grand parents (GP) and according to the Swedish legislation the day-old chicks were not vaccinated against neither Newcastle disease nor IB and kept in quarantine. Ten to fifteen imports were performed every year. About 850 layer farms kept more than 200 layers and the total population was six million. The number of broilers raised was roughly 50 millions in about 200 farms.

Very few farms were producing both eggs and broilers. The poultry farms are concentrated to the south part of the country and highest density is in the two counties Skåne and Halland.

MATERIALS and METHODS

The farmers recorded clinical signs of appetite loss and prominent depressed egg production. Some farmers noted unusual noises from the flock. Mortality was not significant. The egg white was watery and the eggshells thin and easily broken, later on deformed in shape. In a number of layer farms testing positive serologically no clinical signs at all had been noted.

Cloacal and tracheal swabs and internal organs were collected for virus isolation and RT-PCR analyses in the index cases and in cases crucial for the tracing of the origin of the outbreaks. Blood or egg sampling for serology was made at the farms by the owner, the retailer company or occasionally by a veterinarian. The number of samples recommended was 18-20 per flock. In the majority of cases the number sampled was less than recommended but a minimum of 12 samples was considered sufficient. Moving et al. (1998)

For virus isolation suspensions made from cloacal and tracheal swabs and various organs were inoculated in 9-12 days embryonated eggs according to FTW Jordan et al. (1990). After passaging IBV was identified on the chorioallantoic membrane by direct electronmicroscopy and immunohistochemistry using an IBV-specific antibody. Syed A Naqi (1990).

Serological diagnosis was performed using a commercial kit (SVANOVIR™ IBV-Ab ELISA) detecting specific antibodies in serum and egg yolk. V. Moving et al. (1998).

Specimens of trachea and swabs were analysed by RT-PCR performed as previously described. Farsang et al. (2002).

The tracing of the origin of the IBV infection started as soon as the laboratory had confirmed the index case. Information about the farm, the type of poultry, the number of flocks and ages, the date of the debut and the clinical symptoms, the geographical location, direct and indirect contacts with other poultry farms was collected. The organisation of the industry was important to consider as the network of contacts is a map of farms at risk. At these farms poultry were sampled and tested serologically. Positive cases were indicated on maps and a reasonable model for the distribution of the infection based on data collected was made.

In an effort to reveal the origin of the outbreaks during 1994 a plan to sample all commercial egg producing farms was decided by the egg industry and still went on during the first half year of 1995. In the two 1995 outbreaks a careful sampling in the area around the infected farms was made in both commercial poultry and hobby flocks.

RESULTS

In 1994 to 1998 a series of outbreaks with clinical symptoms were diagnosed: in April 1994 layers, in February 1995 a broiler breeder company in Skåne (1995/1), in April the same year a highly poultry populated area in Halland affecting layers, broilers and back yard flocks (1995/2). The broiler breeder company was again infected in April 1997. Finally vaccination against IBV was decided and had started the very same year. Vaccination virus spread to older layer flocks at the farms when young vaccinated pullets arrived (1997-98). See table 1 Summary of outbreaks 1994-1998.

The layer outbreak 1994 emanated from a rearing site for pullets in Halland. The infected birds were delivered to five egg producing farms where the infection spread to older producing flocks and the disease was discovered. From one of these farms the empty cages previous housing the pullets were transported to a sixth farm contracting the infection and spreading the virus further when delivering reared pullets to the seventh farm. From the seventh farm delivered pullets infected additional five farms. In one case the infection was also spread through handling of manure and in one case probably working staff spread the virus from one farm to another. In summary infected pullets spread the infection in eleven cases, empty cages in one, manure in one case and staff in one. As shown in many other instances transporting of animals is a frequent cause of spreading of infectious diseases. The outbreaks of the parents flocks of a broiler hatchery in February 1995 and 1997 were spread to nearby parents flocks at a site 17 km distant but the infection was contained in these houses. The outbreak in Halland in April, 1995/2, comprising both layers, broilers and back yard flocks did obviously not spread from the localised area which is a narrow valley surrounded by woods to the south, east and north, and to the west by a town and the sea. See figure 1 Summary of IB outbreaks 1994-1998.

A screening of layer farms followed the layer outbreak 1994. About 50% were tested and additional 24 farms were found serologically positive. No production problems were noted in these farms.

A molecular analysis of samples collected during the outbreaks was made. Farsang et al. (2002). Unfortunately the RT-PCR on the spike protein gene did not work on samples from the outbreak 1994. An interesting result for 1995 was that a broiler farm in Skåne positive in March showed the same virus as one sample from the outbreak in Halland, which was recorded in April. The distance between the farms is about 150 km. The last farm in the Halland outbreak to be infected was with a virus related to strain D274 and far from the above mentioned. The strain causing the outbreak in broiler breeders in 1997 was related to the vaccine virus Ma5 but not identical. The problems in layer flocks later the same year were clearly related to spreading of the live vaccine virus Ma5 to older flocks on the farms. See figure 2 Molecular epizootology of IB strains 1994-1998 and the vaccine strain Ma5.

In the positive layer farms the birds were euthanised. The parents flocks infected in 1995 were vaccinated with killed vaccine since a stamping out would have been very costly. When this broiler breeding company again was infected in 1997 a decision on vaccinating with live vaccine was already decided. The layer breeders had because of the outbreak to start the IB vaccination earlier than planned.

DISCUSSION

The origin of the outbreaks was a question lively debated in the period 1994-1997. The data on the molecular epizootology of the samples collected showed a relation between the 1995/1 and 2 outbreaks, a relation not possible to show in the trace back investigation made at the time. The second surprise was the two clearly different strains from the outbreak 1995/2 as they were timely and geographically related. After two years another strain appeared in February 1997 which has no other known connections. The origin of the different outbreaks was not clarified but the result of the molecular analysis showed more than one present.
The screening of the layer farms in 1994 showed that IB virus was circulating in the farms obviously with no clinical signs. As the Health Control Programme during 1992-93 sampling layers at slaughter had no positive birds except one isolated farm the result of the screening made at the farms in 1994 was another surprise. In the beginning of 1994 both National Veterinary Institute and the poultry industry were convinced that IB was not a problem in commercial poultry and even more, the infection did not exist. The layer outbreak was seen as an outstanding event and the risk of new outbreaks in the near future was according to the farmer's organisations very low. Politically Sweden was negotiating for EU member ship and the industry, all categories, feared the spreading of new infectious diseases. Additional guarantees for a number of diseases and among them IB were claimed. In this situation it was considered impossible to start vaccination and the stamping out policy was decided. A discussion on vaccination with live vaccine started after the breeder outbreak 1995/2 and was advocated by the broiler industry against the egg producer´s organisation. They feared coming problems in the layer flocks as seen in continental Europe. An attempt to raise a fund for compensation in case of future outbreaks was made but the building up of the capital could not be agreed upon. In the beginning of 1997 the discussion was ended and the authority, National Board of Agriculture, gave their permission to vaccinate with live vaccine. The vaccine company promised there would be no problems, which showed to be not completely true.
Though the outbreaks per se were small in number of cases the consequences drastically changed both the previous conception as commercial poultry free of IBV and the future for the poultry industry now that IB vaccination has to go on. Interestingly both Norway and Finland are still non vaccinating countries.

REFERENCES

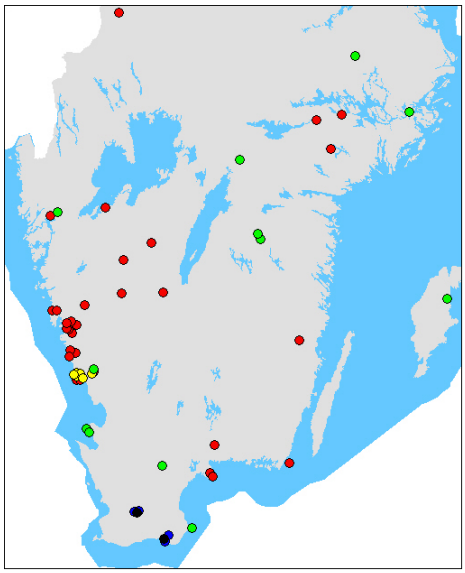
Farsang, C. Ros, Lena H.M. Renström, Claudia Baule, T Soós and S. Belak, Molecular epizootology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain. Avian Pathology 31, 221-236, 2002.

V. Moving, G. Czifra L. Renström, A Monoclonal Antibody Blocking ELISA for the Detection of IBV antibodies in Fowl, Adv. Exp. Med Biol. 440, 495-499, 1998.

Syed A Naqi, A monoclonal antibody-based immunoperoxide precedure for rapid detection of infectious bronchitis virus in infected tissues. Avian Diseases, 34, 893-898.

Table 1: Summary of outbreaks 1994-1998.

Outbreak	Index case	Poultry	Positive farms
1994	April	Layers	39
1995/1	February	Broiler breeders	4
1995/2	April	Layers, broilers	8
1997	April	Broiler breeders	2
1997-98	September	Layers	13



Summary of IB outbreaks 1994-1998.
● 1994 ● 1995/1 ● 1995/2 ● 1997 ● 1997-98

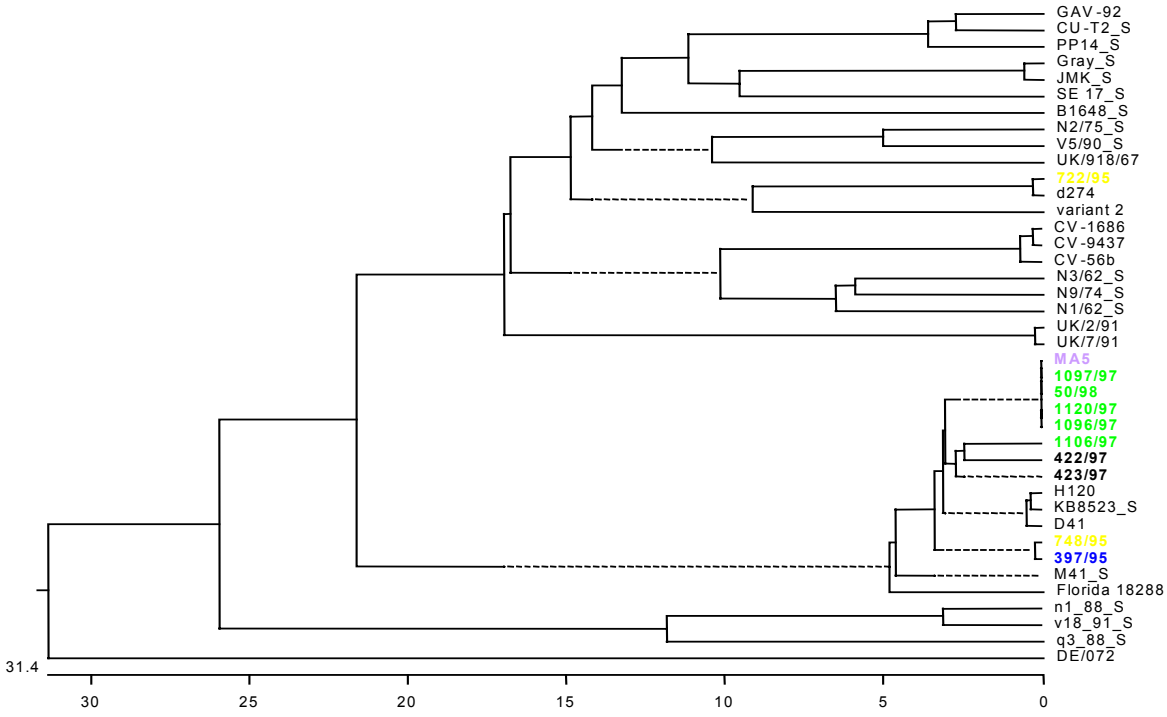


Figure 2
Molecular epizootology of IB strains 1994-1998 and the vaccine strain Ma5.
● 1995/1 ● 1995/2 ● 1997 ● 1997-98 ● Vaccine strain

MOLECULAR STUDIES ON INFECTIOUS BRONCHITIS VIRUS ISOLATED IN POLAND

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SUMMARY

The comparison of the conventional (aimed at the UTR'3 fragment) and nested PCR (aimed at the N gene fragment) -based methods revealed that nested PCR was about 1000 x more sensitive. Molecular differentiation of 793/B, D274 and Mass IBV types in multiplex nested PCR was also achieved. Eight from nine studied Polish isolates had the characteristic band for 793/B and five simultaneously to D274 type. No band was obtained in the case of 162/97 isolate. However, obtained results should be interpreted carefully due to probable mismatch of used primers as well as due to the presence of various strains originated from applied vaccination program. Phylogenetic analysis of nucleotide sequences of IBV isolates from 1997-2006 revealed that 793/B and QX types are present in Poland. Two isolates have unclear relationship with the rest of the strains and formed separate branches of the phylogenetic tree.

INTRODUCTION

Infectious bronchitis seems to be a growing problem in poultry industry in Europe. The presence of various types (sero- and genotypes) was reported recently be several authors. The survey conducted in UK, France, Holland, Germany and Spain revealed that the most commonly found genotypes were IBV variant 793/B (4/91, CR88) and Massachusetts (Mass), accounting for 38% and 27% of studied isolates, respectively. The next most frequently detected genotypes were Italy-02 (It-02), D274 and Arkansas (Worthington et al., 2004). Similarly, several types of IBV in the field of the other European country simultaneously exist. In Italy, from 14 isolated IBV strains, 7 were identified as It-02, six as 793/B and one as Chinese strain QXIBV (Beato et al., 2005). The study conducted in Hungary revealed that their IBV strains belonged to Massachusetts and 793/B serotypes (Bratu et al., 2004). The presence of different IBV serotypes (the most frequent Mass but also D274, B1649, 793B) has been described in Belgium (Meulemans et al., 2001). A study of 20 Turkish IBV

isolates demonstrated the simultaneous presence of two serotypes, 11 of them belonged to 793/B and the rest to Mass (Azad et al., 2005).
In Poland infection by IBV was first serologically demonstrated in 1967 (Karczewski and Cąkała, 1967). Outbreaks of IB with respiratory signs, drop in egg production and egg quality in non vaccinated breeding and particularly in laying hens flocks were recorded in the middle of the 1980s (Bugajak et al., 1987). Since the middle of 1990s the outbreaks of IB-nephritis have been reported in broilers flocks (Minta et al., 1995). The multiplex-PCR with strains isolated between 1997-1998 revealed that the most of them belonged to 793/B type and one to other unknown type (Minta et al., 1998).
The aim of this study was to evaluate i) conventional RT-PCR and nested PCR for detection of IBV, ii) multiplex nested PCR for their differentiation and iii) nucleotide sequencing the fragment of S1 gene of IBV isolates collected between 1997-2006 in Poland for molecular characterization.

MATERIALS AND METHODS

Twelve Polish IBV strains were included in the study: two (236/96, 242/96) isolated in 1996, four (158/97, 162/97, 264/97, 255/97) isolated in 1997, two (29/05, 217/05) from 2005 and five isolated in following years: 1998 (58/98), 1999 (197/99), 2004 (338/04) and 2006 (1377/06). Eight reference IBV (M41, D274, D1466, B1648, D8880, 793/B and Beaudette) as well as seven vaccinal strains (Nobilis IB 4/91 and Nobilis IB Ma5 - Intervet, Gallivac 88 - Merial, Poulvac IB Primer - Fort Dodge, H120 - Abic, Cevac BIL - Ceva Sante Animale, Bronhical II SPF - Pliva) were also included in this study. Viral materials were allantoic fluid harvested from inoculated chicken eggs SPF (dead or frozen) or vaccinal lyophylizates. Commercial kit (Qiagen) for isolation of viral RNA was used. Translation of viral RNA to DNA was done in reverse transcription (RT).
For IBV genome detection two methods were used: conventional aimed at 3'untraslated region (UTR) according the methods by Adzhar (Adzhar et al., 1996) and nested RT-PCR aimed at highly conserved region of N gene according the methods by Farsang (Farsang et al., 2002). The sensitivity of both methods was established by ten-fold diluting of allantoic fluid containing D274 ($10^{4,5}$ ELD_{50}/0.1 ml) and 793B ($10^{2,67}$ ELD_{50}/0.1 ml).
Molecular differentiation of 793B, D274 and Mass IBV types was achieved by using primers specific for this types (S1 region sequences of S protein) in multiplex nested RT-PCR (Capua et al., 1999). Nine RT-PCR products obtained with Polish field isolates in the first RT-PCR (464 base pairs (bp)) were sequenced using commercial services (IBB-PAN, Warsaw Poland) with the XCE1/XCE2 primers. Sequences have been processed and phylogenetic analysis was performed using DNAStar v. 5.01 (DNASTAR Inc.2001)

RESULTS

Using UTR1/UTR2 primers in conventional RT-PCR 21 from 27 isolates produced the characteristic amplicons of 297 bp, however with two sets of primers, N984/N1145 and N794/N1129 in nested PCRs 24 from 27 tested samples gave 380 bp amplicons.

Sensitivity of the conventional PCR based on UTR gene was established at 100 EID_{50}/0,1 ml and the nested PCR based on N-gene - at 0,01 EID_{50}/0,1 ml. No cross reaction was found with other RNA viruses used in the study (AIV, NDV, IBDV, ARV) which revealed high specificity of used primers.
Molecular differentiation of 793B, D274 and Mass IBV types was achieved by using primers specific for these types (S1 region sequences of S protein) in multiplex nested PCR and obtained amplicons were, respectively 154, 217 and 195 bp. The results of this differentiation are shown in Table 1. The specific product for 793B type was obtained in the case of 2 references (793B and D8880), 2 vaccinal (Nobilis 4-91 and Gallivac 88) and 8 from 9 tested field IBV strains (158/97, 254/97, 255/97, 197/99, 338/04, 29/05, 217/05 and 1377/06). The band characteristic for D274 type was present in one vaccianal (Poulvac IB Primer) and 3 reference (D274, D1466, B1648) materials and also 5 field IBV strains. As regards to Mass type, the characteristic band in 5 vaccinal (Poulvac IB Primer, V.H.+H120, Cevac BIL, Nobilis IB Ma5, Bronhical II SPF) and 2 reference (M41, Beaudette) materials was found. No products were present in the case of one strain from 1997 (162/97).
The sequences of the 464 bp fragment of the S1 part of the spike protein gene of nine Polish isolates revealed that most of them belonged to 793/B type (three from 1997-158/97, 254/07, 255/97, one from 1999-197/99 and one from 2004-338/04). Two isolates, 29/05 and 1377/06, were included in the Chinese QX type of IBV. One Polish strain from 1997 (162/97) and one from 2005 (217/05) formed a separate genetic branches of the phylogenetic tree (Figure 1). The comparison of the percent of nucleotides (nt) identity of these strains with the main types of IBV was done (Table 2). The 162/97 isolate showed rather low nt homology with the rest of the strains (ranging from 63,7 to 81,5%) and the second one (217/05) the highest nt homology with 793/B (90,9%) and IT-02 (88,3%). This could suggest that 217/05 isolate belong to 793/B type, however it should be confirmed by more detailed studies (sequencing the whole S1 part of the S protein gene).

DISCUSSION

The aim of the present study was to evaluate two PCR-based methods (the conventional and nested) for IBV detection as well as the multiplex nested PCR for IBV strains differentiation. The preliminary molecular characterization based on the 464 bp fragment of the S1 gene of Polish IBV isolates was also achieved.
There are many different PCR-based methods for IBV detection applications (Cavanagh et al., 1997; Falcone et al., 1997; Jackwood et al., 1992; Kwon et al., 1993). The sensitivities and specificities of these methods are mostly dependent on the sequences of chosen primers. The most frequently used primers to detect infectious bronchitis virus are based on highly conserved regions of N, M or UTR gene. In the present study, two methods were applied and evaluated for diagnostic purpose: one based on UTR-gene according to Adzhar et al (Adzhar et al., 1996) and second one based on N-gene according to Farsang et al(Farsang et al., 2002). Our experimental results confirmed that these two primers sets do not cross-react with other RNA viruses and are suitable for detection of IBV. As one could expect, nested PCR was more sensitive (about 1000 x) than conventional RT-PCR. On the other hand, risk of cross contamination may lead to false positive results in nested PCR (de Wit J.J., 2000).

The results of the multiplex nested PCR, aimed in detecting of 793/B, D274 and Mass IBV type revealed the presence of 793/B in 8 from 9 studied Polish field strains. In 5 cases the band characteristic for D274 type was also found which may suggest vaccinal origin of these strains. No band was identified with 162/97 isolate. The genotyping by multiplex PCR for use in the field could have some disadvantage (de Wit J.J., 2000). The information included in the IBV genome could not be exactly extrapolated into biological features. There are contradictory data about the correlation between genotype and serotype of IBV strains: some reported the high while the other rather diverse correlations between the genotype or sero- and/or protectotype (Capua et al., 1999; Keeler et al., 1998; Kwon et al., 1993). In applied multiplex nested PCR D8880 strain, which is known to belong to B1648 type, was identified as 793/B type and the reference B1648 as D274 type. The reason for this could lie in the sequence of used primers. The sequence of the given type-specific primers could hybridize with irrelevant type and give false positive results. Such possibilities were indicated by Capua (Capua et al., 1999). So the type-specific primers construction as well as an interpretation of differentiation results (complication by used vaccine strains) should be done carefully.
The results of S1 fragment gene sequencing clearly indicate that 793/B and QX types are present in Poland. Two isolates, 162/97 and 217/05 showed differences from mentioned IBV types and formed separate branches of the phylogenetic tree, however the 217/05 isolate had relatively high nt homology with 793/B. Further analysis (sequencing of whole S1 gene) is necessary for the detailed molecular characterization of these strains.

REFERENCES

Adzhar, A.B., Shaw K., Britton P., Cavanagh D. (1996) Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. Avian Pathol 25, 817-836.

Azad G. A., Marandi M. V., Aminae H. K. (2005) Detection and molecular characterisation of infectious bronchitis viruses by RT-PCR/RFLP and sequencing. Abstract Book of 14th WVP Congress, 2005 , Abstract No: 080, p. 136.

Beato, M.S., De Battisti C., Terregino C., Drago A., Capua I., Ortali G. (2005) Evidence of circulation of a Chinese strain of infectious bronchitis virus (QXIBV) in Italy: Vet Rec, 156, p. 720.

Bratu H., Miklo C., Mato T., Benyeda J., Penzes Z., Palya V. (2004) Epidemiology of avian infectious bronchitis virus in Hungary. Identification of a field recombinant IB. Proceedings from IV Internationa Symosium on avian corona and pneumovirus infections. Rauischholzhausen, Germany, 20-23 June 2004, pp. 117-124.

Bugajak P., Karczewski W., Mendeleski E., Musialik M. (1997) Występowanie zakaźnego zapalenia oskrzeli u kur w Polsce w latach 1984-1986. Materiały VIII Kongresu PTNW, p. 50.

Capua I., Minta Z., Karpinska E., Mawditt K., Britton P., Cavanagh D., Gough R. E. (1999) Co-circulation of four type of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). Avian Pathol 28, 587-592. 1999.

Cavanagh, D., Mawditt K., Shaw K., Britton P., Naylor C. (1997) Towards the routine application of nucleic acid technology for avian disease diagnosis: Acta Vet Hung 45, 281-298.

de Wit J.J. (2000) Detection of infectious bronchitis virus. Avian Pathol 29, 71-93.

Falcone E., D'Amore E., Di Trani L., Sili A., Tollis M. (1997) Rapid diagnosis of avian infectious bronchitis virus by the polymerase chain reaction: J Virol Methods 64, 125-130.

Farsang A., Ros C., Renstrom L. H., Baule C., Soos T., Belak S. (2002) Molecular epizootiology of infectious bronchitis virus in Sweden indicating the involvement of a vaccine strain: Avian Pathol, 31, 229-236.

Jackwood M.W., Kwon H. M., Hilt D.A. (1992) Infectious bronchitis virus detection in allantoic fluid using the polymerase chain reaction and DNA probe. Avian Dis 36, 403-409.

Karczewski W., Cąkała A. (1967) Badania serologiczne nad rozprzestrzenieniem wirusa zakaźnego zapalenia oskrzeli kur w Polsce., Medycyna Wet 23, 475-480.

Keeler C.L. Jr., . Reed K.L., Nix W.A., Gelb J.Jr, (1998) Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene., Avian Dis, 42, 275-284.

Kwon, HM, M W Jackwood, T P Brown, D A Hilt, (1993) Polymerase chain reaction and a biotin-labeled DNA probe for detection of infectious bronchitis virus in chickens., Avian Dis, 37, 149-156.

Kwon H. M., Jackwood M.W., Gelb J. (1993) Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction length polymorphism analysis. Avian Dis 37, 194-202.

Meulemans G., Boschmans M., Decaesstecker M., van den Berg T. P., Denis P., Cavanagh D. (2001) Epidemiology of infectious bronchitis virus in Belgian broilers: a retrospective study, 1986 to 1995., Avian Pathol 30, 411-421.

Minta Z., Bugajak P., Daniel A., Tomczyk G., Koncicki A. (1995) Stan epidemiologiczny chorób wirusowych drobiu grzebiącego w Polsce. Materiały Konferencji: Aktualny stan epidemiologiczny i immunoprofilaktyki chorób drobiu" Puławy 1995, pp. 37-40.

Minta Z., Bugajak P., Karpinska E., Gough R., Cavanagh D., Mawditt K., Cox W. J., Bartnicka B., Wiercinski J. (1998) Isolation of a new strains of IBV from broiler chickens in Poland., Proceedings from International Symposium on Avian Corona- and Pneumovirus Infections. Rauischholzhausen, Germany, 15-18 June, pp. 180-185.

Worthington K.J., Savage C., Naylor C.J., Wijmenga W., Jones R.C. (2004) An RT-PCR survey of infectious bronchitis virus genotypes in the UK and selected European countries between 2002 and 2004 and the results from vaccine trial., Proceedings from IV Internationa Symosium on avian corona and pneumovirus infections. Rauischholzhausen, Germany, 20-23 June 2004 , pp. 125-133.

Table 1. Results of IBVs differentiation by multiplex nested PCR.

793/B (154bp)	D274 (217bp)	Mass (295bp)
Nobilis 4-91	Poulvac IB Primer	Poulvac IB Primer
Gallivac 88	ref. D274	V.H.+H120
ref. 793/B	ref. D1466	Cevac BIL
ref. D8880	ref. B1648	Nobilis IB Ma5
158/97	158/97	Bronhical II SPF
254/97	254/97	ref. M41
255/97	255/97	ref. Beaudette
197/99	197/99	
338/04	338/04	
29/05		
217/05		
1377/06		

Figure 1. Phylogenetic tree of the 464 bp fragment of the S1 nt sequence of Polish and GeneBank IBV strains.

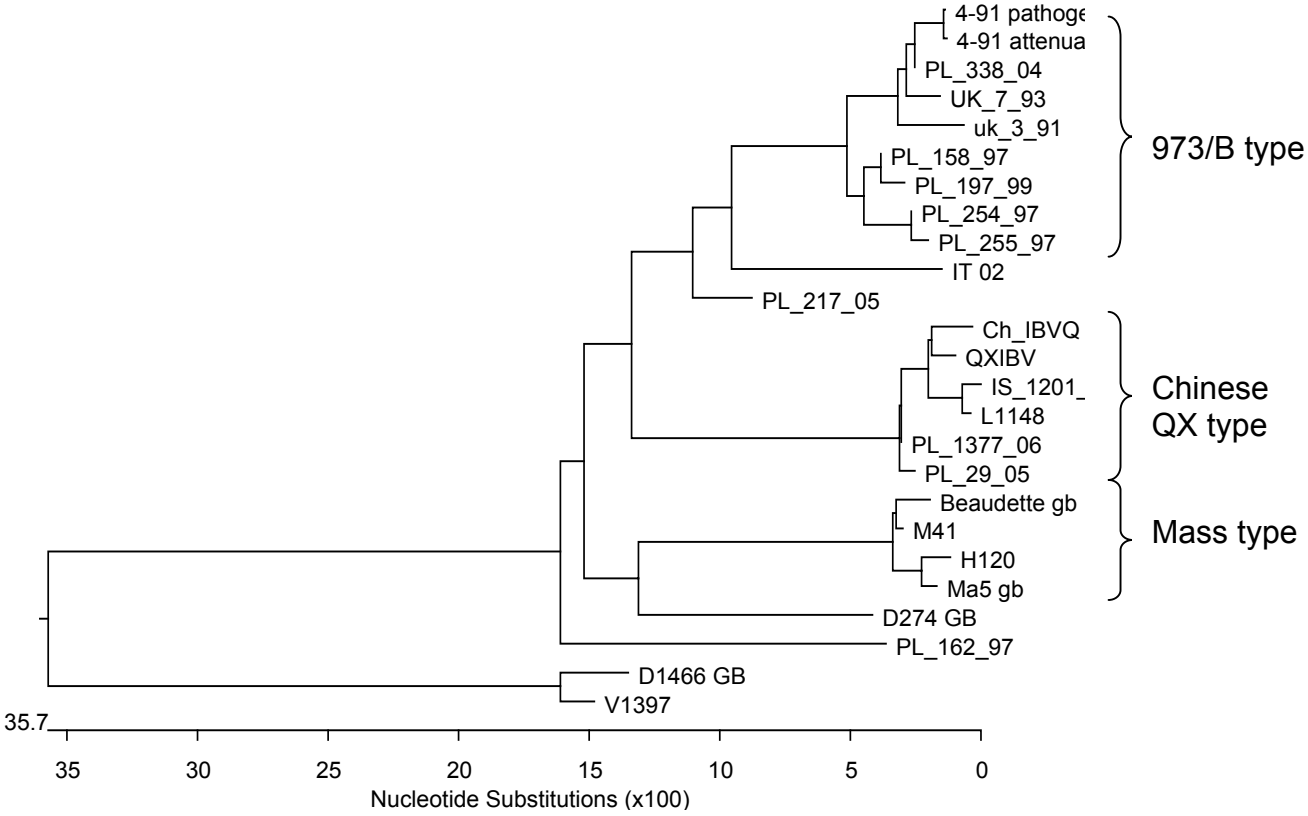

Table 2. Percent identity of Polish 162/97 and 217/05 isolates with the main IBV types.

	PL/162/97	PL/217/05
793/B	77,1	90,9
D1466	63,7	63,5
D274	78,2	81,1
Ma5	79,5	80,0
Beaudette	81,5	80,2
QXIBV	76,7	87,2
IT-02	77,5	88,3

INFECTIOUS BRONCHITIS VIRUS INFECTIONS IN GERMAN CHICKEN FLOCKS- RECENT FIELD OBSERVATIONS

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SUMMARY

Our diagnostic laboratory is regularly detecting infectious bronchitis virus with various methods. The widespread use of different kinds of vaccines is interfering with the diagnosis and is thus limiting the number of cases where field virus can be identified and typed. We could detect IBV field strains belonging to the serogroups 793B, Italy, Massachusetts, D-274, D-1466 and China, respectively, in samples taken from 2004 onwards. Kidney lesions caused by IBV are rare in Germany, the predominant clinical appearance is respiratory and reproductive.

INTRODUCTION

Infectious Bronchits Virus (IBV) is a frequently occurring infectious disease with considerable economic impact in Germany. The vaccination intensity and the use of vaccine strains other than Massachusetts has been continuously increasing during the last years. Still, field infections are not fully controlled. This paper discusses the possibilities and limitations of a routine diagnostic lab for the identification and typing of IBV isolations. Data from recent field cases from is presented and discussed.

MATERIALS and METHODS

IBV was isolated by serial culture of clinical specimens in spf chicken embryos (Lohmann Valo, Cuxhaven, Germany). The allantoic fluid of positive cultures was harvested and the presence of IBV was confirmed by RT-PCR using published protocols (Handberg et al., 1999) and commercial RNA-purification and amplification kits (Quiagen, Hilden, Germany). Further typing was attempted with serotype-specific nested mutiplex RT-PCR using published primers (Cavanagh et al., 1999; Capua et al., 1999) and primer sequences provided by Dr. Clive Naylor. In order to confirm PCR typing results and to identify strains which did not yield PCR products with the available primers, parts of the S1 gene were amplified by PCR and submitted to sequencing through a service laboratory (MWG, Martinsried, Germany). As field virus

could not be differentiated from vaccine virus with the available methods, we tried to exclude vaccine virus isolations from our study by assessing the degree of adaptation to laboratory host systems and using anamnestic data.

RESULTS

Table 1 lists field cases of laboratory confirmed IBV infections in German chicken flocks with the respective typing result.
Most isolations were related to 793B and different types of lesions were observed in conjunction with these infections. Massachusetts, D-274 and D-1466 were detected only once, respectively. Only one case of IBV Italy with typical kidney lesions was observed, supporting the field observation that kidney disease caused by IBV is a rare event in German broilers and pullets. IBV China- like strains were sporadicially detected from both layers and broilers, causing mostly respiratory disease.
We observed the long-known syndrome of “false layers”, which is now termed “hydropic oviduct degeneration”, on several occasions, but could not regularly identify IBV. The antibody titers of affected flocks were not suggesting recent IBV infections either. However, as “false layers” are described to result from early infections with virulent IBV, laboratory findings are not to be expected in mature birds, when these lesions become apparent. So far, in 2006, cases of hydropic oviduct degeneration were extremely rare.

DISCUSSION

Our small-scale survey confirmed that IBV strains described elsewhere in Europe are present in Germany, too. IBV China seems to be emerging and to be involved in cases with respiratory and reproductive pathology rather than kidney lesions. 793B was detected most frequently and often causing severe clinical disease. As a consequence, some demand for autogenous 793B inactivated vaccine developed. Further laboratory investigations are necessary to have a better overview about the epizootiology of IBV-infections in our area. Diagnostic laboratories are having difficulties with the interference of vaccine with field virus, this applies to the agent identification as well as for antibody testing. There is a need for robust and sensitive protocols for the serotype-specific detection of IBV and IBV-antibodies. Ideally, these methods would also allow the discrimination of field from vaccine virus.

REFERENCES

Capua, I; Minta, Z; Karpinska, E; Mawditt, K, Britton, P; Cavanagh, D; and Gough, RE (1999): Co-circulation of four serotypes of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). Avian Pathology (1999) 28, 587-592

Cavanagh, D; Mawditt, K: Britton, P, and Naylor, CJ (1999): Longitudinal field studies on infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathology 28, 593-605

Handberg, KJ; Nielsen, OL; Pedersen, MW; and JØrgensen, PH (1999): Detection and strain differentiation of infectious bronchitis virus in tracheal tissues from experimentally infected chickens by reverse transcription-polymerase chain reaction. Comparison with an immunohistochemical technique. Avian Pathology 28, 327-335

Table 1

Case #	Year	IBV serotype	Kind of chicken	Clinical observation
EK 464	2004	793B	broiler	n.a.*
EK 760	2004	793B	laying hen	w/o findings
K 100	2004	793B	laying hen	n.a.
K 101	2004	Massachusetts	laying hen	production drop, shell discolouration
K 1050	2004	793B	broiler	n.a.
K 1050	2004	D-274	broiler	rales, tracheitis
K 1091	2004	793B	broiler	n.a.
K 1200	2004	D-1466	backyard chicken	severe repiratory disease
K 1270	2004	Italy	broiler	n.a.
K 1352	2004	793B	laying hen	n.a.
K 1353	2004	793B	laying hen	"a lot of non-layers"
K 1477	2004	Italy	broiler	kidney lesions
K 1548	2004	China	laying hen	n.a.
K 1573	2004	793B	broiler	n.a.
K 1608	2004	793B	laying hen	n.a.
K 1650	2004	793B	laying hen	n.a.
K 169	2005	793B	broiler	n.a.
K 25	2005	793B	laying hen	n.a.
K 254	2005	793B	laying hen	n.a.
K 263	2005	793B	laying hen	n.a.
K 342	2005	793B	laying hen	hydropic oviduct dgeneration
L 306-2	2005	China	laying hen	production drop, IB suspected
K 1381	2005	China	broiler	respiratory disease

* no autopsy

CLINICAL DISEASE CAUSED BY THE CHINESE VARIANT OF INFECTIOUS BRONCHITIS (QXIBV) IN BROILER AND LAYER FARMS IN NORTHERN ITALY

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SUMMARY

Infectious Bronchitis (IB) is still causing significant health problems in the poultry industry with a high economic impact. The control of this disease is mainly based on the use of vaccination. The efficacy of this control measure depends on the cross protective characteristics of vaccine strains against the circulating viruses. The presence of several IB serotypes and the emergence of novel ones requires that vaccination programmes must be adapted to the prevalent serotypes. In order to establish which serotypes circulate in a selected areas surveillance programmes are currently considered the most efficacious tools. In Densely Populated Poultry Area (DPPA) of Northern Italy a targeted surveillance programme was carried out over a two year period (2004-2006) in order to isolate and characterise the IBV strains circulating in this area. During this surveillance effort a novel variant of IBV was identified as a Chinese strain (QXIBV). From the first identification of this Chinese strain in 2004, an increase in the incidence of cases and in the severity of clinical signs has been observed in affected flocks. This paper reports clinical disease caused by QXIBV in broilers and layers in Italian farms.

INTRODUCTION

Infectious Bronchitis (IB) still causes significant health problems in the poultry industry with a high economic impact due to the virulence of some strains and the cost of vaccination as a control measure for this disease. The presence of several serotypes make the control of the disease a challenge for the industry. Vaccination has been shown to be an efficacious tool and in most cases is an unavoidable intervention if the infection is to be kept under control. The major limitation of vaccination against IB is that vaccine strains and vaccination schemes must be

adapted according to the epidemiological situation of the affected area. In order to make vaccination against IB efficacious it is essential to monitor the presence of IB field strains and to investigate the antigenic properties of emerging variants.
Between 2000 and 2003 no field and laboratory investigations on IB were carried out in the densely populated poultry areas (DPPA) of Northern Italy, although clinical signs attributable to the presence of IB infection were reported. In order to establish which serotypes are circulating in Northern Italy a surveillance programme was carried out between 2004 and 2006 in a DPPA of Northern Italy, which contains 30% of the Italian poultry production. Two regions, Veneto and Lombardia, including eight provinces were involved in the monitoring programme. A total of 68 flocks including backyard flocks, broilers and layer farms were monitored. Farms were selected on the presence of clinical disease attributable to IB infection or in farms in which such clinical signs had previously been reported.

MATERIALS and METHODS

The surveillance programme was carried out by monitoring flocks through the introduction of sentinel birds. SPF (*Specific Pathogen Free*) chickens were introduced into farms for a period of 7-10 days as sentinels. Tissue samples, collected from sentinels or sick birds (trachea, lung, kidney and cecal tonsils) were directly analysed by RT-PCR and submitted for virological investigations. The tissue homogenates were inoculated into the allantoic cavity of 9-to-11-day-old embryonated SPF eggs (Gelb *et al,* 1998). Following a maximum of four blind passages, the allantoic fluid was harvested and examined by negative contrast electron microscopy for the presence of coronavirus particles (Hayat, 1985). In order to characterise the strains, viral RNA was extracted from the organs and from positive allantoic fluid. The strains were analysed in RT-PCR, with specific primers for the S1-gene (Adzhar *et al.* 1996). This cDNA, was sequenced (Keeler *et al.*, 1998). Isolates were typed on the basis of the sequence analysis.

RESULTS

The most interesting finding of the monitoring programme was the identification of a novel IB strain characterised as QXIBV (Beato et al., 2005) most likely introduced from the Far East.
The first identification of this novel variant was in a backyard flock with a high mortality late in 2004. The age of the animals affected was approximately forty days and the main gross lesion reported by the field veterinarian was nephritis with an increase in kidney volume.
For several months after the first detection of QXIBV, this strain was not detected in poultry flocks. In April 2005 the first identification of QXIBV occurred in intensively reared poultry farms. The incidence of QXIBV infection in the first 9 months of 2005 was mainly in broilers farms.
Eleven out of sixty-eight flocks monitored were positive for QXIBV, of which 3 were backyard flocks. The clinical disease reported in broiler flocks positive for QXIBV included respiratory signs, nephritis and nephrosis, urolithiasis and a mild increase in mortality. Birds affected shown depression, ruffled feathers, wet dropping, and decreased growth. The mortality rates were only 1-2% more than the normal

conditions and in some cases recovery occurred in 2-3 weeks. The age of animals affected was between 2-6 weeks. In September 2005 there was a suspicion of QXIBV in layers farms due to field evidence of “false layers syndromes” known to be associated to QX infection in Netherlands (Landman et al., 2005). In layers farms affected, the clinical disease was in keeping with “false layers” syndrome with presence of abundant liquid in the abdominal cavity. The oviduct morphology was abnormal, in some cases containing multiple cysts.

DISCUSSION

From the first detection of the QX variant in Italy in 2004 to the time of writing the scenario has changed with significant spread of infection and an increased severity of clinical findings.
The isolation of the QXIBV in Italy was possible due to the implementation of a monitoring programme aiming at isolating and characterizing IBV strains present in Northern Italy. The expected outcome of this monitoring programme was to establish what serotypes were prevalent in order to implement appropriate control measures. The need to carry out a surveillance programme was based on the evidence that IB is an evolving disease characterized by the emergence of novel serotypes. As a consequence vaccination programmes need to be adapted to the prevalent strains.
The results of this monitoring programme show that QXIBV is affecting 25% of all IBV positive flocks, which include different categories (backyard, layers, broilers).
QXIBV was first detected in China (Yu et al., 2001; Liu & Kong, 2004), although there are no data that indicated how this serotype reached the European continent and whether there has been one or multiple introductions from Asia. Moreover little is known about the pathogenic characteristics and antigenic properties of this isolate. The clinical signs observed in the Italian outbreaks of QXIBV are similar to those previously described in other countries with a high kidney tropism and the capacity to cause disease in layers (Liu and Kong 2004; Landman et al., 2005).
The widespread occurrence of QXIBV across Europe highlights the importance of implementing coordinated and continuous surveillance programmes, which aim at the identification of novel subtypes and of the understanding of the transboundary spread of evolving diseases such as IB.

REFERENCES

Adzhar, A., Shaw, K., Britton, P., Cavanagh, D., (1996). Universal oligonucleotides for detection of infectious bronchitis virus by the polymerase chain reaction Avian Pathology 25:817-836.

Beato MS, De Battisti C, Terregino C, Drago A, Capua I, Ortali G. (2005) Evidence of circulation of a Chinese strain of infectious bronchitis virus (QXIBV) in Italy. Veterinary Record. May 28;156(22):720.

Gelb, J., Jackwood, M.W., (1998). Infectious Bronchitis. In: Isolation and identification of avian pathogens, (Fourth ed.), The American Association of Avian Pathologists, pp 169-174.

Hayat, A.M., (1986). Basic techniques for transmission electron microscopy. Academic Press, Inc.

Keeler, C.L., Reed, K.L., Nix, W.A., Gelb, J., (1998). Serotype identification of avian infectious bronchitis virus (IBV) by RT-PCR of the peplomer (S-1) gene. Avian Disease 42(2):275-84.

Landman, W.J.M., Dwars, RE.M., de Witt J.J. (2005). High incidence of false layers in (re)production hens supposedly attributed to a juvenile infectious bronchitis virus infection. Proceedings of the fifty-fourth westerns poultry disease conference. April 25-27, Vancouver, Canada.

Liu S. &, Kong X. (2004). A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. *Avian Pathology* **33,** 321-327

Yu L, Jiang Y, Low S, Wang Z, Nam SJ, Liu W, Kwangac J. (2001) Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Diseases Apr-Jun;45(2):416-24.

AVIAN INFECTIOUS BRONCHITIS: ISOLATION OF AN UMPTEENTH NEW SEROTYPE OF VIRUS IN ITALY.

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SUMMARY

The isolation of a new serotype of avian infectious bronchitis virus in Italy is reported. The characteristics of the strains were investigated by molecular and serological tests, in comparison with the most common IBV serotypes, spread in these last 15 years in Europe, besides the Massachussetts and Arkansas serotype.
Two isolates, AZ-40/05 and FO-70856/05, would be very related from molecular point of view (nucleotides 98%, amino acids 100% homology), to the Chinese QX strain in the fragment S1 of the spike protein, but different from other considered serotypes (from 77 to 86%, regarding amino acids).
The same isolates, plus others two (1730/05 and 1732/05), presented antigenic characteristics different from all previously considered serotypes: 8 virus strains and a panel of 14 antisera, in which it was not possible to include the QX serum, were compared.
An interesting aspect is that all isolates adapted very soon (2 passages) to embryonated egg, reaching high titres (>$10^{7,5}$ EID_{50}) in 6-7 passages, with clear lesions in the embryo.

INTRODUCTION

For some decades, the appearance has been recorded of ever new serotypes or variants of infectious bronchitis virus (IBV) in Italy, as well as in the rest of Europe and other Continents. Up to now, over 60 different serotypes of virus worldwide and at least 13 in Italy, have been reported (7,10,16,17). Most of thm have been sporadic isolations, but sometimes their frequency has been quite high, persistent and widespread, as in case of Massachusetts, Australia T, Arkansas DPI, AZ-23/74, D-274, 793 B, AZ-27/98 and some other strains (5,14,17). The isolates, which have

spread in the last few years to many European countries and have often been reported as the “It-02” variant (12), seem to belong antigenically , or are very similar, to the AZ-27/98 serotype, isolated in Italy since 1997-98 (15,17).
The main reason for such antigenic instability in IBV would derive from the particular mechanism of coronavirus replication, which occurs by discontinuous transcription of RNA (6 mRNAs), with consequent changes in nucleotide and, consequently, in amino acid sequences of the S1 part of the virus spike protein (3). It is believed that, if a cell becomes infected by two different virus particles, new recombinants may be generated. However, the possibility of spontaneous appearance of a latently pre-existent serotype cannot be excluded. Biomolecular research has shown that a new serotype can emerge as a result of just a few changes in amino acid composition of the S1 spike protein, with the majority of the viral genome remaining unchanged (4).
The tropism of the different strains of virus is also becoming broader and more variable: respiratory, urogenital, gastrointestinal or mixed, both in layers and in broilers. Moreover, coronaviruses of the same group, to which IBV belongs (3rd group), have been isolated from other wild and domestic birds; they are currently being studied (5).
The objective of the present paper is to report on certain recent isolations of IBV from chickens with severe kidney lesions, seeming to be similar or identical to each other, but apparently different from the other main serotypes, already known and widespread in Italy and throughout the world.

MATERIALS and METHODS

Clinical outbreaks

Light six-week-old pullets, vaccinated as one-day-old with Mass H120, showing mild respiratory and enteric symptoms, but very severe kidney lesions, with mortality varying from 0.2 to 1.0 % over two weeks in 3 different houses with 3 different breeds (isolate AZ-40/05);
broilers of three separate farms, five-wk-old, previously vaccinated as one-day-old with Mass H120 and as 18/20-day-old with UK-4/91, showing depression, respiratory and enteric symptoms with wet litter and very severe kidney lesions and a mortality varying from 0.5% to 3.5% (1730/05, 1732/05 and FO-70856/05 isolates).

Substrate

9/11 day-old specific pathogen free (SPF) embryonated chicken eggs (Valo or Hyvac).

Virus isolation

Samples of kidney tissue, from chickens showing lesions, were homogenized in phosphate buffered saline (1:5 w/v), treated with antibiotics and inoculated into allantoic sac of 10 embryonated eggs. After incubation at 37°C for 8 days, the eggs were candled daily and examined for specific IBV lesions(15). Allantoic fluids from a number of eggs were harvested 48-72 hours post- inoculation. At least eight serial passages were performed before to do the cross neutralization test.

IBV strains
In addition to the mentioned isolates, also the Mass.41, AZ-23/74, Arkansas DPI, UK-4/91 and AZ-27/98 serotypes were used .

IBV monospecific antisera
A panel of specific polyclonal antisera was used: AZ-40/05 (new isolate), AZ-27/98, FO-4682/99 (It -02), AZ-23/74, Mass.41, Arkansas DPI, Gray, Australia T, UK-4/91 (793 B), AZ-446/66, 624I, PV-1731/65 and BS-216/01. All sera were raised in 4 to 6-wk-old SPF chickens, kept in isolation units, inoculated twice at an interval of 21 days and bled 2 wk later; the sera were filtered through Millipore 0.22 □m membrane, inactivated at 56°C for 30 min., lyophilized and stored at -20°C (15).

VN test
To establish the possible correlation of new field isolates with some of the most common European, American, and Australian IBV serotypes, VN tests were performed in embryonated eggs, according to the method variant virus–constant serum ($\log_{10}$ virus dilution and 1:5 serum dilution). Virus and serum were kept in contact for 1 hr and then the mixture was inoculated in eggs (five eggs per dilution). The embryos were candled and examined for specific lesions within 8 days. The neutralization indexes (NIs) were calculated according to the well-known method of Reed and Muench. Only values of NI> 2 $\log_{10}$ were considered positive. In the test a negative serum was always included.

Molecular characterization
Reverse transcription-polymerase chain reaction (RT-PCR) test was performed according to described methods (4,6) on allantoic fluids, using primers that recognize the following most common European IBV types: Mass.41, 793B and FO-4282/99 (It 02). RNA was extracted from allantoic fluids and purified by the method of Chomezynski and Sacchi (6), with minor modifications and stored at –20°C. RNA was detected by RT-PCR assay, with XCE1+(5' CTC TAT AAA CAC CTT ACA 3') and XCE3– (5' CAG ATT GCT TAC CAA ACC ACC 3') primers, which are able to amplify a fragment of 383 bp of hyper variable region,, common to all IBV types. Then, a nested PCR was performed combining XCE3– with BCE1+ (5' AGT AGT TTT GTG TAT AAA CCA 3') and MCE1+ (5' AAT ACT ACT TTT TAG GTT ACAC 3') primers, specific respectively for 793B and Mass.41 IBV strains, able to amplifly cDNA fragments respectively of 154 and 295 bp (4). In addition, a nested PCR was performed using It-O2-F (5' TCA AAC GAA AGT AAT GCT CCA 3') and It-O2-R (5' GTA TAA ACA CCC TTA CAC TTA GT 3') primers specific for FO-4682/99 (It-O2) strain, able to amplify cDNA fragment of 330 bp. The amplified fragments were analyzed by 1.7% agarose gel electrophoresis, stained with ethidium bromide and observed with an ultraviolet transilluminator. A molecular weight standard was added and used to determine the size of the cDNA fragments.
Direct sequencing of various strains was performed on PCR products after gel purification with Qiaquick gel extaction kit (QIAGEN) with primers XCE1+ and XCE3- (ref. 15) by means of a Big Dye Terminator DNA Sequencing Kit on ABI Prism 310 Automatic Sequencer (Applied Biosystem, Foster City, CA). Comparative analysis of nucleotide and deduced amino acid sequences was carried out with ClustalW Package of DNAStar (MegAlign v.5, 2001; DNAStar inc., Madison, WI).

RESULTS

VN test

The AZ-40/05 1730/05, 1732/05 and FO-70856/05 isolates showed neutralization (NI> 6.5 $\log_{10}$) by an antiserum obtained from convalescent birds infected with AZ-40/05 isolate and from SPF chickens inoculated with the 8th passage of such virus, at 3 and 7 wks of age While, they were not neutralized (NI<2.0 $\log_{10}$) by any other antisera against many previously-reported serotypes. The AZ-40/05 antiserum did not neutralize any other better-known IBV serotype. However, the complete results are shown in table 1.

RT-PCR test and sequence analysis

RT-PCR tests showed that the AZ-40/05 isolate seems to be related to Chinese QX isolate, but unrelated to the most common European IBV types 793B, AZ-27/98, FO-4882/99 (It-02), as well as Mass.41.

Nucleotide sequence analysis of partial genomic S1 region (127 nucleotides) revealed that AZ-40/05 seems to be related to QX isolate, showing 97.9% homology; as far as it concerns amino acid sequence, AZ-40/05 isolate showed no substitution compared to QX isolate (100% homology).

From the nucleotide and amino acid sequence analysis, the AZ-40/05 isolate showed very high homology with the QX Chinese reference strain (10), against only c. 86% to 77% with other IBV types previously reported. Anyway, the complete results of amino acid sequences of S1 spike protein of six different strains of virus are reported in table 2.

DISCUSSION

A new serotype of IBV was isolated during 2005 in some poultry-intensive areas of Italy (1,18). As regards our isolates, AZ-40/05, 1730/05, 1732/05, FO-70856/05, from the immunological point of view, they differ completely from the serotypes or variants reported prior to 2004 in Europe and from some of the most common serotypes found in the USA and in Australia. However, very little is known as regards the most recent isolates in Asia, of which not much is known. Some recent isolates in Italy, including the AZ-40/05 isolate, seem to be related, from the molecular point of view (100% amino acid homology) to the Chinese QX strain (9) with regard to the S1 fragment of the spikes; while, with regard to the most common European serotypes, this is only about 86% and only 77% for the Massachusetts serotype. Similar strains have been isolated in some European countries, with the first described appearance (D-388 strain) in The Netherlands in 2004 (8, 13). Nevertheless, it seems to be rash to consider them as a strain coming from China, on the grounds of the genetic characteristics of a part of the spike, as has been asserted during some meetings or in certain papers. It seems more appropriate to consider them as QX-like. So far, serological tests have not yet been reported for other QX-like isolates.

All four strains isolated in our laboratories adapted unusually quickly (two passages) to embryonated eggs, inducing classic very severe lesions to the embryo (stunting, dwarfing, curling, mesonephros persistence) and very rapidly reaching high titres (> 7.5-8.5 $\log_{10}$ EID_{50}/0.1 ml), after 6-8 passages (18). Such a situation never occurred,

at least regarding our previous isolations, except in case of vaccinal strains, as occurred with many isolates of Dutch variants D-274 and D-1466 and English strain UK-4/91, after their introduction to Italy as vaccines (17). This behaviour could lead to also consider a possible new recombination between a vaccinal strain well-adapted to embryo and another pre-existent strain. Further investigations, which should be extended at least to the European Continent, could allow the origin and spread of these new types to be determined, together with their exact relationship to the Chinese QX strain. On this matter, research conducted in Quebec (Canada), has shown that certain IBV isolates, which bound two different monoclonal antibodies considered specific to the Massachusetts and Arkansas serotypes, after sequencing showed a part of the S1 spike protein gene, which was typical of the Massachusetts serotype, whereas the remainder was similar to the Arkansas serotype. Because the birds had been vaccinated with Massachusetts strain, a recombination could have occurred between the two viruses (11). A mosaic of Mass+B1648+793B sequences was also reported in Hungary, revealing a potential field recombinant (2). It follows that sequencing of various isolates should be broadened in order to better define the right type of virus and its eventual origin and relation to other types

More detailed molecular biology and immunological studies lie ahead in various laboratories. Nevertheless, broader cooperation and comparison of work between different laboratories would be appropriate and desirable to obtain more profitable results and draw more accurate and unequivocal conclusions on such an important and, as regards certain aspects, complex infectious disease.

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REFERENCES

1. Beato, M.S.,C. De Battisti, A. Drago, S. Fassino, C. Terragino, G. Cattoli, and I. Capua. Circulation of IBV strains in Northern Italy. Proc. 14th WVPA Congress, Istanbul, 22-25 August 2005, p 262,2005
2. Bratu, H., C. Miklo, T. Matò, J. Benieda, Z. Pénzes, and V. Palya. Epidemiology of avian IBV in Hungary. Proc. 4th International Symposium on Avian Corona-and Pneumovirus Infections, Rauishholzhausen, Germany 20-23 June 2004. pp. 117-124, 2004.
3. Cavanagh D.,P. J. Davis, and J.K.A. Cook. IBV: evidence for recombination within the Massachusetts serotype. Avian Pathol., 21: 401-408, 1992.
4. Cavanagh, D., D.K. Mawditt, P. Britton, and C. J. Nylor. Longitudinal field studies of IBV and avian pmeumovirus in broilers using type specific polymerase chaine reactions. Avian Pathol. 28:593-605, 1990.
5. Cavanagh, D. Susceptibility of domestic and other birds to coronavirus. Proc. 14th WVPA Congress, Istanbul , 22-25 August 2005, pp. 74-81,2005.
6. Chomezynski, P.,and N. Sacchi. Single- step method of RNA isolation by acid guanidinium thiochanate-phenol-cloroform extraction. Anal. Biochem.162:156-159,1987.

7. Cook J.K.A.Infections Bronchitis–worldwide situation: publications on IB since 1998. Proc. 4th International Symposium on Avian Corona-and Pneumovirus Infections, Rauishholzhausen, Germany 20-23 June 2004. pp. 1-7,2004.
8. Landman, W.J.M, R.M. Dwars, and J.J. de Wit. High incidence of false layers in (re)production hens supposely attribuited to a juvenile IBV infection. Proc. 14th WVPA Congress, Istanbul, 22-26 August 2005, p. 369 (poster),2005.
9. Liu, S., and Kong, X. A new genotype of nephropathogenic IBV circulating in vaccinated and non vaccinated flocks in China. Avian Pathol. 33: 321-327,2004.
10. Rinaldi,A., A. Crespi, G. Cervio, and G. Mandelli. Isolamento di un ceppo nefropatogeno del IBV del pollo. Selezione Veterinaria, 7; 284-289, 1966.
11. Silim, A., and Marandi M.V. Molecular characterization of a new strain of IBV in Quebec. Proc. 3th International Symposium on Infectious Bronchitis and Pneumovirus Infections in Poultry. Rauishholzhausen, Germany 15-18 June 1998, pp. 226-228,1998.
12. Worthington, K.J,C. Savage, C.J. Nylor, W. Wiymenga, and R.C. Jones. An RT-PCR survey of IBV and avian pneumovirus in Europe between 2002 and 2005. Proc. 14th WVPA Congress, Istanbul, 22-26 August 2005, p 252, 2005.
13. Worthington K.J, C. Savage. C:J. Nylor, W. Wiymenga, and R.C. Jones. An RT-PCR survey of IBV genotypes in UK and selected european countries between 2002 and 2004 and the result of a vaccine trial. Proc. 4th International Symposium on Avian Corona-and Pneumovirus Infections, Rauishholzhausen, Germany 20-23 June 2004. pp. 125-133, 2004.
14. Zanella, A., and P. A. Martino. IBV in Italy: persistence of nephropathogenic strains related to serotype AZ-23/74. Proc. 3th International Symposium on Infectious bronchitis and Pneumovirus Infections in Poultry. Rauishholzhausen, Germany 15-18 June 1998, pp. 189-197, 1998
15. Zanella, A.,A. Lavazza, R. Marchi, A. Moreno Martin, and F. Paganelli. Avian Infectious Bronchitis: characterization of new isolates from Italy. Avian Diseases, 47: 180-185,2003.
16. Zanella, A.,R. Ceruti, and L. Gavazzi. Correlazione antigenica tra sierotipo Fa-6881/97 e AZ- 27/98 ed i ceppi “It 02“ di IBV isolati più recentemente in Europa. Atti XLIII Convegno S.I.P.A., Forlì 7-8 Ottobre 2004. pp 59-61,2004 and World Poultry 21:37,2005.
17. Zanella, A., R. Ceruti, and L. Gavazzi. Infectious Bronchitis: an overview of European epidemiological situation. Proc. 54th Western Poultry Disease Conf., Vancouver, Canada, 25-27 April, 2005. pp.94-97,2005.
18. Zanella, A., and L. Gavazzi. Bronchite infettiva aviare:isolamento di un ennesimo nuovo sierotipo del virus in Italia. Atti XLIV Convegno S.I.P.A., Forli’, 29-30 Settembre 2005 pp. 12, 2005.

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Table 1: Virus-neutralization test among recently-isolated IBV strains (AZ-40/05 and FO-70856/05) and numerous serotypes previously isolated. Values are expressed as neutralization index (NI).

A— = NI < 2 $\log_{10}$; B n.d.= not done

Antisera	Virus strains							
	AZ-40/05	FO-70856/05	AZ-27/98	FO-4682/99 (It-02)	UK 4/91	AZ-23/74	Ark DPI	Mass. 41
AZ-40/05	≥ 6.5	≥ 6.2	**---**	---	**---**	**---**	**---**	**---**
AZ-27/98	---A	---	≥ 7.0	5.0	2.7	---	---	---
Fo 4682/99 (It 02)	---	---	5.4	6.0	---	---	---	---
Bs 216/01	---	n.d.B	---	---	---	---	---	---
Mass. 41	---	---	---	---	n.d.	---	---	≥ 6.0
PV 1731/65	---	---	2.5	n.d.	n.d.	---	n.d	n.d.
AZ-446/66	---	n.d.	---	n.d.	---	---	n.d.	n.d.
AZ-23/74	---	---	---	---	---	≥ 6.0	---	---
624 I	---	n.d.	---	---	n.d.	3.0	n.d.	n.d.
Gray	---	---	---	n.d.	n.d.	---	---	---
Australia T	---	---	---	---	---	---	---	---
Arkansas DPI	---	n.d.	---	---	---	---	≥ 6.5	---
D 274	---	---	---	---	---	---	---	---
UK 4/91	---	---	---	---	5.2	---	---	---

Figure 1: Amino acid sequence of the S1 protein. Partial sequences of strain M41, 793/B FO-4682/99 (It.02), AZ-27/98 and QX-IBV are compared to AZ-40/05. Dots indicate sequence identy.

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                          10        20        30        40        50
M41                T G N F S D G F Y P F I N S S L V K Q K F I V Y R E N S V N T T F T L H N F T F H N E T G A N P N P
793/B              . . . . . . . . . . . T . . . . . . D R . . . . . . S . T . . . L E . T . . . . T . V S N . S . . S
4682/99=Italy 02   . . . . . . . . . . . T . A . . . . D R . V . . . . S . . . . . L E . T . . . . S . . S N . P . . S
AZ-27/98           . . . . . . . . . . . T . . . . . . D R . . . . . . S . I . . . L E . T . . . . T . V S N . A . . S
QX-IBV             . . . . . . . . . . . T . . T . . R E . . . . . . . S . . . . . L A . T . . . . T . V S N . Q . . S
AZ-40/05           . . . . . . . . . . . T . . T . . R E . . . . . . . S . . . . . L A . T . . . . T . V S N . Q . . S

                          60        70        80        90       100
M41                S G V Q N I L T Y Q T Q T A Q S G Y Y N F N F S F L S S F V Y K E S N F M Y G S Y H P S C N F R L E
793/B              G . . D T F Q L . . . H . . . D . . . . . . L . . . . . . . . . . P . D . . . . . . . . . N . . . . P .
4682/99=Italy 02   G . . N T F V L . . . H . . . . . . . . . . L . . . . . . . . . . P . D . . . . . . . . . K . . . . P .
AZ-27/98           G . . D T F Q L . . . H . . . D . . . . . . L . . . . . . . . . . S . D . . . . . . . . . K . . . . P .
QX-IBV             G . . N T F H L . . . . . . . . . . . . . . . . L . . . . Q . . . . A . D . . . . . . . . . . . S . . P .
AZ-40/05           G . . N T F H L . . . . . . . . . . . . . . . . L . . . . Q . . . . A . D . . . . . . . . . . . S . . P .

                         110       120
M41                T I N N G L W F N S L S V S I A Y G P L Q G G C K Q S D
793/B              N . . . . . . . . . . . . . . L T . . . I . . . . . . . .
4682/99=Italy 02   N . . . . . . . . . . . . . . L T . . . I . . . . . . . .
AZ-27/98           N . . - . . . . . . . . . . . L T . . . I . . . . . . . .
QX-IBV             . . . S . . . . . . . . . . . L T . . . . . . . . . . . .
AZ-40/05           . . . S . . . . . . . . . . . L T . . . . . . . . . . . .
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EVOLUTION OF 4/91 INFECTIOUS BRONCHITIS VIRUS GENOTYPE IN SPAIN

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SUMMARY

Preliminary results of a molecular epidemiologic survey of IBV showed 4/91 genotype to be one of the most common IBV genotypes in Spain. In order to reach a better understanding of the epidemiologic evolution of this genotype, taking into account its epidemiologic relevance and the lack of information with regards to 4/91 genotype in Spain, it was considered important to further characterize those isolates. The Sequence analyses and phylogenetic studies were carried out on the complete S1 gene sequences of the 13 isolates. Moreover cross VN was implemented in the Spain/99/319 isolate. Phylogenetic analyses suggested that Spanish isolates could have evolved from distinct ancestors. However, cross VN indicated that unique evolutive processes might have occurred leading a decreased antigenic relatedness between some Spanish isolates and 4/91 reference strain.

INTRODUCTION

Infectious bronchitis (IB) is an acute, highly contagious, worldwide distributed respiratory disease in chickens. In 2002, an epidemiologic survey among the most important poultry companies in Spain pointed out IB as one of the poultry diseases with higher economic impact in poultry production. Because of this fact, a molecular epidemiological study to investigate the past and current situation of IB viruses (IBV) in Spain was started. Preliminary results based on sequence analysis of a little region of the S1 protein showed 4/91 IBV genotype to be one of the most common isolated in Spain.

4/91 serotype, also named as 793/B, was first described in UK in the early nineties associated with respiratory disease and also with bilateral myopathy in layer hens (Gough et al., 1992; Parsons et al., 1992). In subsequent years, this serotype was demonstrated to be widespread not only thorough UK, but also to several other European and Asiatic countries. However, recent published studies have

demonstrated the presence of 4/91 strains in France in the mid eighties, and a close relationship between these isolates and those isolated in UK in the nineties (Cavanagh et al., 2005).
Although vaccination programs against 4/91 serotype have been implemented in Spain, scientific information about the situation of Spain regarding this genotype is still unavailable. Moreover, taking into account that these strains were isolated during a nine years period it was thought to be interesting to further investigate their sequences to reach a better understanding of IBV epidemiology in Spain. The aim of the present study was to further molecularly characterize Spanish 4/91 strains in order to determine the evolution of 4/91 genotype in Spain.

MATERIALS and METHODS

Viruses

Thirteen Spanish strains isolated between 1992 and 2005 were included in this study. Epidemiological information of these isolates is summarized in Table 1. All these isolates were isolated from chicken tissues and passaged in embryonated chicken eggs. Allantoic fluid samples were kindly provided by CESAC (Centre de Sanitat Avícola de Catalunya I Aragó). The reference IBV serotypes M41, D274, D1466 and 4/91, used in the VN assay, were acquired from the Animal Health Centre, Deventer (The Netherlands).

S1 gene sequencing

Viral RNA was extracted from allantoic fluid samples with the Nucleospin RNA Virus Kit (Macherey-Nagel) following manufacturers instructions. An RT-PCR to amplify the complete S1 gene was carried out as previously described (Adzhar et al., 1996). The 1800-bp RT-PCR products were purified by QIAquick gel extraction Kit (Qiagen) according to the manufacturer's instructions. Purified RT-PCR products were sequenced using ABI PRISM BigDye® Terminator v3.1 Cycle Sequencing Kit (PE Biosystems) as described by the manufacturer. Sequences were analyzed with an automated nucleic acid analyser (ABI PRISM 3100 Avant; PE Biosystems)

Nucleotide and amino acid deduced sequence analyses

Nucleotide and amino acid deduced sequences were aligned using ClustalX software included in BioEdit 5.0 package. Neighbour-Joining method with 1000 bootstraps replicates within the software *MEGA* version 3.0 (Kumar et al., 2004) was used to perform phylogenetic analyses. The average ratio d_S/d_N for the S1 gene was conducted by the Nei and Gojobori method (Jukes Cantor distance) also included in the software *MEGA* version 3.0.

GenBank accession numbers

S1 gene nucleotide and amino acid deduced sequences of the 13 Spanish field isolates were submitted to the GenBank nucleotide database. GenBank accession numbers for these isolates are presented in Table 1. GenBank accession numbers of the IBV isolates used in the phylogenetic comparisons included: FR-85131-85 (AJ618985); FR-CR88061-88 (AJ618986); FR-96047-94 (AJ818987); IR-1062-GA (AY544777); IR-3654-VM (AY544776); IR-1061-PH (AY544778); UK/2/91 (Z83976);

UK/3/91 (Z83977); UK/5/91 (Z83978); UK/7/91 (Z83975); UK/7/93 (Z83979); UK-1233-95 (AJ618984); 4/91 pathogenic (AF093794).

Cross-VN analysis

Field isolate Spain/99/319 was serologically characterized by reciprocal VN test against the main reference IBV serotypes M41, 4/91, D274 and D1466 by the beta procedure as previously described (Thayer and Beard, 1998). Monospecific antisera against field strains Spain/99/319 was raised by immunizing twice 4-weeeks-old specific pathogen free (SPF) chickens as previously described (Gelb and Jackwood, 1998). Monospecific antisera against the main reference IBV serotypes were purchased from the Animal Health Centre, Deventer (The Netherlands). IBV strains were titrated in ten-day-old to 11-day-old embryonated SPF chicken eggs by standard procedures (Villegas, 1998). Endpoint titers were calculated by the Reed and Müench method. The VN titers against homologous and heterologous were used to calculate the antigenic relatedness (*r*-value) by the formula of Archetti and Horsfall (Archetti and Horsfall, 1950).

RESULTS

Nucleotide and amino acid deduced sequence analyses

Phylogenetic analyses comparing the complete S1 gene sequences of the 13 Spanish field isolates and 4/91 British, French and Iranian isolates, previously published in the GenBank database revealed that Spanish isolates were clearly clustered in two distinct groups identified as Group 1 (including 11 isolates) and Group 2 (including 2 isolates) (Figure 1). While Group 1 formed a new own separated cluster, Group 2 was branched with French isolate FR-94047-94.

S1 gene amino acid divergence among isolates in Group 1 ranged from 0,4% to 8,6%, while both isolates in Group 2 diverged 2,1% at amino acidic level. Average nucleotide and amino acid identities shared by both Groups were 93,1% and 91,3% respectively. The minimum amino acid divergence observed between two isolates from the same year was 0,4% (between Spain/92/185 and Spain/92/51), whereas the maximum was 9,9% (between Spain/99/319 and Spain/99/327).

Both Group 1 and Group 2 isolates showed maximum average nucleotide (96% and 95,3% respectively) and amino acid (93,5% and 94,4% respectively) identities with French isolate FR-94047-94. Amino acid identities between Group 1 and the other 4/91 isolates included in the comparison ranged from 89,7% to 93,8%. S1 sequences from Group 2 isolates had 89,6%-93,2% amino acid identities with the rest of 4/91 sequences.

Comparison of Spanish field isolates sequences revealed a common unique deletion at amino acid position 58 (located in HVR1) in 6 isolates included in Group 1. Moreover 4 isolates within this same group shared an amino acid deletion at position 340, located in HVR3. Both isolates included in Group 2 showed 8 unique amino acid substitutions. In addition, isolate Spain/99/327 in Group 2 showed 3 amino acid deletions at positions 55, 56 and 57 (located in HVR1).

The average ratio d_S/d_N within the 13 Spanish filed isolates was estimated to be 3,43. No cumulative changes were observed among Spanish isolates.

Cross-VN analysis

Isolate Spain/99/319 shared a 59,8% antigenic relatedness with 4/91 reference IBV strain. Antigenic relatedness values shared by Spain/99/319 isolate with the rest of IBV reference strain were lower than 50%: 27,3% with M41, 46,3% with D274 and 33,8% with D1466.

DISCUSSION

Since it was first described in the early nineties, 4/91 IBV serotype has spread over many other countries and has become one of the most predominant serotypes in Europe. The present study has demonstrated that 4/91 genotype has been circulating in Spain for at least a nine years period. Although IB viruses have been isolated from suspect cases submitted to our laboratory after 2000, no 4/91 genotype strains have been isolated in recent years. This observation might be related with the fact that vaccination programs against this serotype were implemented in Spain since last nineties.

Nucleotide and amino acid deduced identities and phylogenetic analyses clearly indicate that Spanish field 4/91 genotype isolates can be split in two major genetic clusters. Moreover, sequence identity data showed high genetic diversity among Spanish 4/91 isolates, even those grouped in the same phylogenetic cluster. This finding suggests that 4/91 genotype has gone through evolutive changes during these years. However, our results also demonstrated that two Spanish 4/91 strains isolated the same year diverged by 9,9% in amino acid sequences, and were clustered in separate phylogenetic groups. Although a very rapid evolutive rate from a common ancestor might have lead to a similar situation, no cumulative changes were observed among Spanish 4/91 isolates and unique amino acidic changes observed in one of these two groups were not shared by the other. Moreover, d_S/d_N ratio within 4/91 Spanish isolates was significantly higher than 1, indicating not only that genetic changes observed in these isolates were not under positive selection pressure, but also that the evolution rate was not very high. Therefore, taking into account these data it could be suggested that 4/91 strains isolated in Spain have evolved from at least two distinct evolutive lineages probably originated in two different ancestors. Similar scenarios have been previously described in other countries (Adzhar et al., 1997).

Although there were little unique amino acid changes shared in each group, some of these substitutions were located within S1 regions identified as neutralizing epitopes (Cavanagh et al., 1988; Kant et al., 1992). In order to determine possible antigenic changes induced by these amino acid substitutions, cross VN assay was carried out with field Spanish isolate Spain/99/319. Surprisingly, cross VN results between reference 4/91 strain and Spain/99/319 isolate showed an antigenic relatedness of 59,8%. This result indicates that although this isolate still belongs to the 4/91 serotype, it has undergone unique evolutive processes that have decreased its antigenic relatedness with the 4/91 serotype and the future possibility to emerge as a new serotype could not be excluded.

REFERENCES

Adzhar, A., Gough, R.E., Haydon, D., Shaw, K., Britton, P., Cavanagh, D., 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. Av Pathol 26, 625-640.

Adzhar, A., Shaw, K., Britton, P., Cavanagh, D., 1996. Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction. Av Pathol 25, 817-836.

Archetti, I., Horsfall, F.L., 1950. Persistent antigenic variation of influenza A viruses after incomplete neutralization in ovo with heterologous immune serum. J. Exptl. Med. 92, 441-462.

Cavanagh, D., Davis, P.J., Mockett, A.P., 1988. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res 11, 141-150.

Cavanagh, D., Picault, J.P., Gough, R., Hess, M., Mawditt, K., Britton, P., 2005. Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathol 34, 20-25.

Gelb, J., Jr., Jackwood, M.W., 1998. Infectious Bronchitis. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A laboratory manual for the isolation and identification of avian pathogens. The American Association of Avian Pathologists, Pennsylvania, pp. 169-174.

Gough, R.E., Randall, C.J., Dagless, M., Alexander, D.J., Cox, W.J., Pearson, D., 1992. A 'new' strain of infectious bronchitis virus infecting domestic fowl in Great Britain. Vet Rec 130, 493-494.

Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A., van der Zeijst, B.A., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J Gen Virol 73 (Pt 3), 591-596.

Kumar, S., Tamura, K., Nei, M., 2004. Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5, 2.

Parsons, D., Ellis, M.M., Cavanagh, D., Cook, J.K., 1992. Characterisation of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet Rec 131, 408-411.

Thayer, S.G., Beard, C.W., 1998. Serologic procedures. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A laboratory manual for the isolation and identification of avian pathogens. The American Association of Avian Pathologists, Pennsylvania, pp. 255-266.

Villegas, P., 1998. Titration of biological suspensions. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M. (Eds.), A laboratory manual for the isolation and identification of avian pathogens. The American Association of Avian Pathologists, Pennsylvania, pp. 248-254.

Table 1. Epidemiological information of Spanish field isolates included in the study. Isolates are sorted by year of isolation.

ISOLATE	ISOLATION YEAR	TYPE OF BIRD	AGE	TISSUES FOR ISOLATION	REGION	GENBANK ACCESSION NUMBER
Spain/92/35	1992	Broiler		Trachea		DQ386091
Spain/92/51	1992	Broiler		Trachea		DQ064801
Spain/92/185	1992	Broiler		Trachea		DQ386092
Spain/95/193	1995	Broiler		Trachea		DQ386093
Spain/95/194	1995	Broiler		Trachea		DQ064802
Spain/96/312	1996	Broiler	55d	Trachea	Girona	DQ064803
Spain/96/330	1996	Broiler	32d	Trachea, Kidney	Galicia	DQ386094
Spain/97/307	1997	Broiler	43d	Trachea	Girona	DQ064805
Spain/98/315	1998	Broiler	35d	Trachea, Kidney, Tonsil	Levante	DQ386095
Spain/98/328	1998			Trachea, Kidney		DQ386096
Spain/99/319	1999	Label	38d	Trachea, Kidney, Tonsil	Tarragona	DQ064810
Spain/99/327	1999	Broiler	49d	Trachea	Lleida	DQ386097
Spain/00/336	2000	Broiler	44d	Trachea	Tarragona	DQ386098

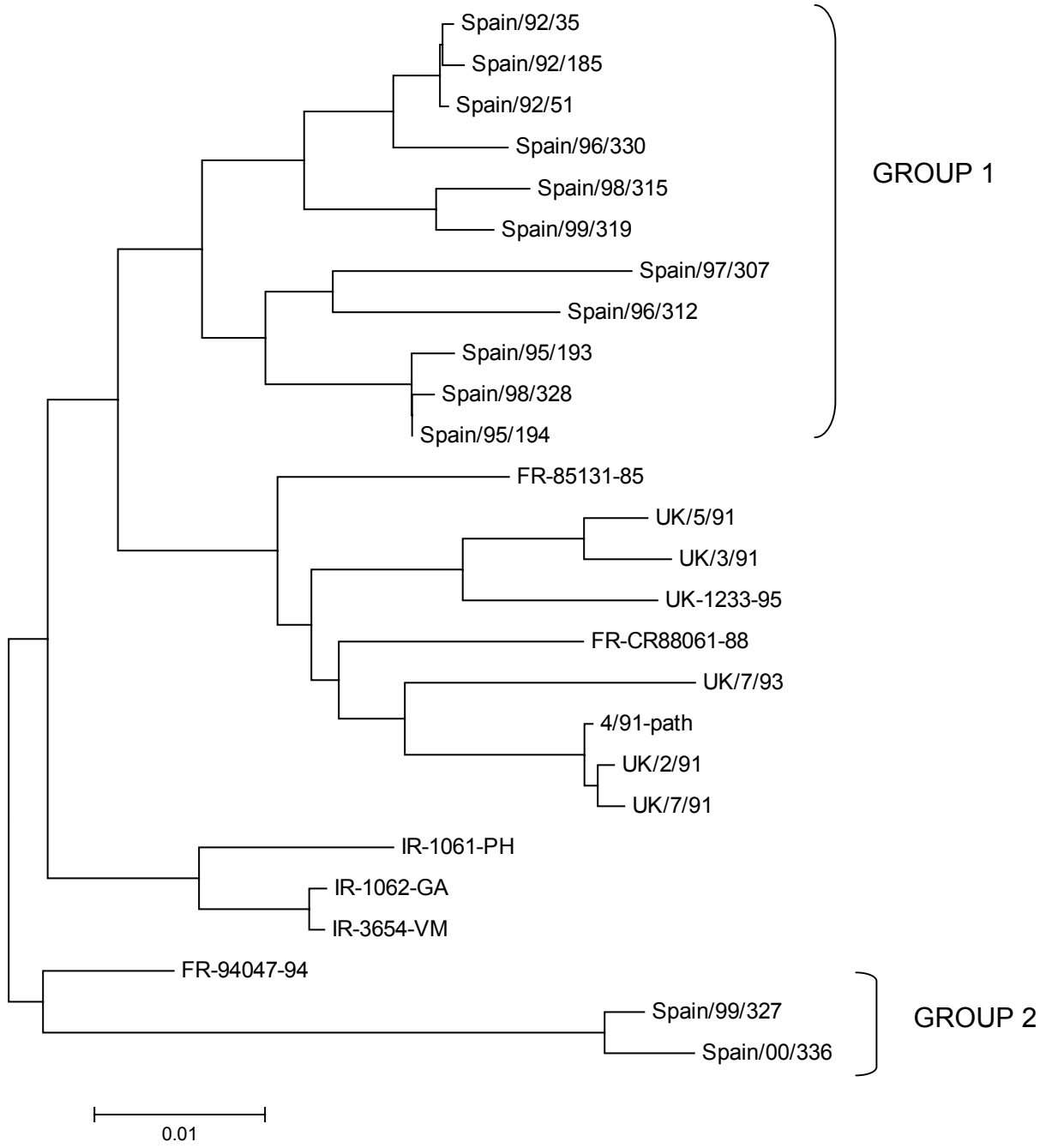

Figure 1. Neighbor-Joining with 1000 bootstraps replicates phylogenetic tree. Distances were estimated by the Kimura-2 parameter method. The lenght of the bar indicates number os substitutions per site. Nucleotide sequences from 13 Spanish isolates and 4/91 genotype strains previously published in the GenBank database were included in the comparison.

AN UPDATE ON THE EUROPEAN RT-PCR IBV SURVEY AND RECENT FINDINGS ON A NOVEL IBV GENOTYPE.

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SUMMARY

A survey to detect infectious bronchitis virus (IBV) by RT–PCR in poultry flocks in selected countries in Western Europe was started in March 2002. To date 3205 samples have been processed and 1901 (59%) were positive. The predominant genotypes detected were 793B and Massachusetts, both of which are used extensively in commercially available vaccines. In 4th and 5th place were two important field types, Italy-02 and a virus similar to genotypes found in China. When the distribution of genotypes in each country with time was examined it could be seen that since 2004 the prevalence of Italy-02 was declining in all countries except Spain, where it is still the predominant genotype. Since the beginning of 2004 we have been detecting an IBV genotype in Holland, Germany, Belgium and France which is similar to several Chinese genotypes, one of which is called QX-IBV and the incidence is increasing. At the time of writing we have not detected it in the UK or Spain. Conventional vaccine strategies give some protection against QX-like challenge but dual vaccination is required for greater than 80% protection.

INTRODUCTION

It is well established that the main problem in control of infectious bronchitis is the ability of the virus to generate variants, due to mutation of the S1 spike gene. The spike protein is responsible for cell attachment, for a large component of immunity and is important in virus neutralisation tests, which have been used traditionally to determine serotyping of IB viruses.[1] Small changes in the amino acid sequences of the spike gene can result in the generation of new antigenic types which may be quite different from existing vaccine types.

No systematic survey of the genotypes of IBV in Europe has previously been made. Such a study is a useful indicator of what strains of virus currently exist in the field and may alert the poultry industry to new and potentially important viruses which could require adjustments to vaccination strategies.

Since 2002, Fort Dodge Animal Health has funded a survey of genotypes of IBV undertaken at Liverpool University. The study has been based on the submissions of dry swabs to this laboratory, which have been examined by reverse transcriptase-polymerase chain reaction (RT-PCR) which initially detects IBV in the samples. Products from positive samples were sequenced and these were then compared to those on a database in order to identify the strain of IBV detected.
This report describes the outcome of the study to date, the discovery of two economically important field strains genotype which have proved to be widespread in Western Europe and the protection offered by existing IBV vaccines against challenge with one of these viruses.

MATERIAL and METHODS

RT-PCR survey
Sets of 10 dry swabs were submitted by veterinarians or farm managers from selected farms in the UK or Western Europe, which were using Fort Dodge poultry vaccines as a routine. Information concerning the flock in question was requested, but not always fully submitted. Details asked for included: type of flock, age, disease status and relevant vaccination history. Any other details of relevance were to be included. Samples were sent from flocks of broilers, broiler breeders, layers and occasionally rare breeds.
On receipt, the swabs were pooled for examination, RNA was extracted and a reverse-trancriptase nested polymerase chain reaction (RT-PCR) was carried out according to the method of Cavanagh et al [2] to detect IBV. This method will detect both live virus and remnants of RNA from non-viable virus. The IBV oligonucleotide primers used were common for most of the known strains of infectious bronchitis virus (but not D1466), spanning a region of the S1 gene. The region of the gene within the universal primers was variable for each individual strain of IBV. Products from positive samples were then sent to another laboratory to be sequenced. These were compared to known IBV sequences in order to identify the strain of IBV detected. Since 2005 we have also been performing a separate test to detect D1466 using specific oligonucleotide primers [2].

Virus isolation and full S1 spike sequencing
Various tissues from poultry flocks from which QX-like IBV was detected were received for the purpose of virus isolation. Tissues were homogenised in sterile nutrient broth, frozen, thawed and centrifuged to remove cell debris and the supernatant was used to inoculated 19 day old fertile SPF eggs. Each sample was passaged twice and the presence of virus was confirmed by typical IBV embryo effects or embryo death and RT-PCR. Full S1 spike sequencing was carried out according to the method of Adzar et al [3, 4]

Vaccine Trials:
1. Efficacy of IBMM+Ark or IB Primer against challenge with QX-like IBV.
At day old, two groups of 25 specific pathogen free chicks were vaccinated by eye-drop with the commercial dose of either IBMM+Ark or IB primer vaccines (both from Fort Dodge Animal Health). A group of 35 unvaccinated chicks was maintained as controls. Each group was housed under isolation conditions in separate rooms. At

three weeks of age 20 birds from each of the three groups were challenged via intraocular and intranasal routes with $10^{3.8}$ EID^{50} per bird of strain L-1148 the European QX-like IBV (Worthington K.J, & Jones R.C. Accession No. DQ 431199).

2. Efficacy of dual vaccination against challenge with QX-like IBV.

50 one day old SPF chickens were vaccinated by eye-drop with the commercial dose of IB primer vaccine. A group of 35 unvaccinated chicks were maintained as controls. Each group was housed under isolation conditions in separate rooms. At 2 weeks of age the vaccinated chicks were split into two equal groups and housed separately. One group was vaccinated by eye-drop with the commercial dose of IBMM + Ark vaccine, and the second group with Nobilis 4-91 vaccine. Three weeks after the second vaccination, 10 unvaccinated, 5 week old birds (positive controls) were introduced into each vaccinated group. All the birds were then challenge by eye-drop with L-1148, the European QX-like IBV at a dose $10^{3.8}$ EID_{50} per bird.

Birds were monitored daily for general health and particularly respiratory signs after vaccination and challenge. On days 5 and 6 post-challenge all the birds were killed and the tracheas were examined for ciliary activity. From each trachea, 10 transverse rings were cut (three upper, four mid and three lower) and immediately examined for ciliary activity under an inverted microscope. Each ring was rated for degree of ciliary activity, on a 0 (none) to 4 (maximum) scale. Each chick was scored out of 10 rings, with the maximum possible score therefore being 40. Protection against challenge was calculated according to the method of Cook et al. [5].

RESULTS

RT-PCR survey

1. Total IBV Detected

In the four year period commencing in March 2002, 3205 samples have been examined and of these 1901 (59%) were found to be positive for IBV. The results are shown in Figure 1. The most common genotype found was the IBV variant 793B (otherwise called 4/91 or CR88), followed by Massachusetts type viruses (M41, H120, IBMM, etc.). The third most frequently detected genotype was a field strain Italy-02, first described in 2004 by Worthington et al [6] and in 2005 by Jones et al [7, 8]. In fourth place was a virus similar to a Chinese strain and called QX-like IBV. It has only been detected in Europe since the beginning of 2004 and now contributes to 8.6% of the total. In fifth and sixth place were D274 and Arkansas both of which are used extensively in vaccines. Arkansas field strain is not indigenous in Europe and was only detected in flocks which had received the IBMM + Ark vaccine. Low levels of B1648 and D1466 were also detected. When the distribution of genotypes with time was examined (Figure 2) it can be seen that of the field strains, Italy-02 has been decreasing since 2003 and the QX-like virus has been increasing since 2004

2. The UK

The distribution of IBV in the UK is shown in Table 1 and Figure 3 . The predominant genotype is 793B and the field isolate Italy-02 is in second place, being more prevalent than the Massachusetts type. However it can be seen that the prevalence if Italy-02 has been decreasing since 2004 during which year it was the predominant

genotype detected This may be due to the increased use of IB primer or IBMM+ Ark vaccines [8] as the incidence of both Arkansas and D274 has been increasing.

3. France

The results for France are shown in Table 1 and Figure 4. The predominant genotype is 793B followed by Mass type and Itlay-02. However Italy -02 has been declining in France since 2003, in contrast in 2006 the QX-like virus has increased dramatically. It was first detected in France in Sept 2004 in a flock in the North East, close to the Belgium border and thought to be related to use of layer litter compost coming from the Netherlands. In 2005 it was detected in 3 more flocks in the same location. However since December 2005 we have been detecting it in broiler flocks in the North West of France and the incidence has increased dramatically. So far in 2006 approximately 1/3rd of all positive IBV samples from France have been QX-like IBV.

4. Germany

In Germany 793B and Mass type IBV was found in approximately equal numbers (Table 1). In third place is the field strain QX-like IBV and this has been detected in Germany since 2004 (Figure 5). In 2005 to the present time it is the predominant genotype detected. Very little Italy-02 was found in Germany

5. Holland

We see a similar situation in Holland (Table 1 and Figure 6) to that found in Germany except that we detected more Italy-02 and D1466 appears to be increasing in 2006. QX-like IBV reach a peak in 2005 (approximately 1/3rd of all IBV positives) but at the time of writing it appears to be declining but the number of samples tested is low.

6. Belgium

In Belgium we have tested fewer samples but of these Mass type is the predominant type followed QX-like IBV and D274, which are in equal second place (Table 1 and Figure 7).

7. Spain

In Spain Italy-02 remains the predominant type and we have not detected any QX-like IBV (Table 1 and Figure 8), however, the total number of samples received from Spain is lower than any of the other countries.

Virus isolation and full S1 spike sequencing.

Five European isolates of QX-like IBV have been fully sequenced (Table 2). The S1 spike sequence of one of these, ref L-1148T, has been submitted to the NCBI genebank (Accession no. DQ 431199). This was isolated from 24 day old broilers exhibiting respiratory signs. The nucleotide identity between these 5 European isolates was greater than 99.6%. Recent submissions to the NCBI genebank have identified one Israeli, two Korean and one Chinese IBV strains to have approximately 98.6% identity with L-1148T. Other Chinese strains, including QX IBV have between 96% to 97% identity with L-1148T. The other common European genotypes of IBV all have less than 79% identity with this virus (Figure 9).

Disease associated with the European QX-like IBV

The virus has been detected in flocks of broilers, broiler breeders and commercial layers, between the ages of 18 days and 52 weeks. From these flocks 86% had respiratory signs, 22% had wet litter or enteric problems, 14% had increased mortality, 2% swollen kidneys and 2% arthritis. In 60% of layers there was a loss of egg production or a reduction in egg quality.

Vaccine Trials

The protection against QX-like challenge following single vaccination at day old was 72% in the IBMM + ARK group and 69% in the IB primer group. The protection was greater following dual vaccination, there being little difference between the groups given IBMM+ARK (87%) or Nobilis 4-91(88%) as second vaccine (Table 3).

DISCUSSION

The continuous monitoring of IBV genotypes in selected countries in Western Europe has to made it possible to identify new field strains as soon as they emerge and potentially be the cause of problems. This was the case with Italy-02 IBV which was first identified as causing problems by this survey (Jones et al 2005). Now we are seeing a new European strain which is causing extensive disease in both broilers and layers in Holland, Germany, Belgium and France. A recent report by Beato et al, in 2005 [9] also confirms its presence in Italy.

The European strain is similar to those previously detected in China, many of which are nephropathogenic as well as causing respiratory problems. A recent report by Liu et al in 2006 [10] has classified various Chinese IBV strains detected in China between 1995 and 2004. The European isolate L-1148 is closely related to Group 1 of the Chinese genotypes. When we first detected this isolate in Europe in January 2004 a search of the data base showed QX-IBV to be the closest match being 98.1% similar over the section of the S1 spike that was sequenced. This virus was detected in the Qingdao area of China in 1996/7 (Accession no. AF193423) and was reported to cause proventriculitis. Subsequent analysis of the full S1 spike shows a nucleotide and amino acid similarity of 97% with no gaps.

There have been more recent submissions to the genebank that show a greater similarity to the European L-1148 isolate, two from Korea, another from China and one from Israel (Figure 9). There are also several other Chinese strains very similar genetically to QX-IBV. Many of these strains are reported to be nephropathogenic and were isolated from the kidney. Interestingly reports of nephritis from flocks in which L-1148 was detected was only low (2%). However we did not always receive full details of clinical disease and in the majority of cases post-mortems were not performed. The nomenclature of all these isolates is different, QX-like to describe the European isolate was adopted because it was one of the first 'hits' we had when doing a genebank search and compared to many other very similar Chinese strains this name (QX) was the most user friendly. This genotype has also been named D388 (J. J. De Witt, unpublished). This emphasises the need for a universally agreed convention for the naming IBV isolates, particularly in respect of 'new ' isolates or genotypes. [11]

The two vaccine trials that were carried out showed that only after dual vaccination at day old and two weeks of age was the protection against challenge with QX-like IBV greater than the required 80%. Single day old vaccination with either IB primer (H120 + D274) or IBMM + Ark did offer protection greater than 69%, so is of some benefit These findings differ from those with Italy-02 IBV when these conventional vaccine strategies were show to give adequate protection [8]. These results also indicate that there may be need for the development of a type specific vaccine particularly as the prevalence of the European QX-like strain appears to be increasing dramatically in Europe.

REFERENCES

1. Cavanagh, D., M.M. Ellis, and J.K.A. Cook, *Relationship between sequence variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection in vivo.* Avian Pathology, 1997. **26**(1): p. 63-74.
2. Cavanagh, D., K. Mawditt, P. Britton, and C.J. Naylor, *Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions.* Avian Pathology, 1999. **28** (6): p. 593-605.
3. Adzhar, A., K. Shaw, P. Britton, and D. Cavanagh, *Universal oligonucleotides for the detection of infectious bronchitis virus by the polymerase chain reaction.* Avian Pathology, 1996. **25**: p. 817-836.
4. Adzhar, A., R.E. Gough, D. Haydon, K. Shaw, P. Britton, and D. Cavanagh, *Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain.* Avian Pathology, 1997. **26**(3): p. 625-640.
5. Cook, J.K.A., S.J. Orbell, M.A. Woods, and M.B. Huggins, *Breadth of protection of the respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes.* Avian Pathology, 1999. **28**(5): p. 477-485.
6. Worthington, K.J., C. Savage, C.J. Naylor, W. Wijmenga, and C. Jones R. *An RT-PCR survey of Infectious Bronchitis genotypes in the UK and selected European countries between 2002 and 2004 and the results from a vaccine trial*. in *IV. International Symposium on Avian Corona- and Pneumovirus Infections*. 2004. Rauischholzhausen, Germany: VVB Laufersweiler Verlag, Wettenberg.
7. Jones, R.C., K.J. Worthington, and R.E. Gough, *Detection of the Italy 02 strain of infectious bronchitis virus in the UK.* Veterinary Record, 2005. **156**(8): p. 260-260.
8. Jones, R.C., K.J. Worthington, I. Capua, and C.J. Naylor, *Efficacy of live infectious bronchitis vaccines against a novel European genotype, Italy 02.* Veterinary Record, 2005. **156**(20): p. 646-647.
9. Beato, M.S., C. Terregino, R. De Nardi, A. Toffan, G. Cattoli, and I. Capua, *Epidemiological study on circulation of infectious bronchitis virus strains in North Eastern Italy.* Italian Journal of Animal Science, 2005. **4**(3): p. 263-265.
10. Liu, S.W., Q.X. Zhang, J.D. Chen, Z.X. Han, X. Liu, L. Feng, Y.H. Shao, J.G. Rong, X.G. Kong, and G.Z. Tong, *Genetic diversity of avian infectious bronchitis coronavirus strains isolated in China between 1995 and 2004.* Archives of Virology, 2006. **151**(6): p. 1133-1148.

11. Cavanagh, D., *A nomenclature for avian coronavirus isolates and the question of species status.* Avian Pathology, 2001. **30**(2): p. 109-115.

Table 1.
Geographical distribution of IBV genotypes, expressed as a percentage of the number of IBV positive samples per country

	UK	***France***	***Germany***	***Holland***	***Belgium***	***Spain***
Total No. tested	1274	675	501	451	165	139
No. IBV Positive	817	334	276	293	103	78
793B	**32.2%**	**56.0%**	**30.4%**	27.3%	14.6%	28.2%
Massachusetts	21.2%	23.7%	29.3%	**29.4%**	**25.2%**	19.2%
Italy-02	24.5%	9.3%	1.8%	7.2%	2.9%	**47.4%**
QX-like	0	6.6%	22.1%	19.8%	22.3%	0
D274	6.7%	0.9%	10.9%	11.6%	22.3%	5.1%
Arkansas	10.4%	0	0	1.7%	7.8%	0
B1648	0	1.5%	0.4%	0	0	0
D1466*	1.1%	0.9%	1.8%	2.0%	2.9%	0
Other **	3.9%	1.2%	3.3%	1.0%	1.9%	0

* *only since Jan 2005*
** *Includes rare, novel and mixed IBV samples*
Predominant genotype

Table 2 Isolates of European IBV similar to some Chinese strains

European QX-like IBV

Reference	Country of Origin	Date of sample	Tissue	Species	Genebank Accession No.
L-1148T	Holland	Jun-04	Trachea	Broiler	DQ 431199
L-1449T	Holland	Oct-04	Trachea	B. breeder	
L-1449K	Holland	Mar-04	Kidney	Broiler	
L-1450T	France	Jan-05	Trachea	Broiler	
L-1450L	France	Jan-05	Lung	Broiler	

Table 3
Protection against challenge with the European QX-like IBV following single or dual vaccination.

Vaccination	Challenge (QX-like IBV)	Protection
Single vaccination		
IBMM + Ark @ day old	L-1148 @ 3 wk	72%
IB Primer (H120 + D274) @ day old	L-1148 @ 3 wk	69%
Dual vaccination		
IB Prime r@ d o & IBMM + Ark @ 14 d	L-1148 @ 5 wk	87%
IB Primer @ d o & Nobilis 4-91 @ 14 d	L-1148 @ 5 w	88%

Figure1
IBV Genotypes detected in Europe between 2002 and 2006 (no. tested/no.IBV pos = 3205/1901)

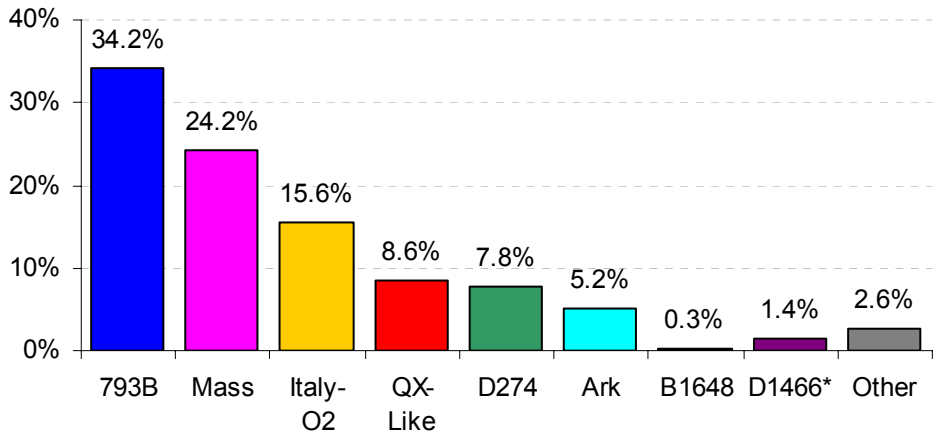


Figure 2
EUROPE: Distribution of IBV genotypes with time

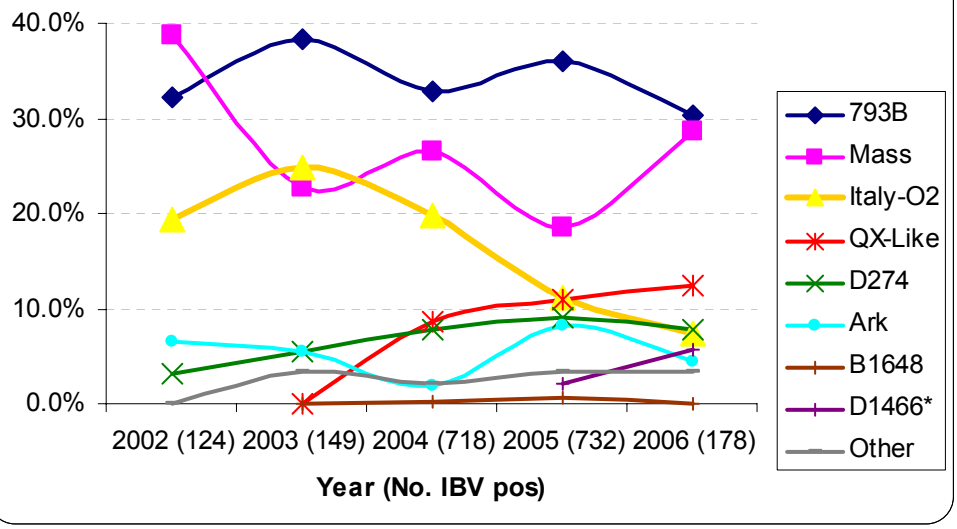


Figure 3.
UK: Distribution of IBV genotypes with time

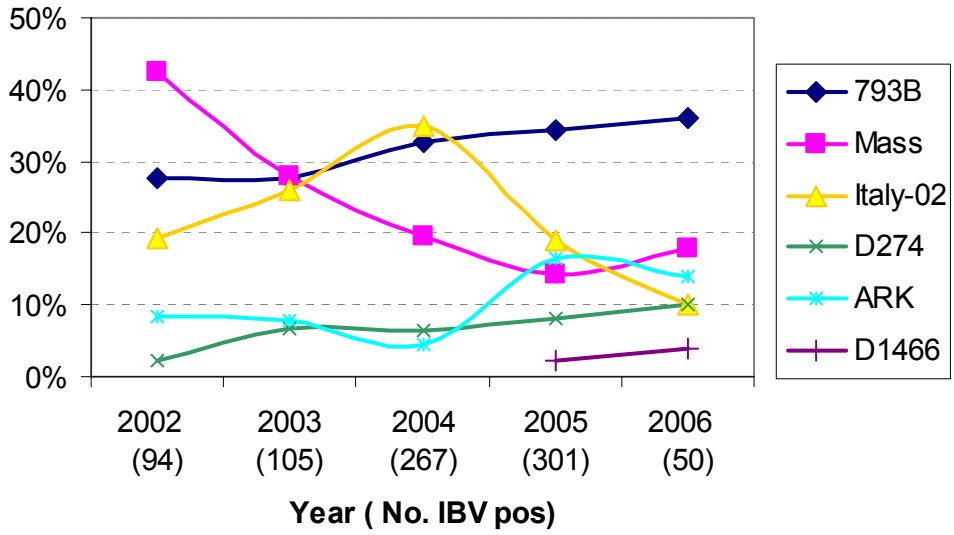


Figure 4
FRANCE: Distribution of IBV genotypes with time

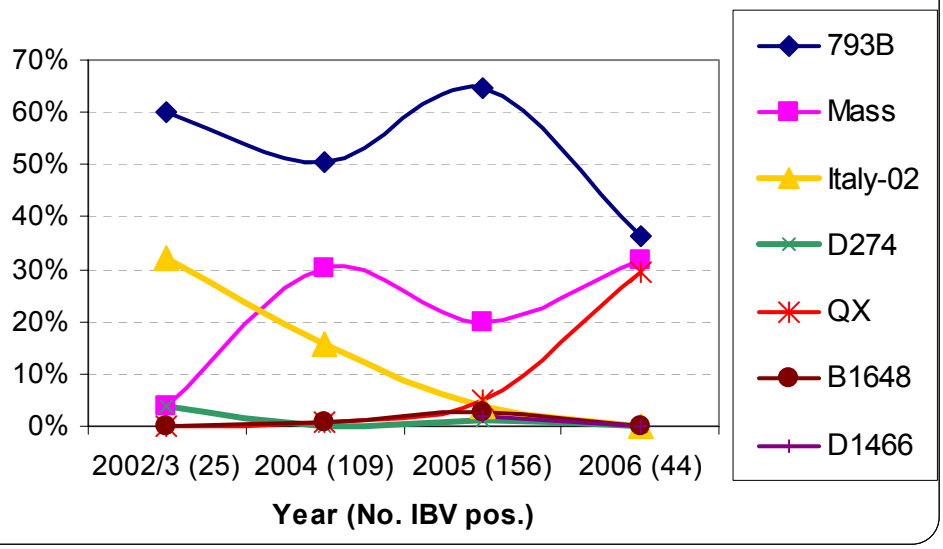


Figure 5.
GERMANY: Distribution of IBV genotypes with time

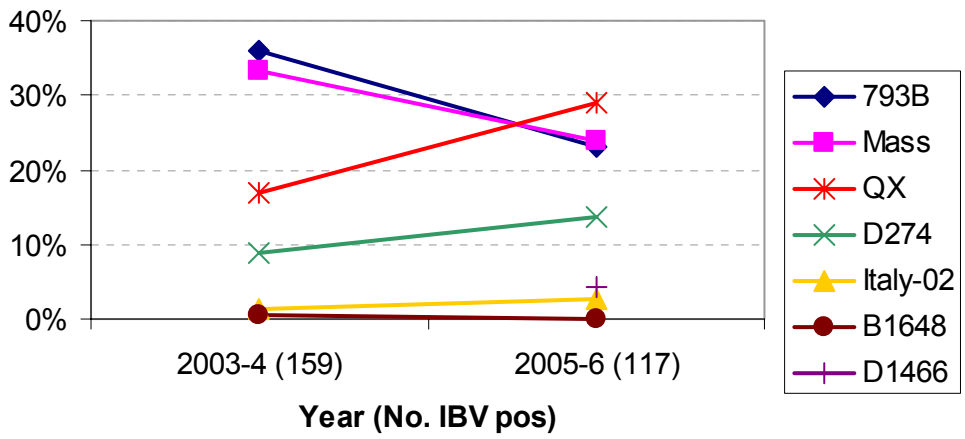


Figure 6
HOLLAND: Distribution of IBV genotypes with time

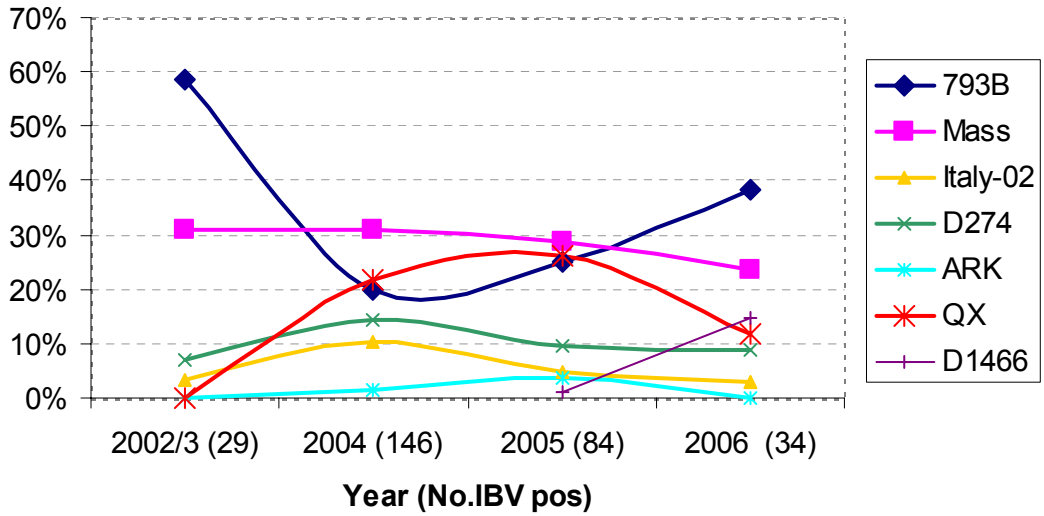


Figure 7
BELGIUM: Distribution of IBV genotypes with time

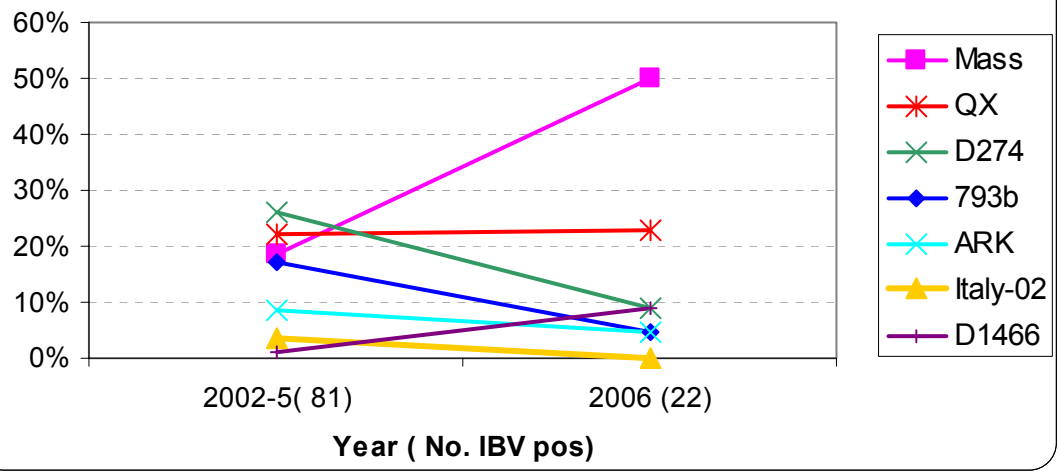


Figure 8
SPAIN: Distribution of IBV genotypes with time

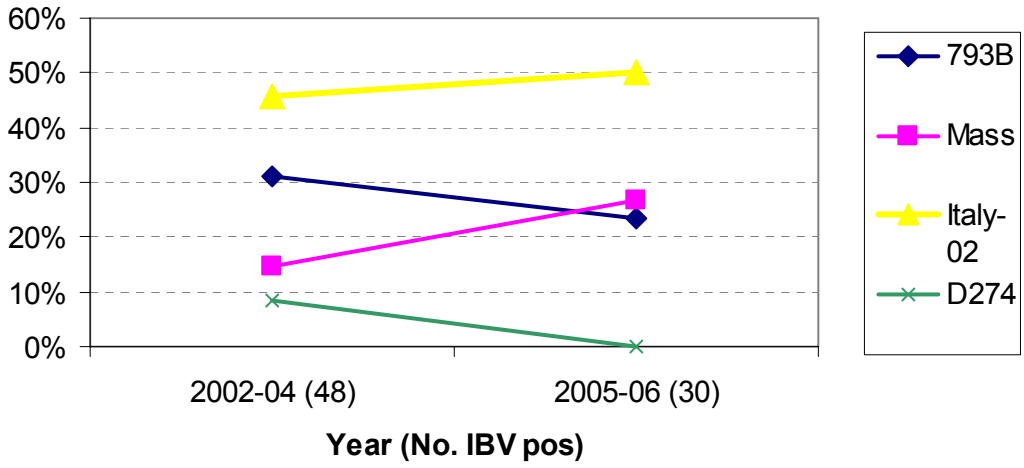
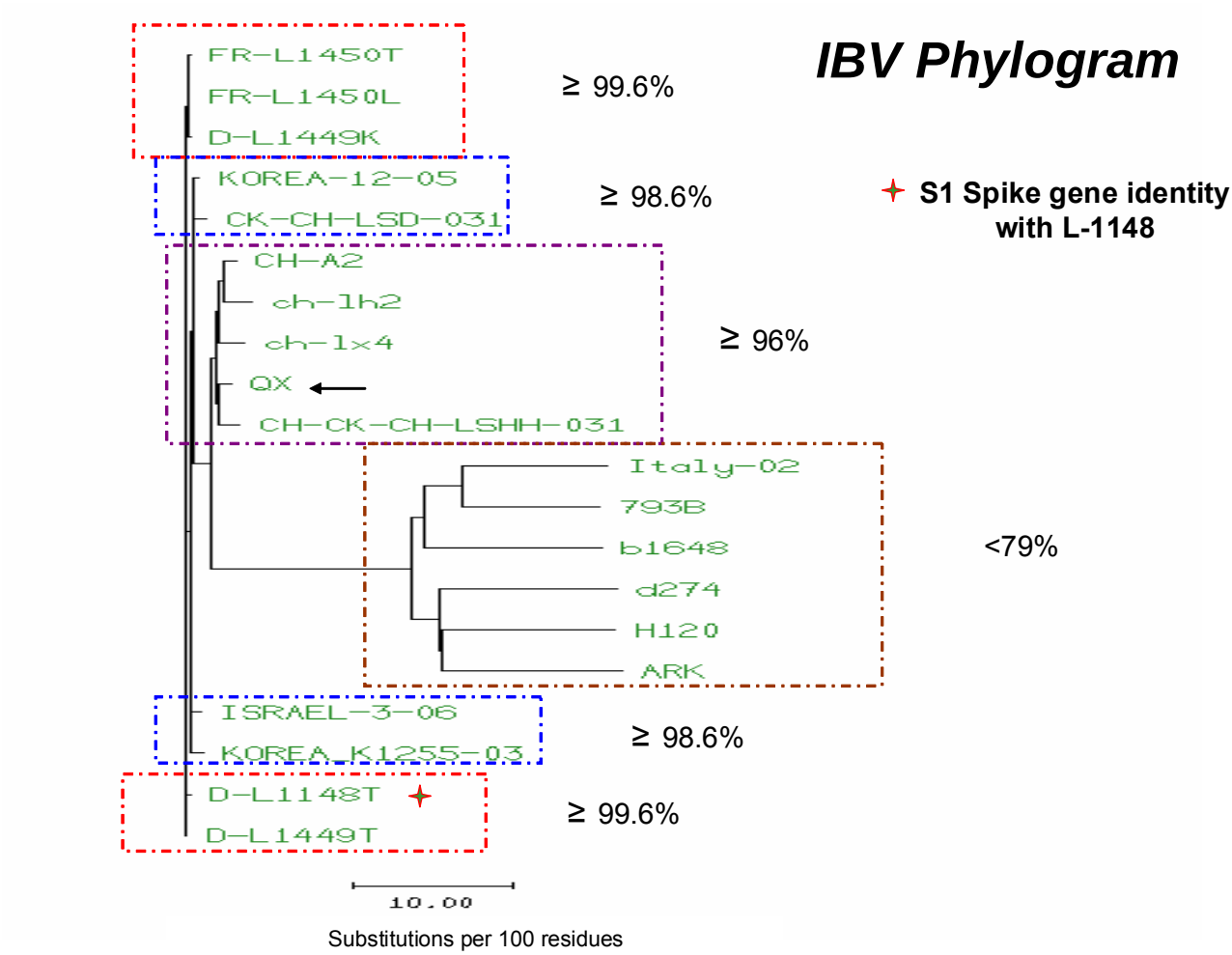


Figure 9
Phylogram to show the reationship between several IBV genotypes



PREVALENCE OF CORONAVIRUS AND ASTROVIRUS IN TURKEYS IN GREAT BRITAIN

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SUMMARY

The prevalence of turkey coronavirus (TCoV) and turkey astrovirus (TAstV) in grow-out turkey flocks in England and Wales was shown to be at least 52% and 70%, respectively. The detection of TCoV by RT-PCR was always associated with scouring. Although that was not always the case for TAstV, the turkeys usually exhibited other signs of illness: depression, huddling, dehydration, stunted growth and loss of appetite. Two-thirds of the astrovirus detections were within the first four weeks of life, including the first week. TCoV was not detected during the first two weeks of life, though it was often present for many successive weeks, including up to 18 weeks of age. In a particularly serious case of scouring mortality exceeded 20% in poults, both viruses were detected, and 'attaching and effacing' enterpathogenic *Escherichia coli* (EPEC)was present. The phenomena on these farms was consistent with poult enteritis and mortality syndrome described in the UAS. The high incidence of both viruses, associated with disease, and in some cases with EPEC present, strongly suggests that both of these viruses play an important role in enteric disease in England and Wales.

INTRODUCTION

A coronavirus was shown thirty years ago to be involved with enteritis of turkeys in the USA where it has been most thoroughly studied. In young poults it can be associated with mortality whilst in older birds it is debilitating, resulting in underperformance with regard to meat and egg production (Guy, 2000, 2003; Guy et al., 2000). The virus, turkey coronavirus (TCoV) was confirmed as being in the UK in 2001 (Cavanagh *et al.*, 2001) and has subsequently been demonstrated in Brasil and Italy. We undertook a systematic investigation of the prevalence of TCoV in England and Wales in 2003-2005, using RT-PCR to detect viral RNA, and immunofluorescence to detect anti-TCoV antibodies. We took advantage of the fact that TCoV is genetically (Breslin *et al.*, 1999a, b) and antigenically (Weissman *et al.,*

1987; Guy *et al.*, 1997, 2002; Breslin et al. 2000) closely related to IBV, confirmed elsewhere (Ismail *et al.*, 2001; Cavanagh et al., 2001), reviewed by Guy (2000).
At the same time we used RT-PCR to detect turkey astrovirus (TAstV), as this virus has also been associated with enteritis with turkeys in North America (reviewed by Koci and Schulz-Cherry, 2002). Astroviruses are known to have been in turkeys in the UK for decades (McNulty et al., 1980), and it is likely that TCoV has been present for a similar time, given the ubiquity in the UK of coronaviruses in other galliform birds: infectious bronchitis virus in domestic fowl, and pheasant coronavirus in pheasants (Cavanagh et al., 2002).

MATERIALS AND METHODS

Procedures will be described more fully elsewhere.

Field samples
We used two sampling approaches: faeces (or ileo-caecal material from very young poults) collected on a weekly basis for up to 20 weeks during longitudinal studies of pre-selected farms, and from faeces collected from many other farms at times of scouring (watery faeces) – "one-off" samples. Ten faecal samples were collected and pooled at each time point.

RNA extraction and RT-PCR
RNA extraction from 0.2 g (frozen) faeces was done using the Qiagen Stool Kit. The presence of turkey coronavirus (TCoV) was demonstrated oligonucleotide primer set UTR 11-/44+, corresponding to the highly conserved part of the 3' untranslated region of the genome (Cavanagh et al., 2001). TAstV was detected using a pair of primers corresponding to a region of gene 1 conserved amongst avian and mammalian astroviruses (Culver et al., 2006).

Immunofluorescence
IBV-Beaudette-infected Vero cells were used to detect TCoV antibodies in sera collected from turkeys at one day of age and/or 10 weeks of age and/or 20 weeks of age. Ten blood samples were collected and pooled at each time point.

RESULTS AND DISCUSSION

The results of our investigation will be described in full elsewhere. We estimated that the incidence of the viruses in a flock at each time point would have to be ~25% for a 95% chance of detection.

Detection of TCoV by RT-PCR
RT-PCR products were sequenced. This confirmed that the PCR products had originated from RNA extracted from faecal material, and not from infectious bronchitis viruses (IBV) used in our laboratory. We also confirmed, using an *in vitro*-transcribed IBV RNA corresponding to the 3'UTR, that the RNA extraction procedure had removed any potentially RT-PCR-inhibiting materials. The RT-PCR was able to detect approximately 1 plaque forming unit of IBV. (We did not have an isolate of TCoV).

- Of the 17 flocks examined in longitudinal studies, 5 (29%) were positive for TCoV.
- 20% of the 'one-off' samples (9/45) from scouring flocks were TCoV-positive.
- Of the 84 flocks examined by RT-PCR, TCoV was detected in 16 (19%).

All the positive samples were from turkeys aged 3-18 weeks of age, and all from flocks that were scouring.

Detection of TCoV by immunofluorescence (IF)
Of the 56 flocks examined by IF, 29 (52%) were seropositive for TCoV.
Thus detection of TCoV-infection by IF was approximately twice as sensitive as by RT-PCR. In part this was probably due to the endurance of the anti-viral antibodies within the birds, whereas virus would probably have persisted for shorter lengths of time within individual turkeys.

Detection of TAstV by RT-PCR
- Of the 17 flocks examined in longitudinal studies, 12 (70%) were positive for TCoV.
- 27% of the 'one-off' samples (12/45) from scouring flocks were TAstV-positive.
- Of the 84 flocks examined by RT-PCR, TCoV was detected in 32 (38%).

All positive samples were from turkeys 1-12 weeks of age. Interestingly, 67 % of TAstV+ samples were in birds aged 4 weeks and under.
Unlike TCoV, TAstV-detection was not always associated with scouring, although the flocks usually had other clinical signs: depression, huddling, dehydration, stunted growth and loss of appetite.

Poult enteritis and mortality syndrome (PEMS)
We investigated a turkey enterprise that was experiencing high mortality associated with scouring (Culver et al., 2006). The management system involved mainly brood-and-move but some of the brood farms also had barns in which older birds were grown. Individual flocks became ill at varying times between 2 and 8 weeks of age. The most obvious clinical signs initially were lack of activity, reduced feed and water intake, scouring and 'huddling'. Deaths mostly occurred overnight and carcases were found in groups with the appearance of smothering.
Post-mortem examination of representative birds showed marked accumulation of liquid in the small intestine, liquid and/or frothy caecal contents, and wet rectal contents. Many of the older flocks showed marked thymic atrophy, to the point of almost total loss of thymic tissue. Some birds showed evidence of inflammation of the cloacal bursa. Crop mycosis was also common. Affected flocks ceased growth and often lost weight for an extended period. This resulted in a marked loss of flock uniformity and stunting of a significant proportion of the birds. Losses from mortality and culling exceeded 20% in a number of flocks. Healthy sentinel birds introduced into diseased flocks rapidly developed similar clinical symptoms.
We analysed caecal contents and tissue samples (spleen, kidney, thymus, bursa of Fabricius and caecal tonsils) for both TCoV and TAstV. Caecal contents from the index cases, and caecal tonsil and bursa of the sentinel birds were positive for TCoV, negative for TAstV. We then sampled three other farms in the same ownership,

which were experiencing similar losses. In total, 10 samples (8 caecal contents, 2 bursa of Fabricius) from 4- to 6-week-old poults within the four farms were positive for TCoV. Two samples (caecal contents) from two of the farms were positive for TAstV, one being positive for both viruses. Sequencing revealed that the PCR products derived from TCoV and TAstV, had 98% and ~90% nucleotide identity, respectively, with the corresponding sequences of US isolates, confirming the identity of the British viruses.

In the USA enteropathogenic *Escherichia coli* (EPEC) are frequently isolated from PEMS cases (Guy et al., 2000; Pakpinyo et al., 2002). Furthermore, experimental dual infection of turkeys with TCoV and an EPEC strain resulted in severe enteritis, high mortality and growth depression (Guy et al., 2000). The symptoms were more severe than if TCoV or EPEC were administered separately, indicating a synergistic effect. We readily isolated *E. coli* from pooled intestinal contents from both index and sentinel birds on Sorbitol MacConkey agar supplemented with 2.5 □g/ml potassium tellurite. By PCR using universal intimin primers (Bachelor et al., 1999) we detected the *eae* gene characteristic of 'attaching & effacing' (AE) strains. PCRs for Shiga toxin 1 and 2 genes (Stevens et al., 2004) were negative, despite positive results with controls, indicating that the isolate was not an enterohaemorrhagic *E. coli*. Serotyping revealed the isolate to be O26:K60. In a standard HeLa cell adherence assay (Stevens et al., 2004), the isolate exhibited localised adherence typical of EPEC elaborating bundle-forming pili. In a fluorescent actin staining test (Knutton et al., 1989) the isolate nucleated actin under the site of attachment, confirming AE activity.

The coincidence of TCoV and/or TAstV, EPEC, the clinical signs and the high degree of mortality were consistent with PEMS

Final remarks

In our investigation approximately one-third of 'one-off' samples i.e. taken from flocks at the time of scouring (excluding those collected during longitudinal studies) were associated with TCoV or TAstV. As many viruses have been shown to be associated with enteritis in turkeys we cannot be sure that the scouring observed in our study was caused by TCoV or TAstV. That said, it is likely that these viruses contributed to the diseased state of the birds, particularly in the case of TCoV, as all detections were associated with scouring, and the virus was detected in birds of several months of age, not just in the early weeks when enteric viruses are commonly found.

The incidence of both viruses, as evidenced by the longitudinal studies, was high, at least 52% and 70% for TCoV and TAstV, respectively. Interestingly, TAstV was frequently detected very early in the life of the flock, include the first week. This raises the question as to whether this virus might be spread vertically. In contrast, we never detected TCoV in the first two weeks of life. That might have been due to (a) a prevalence of <25%, in which case we would have been unlikely to detect it; (b) the effectiveness of maternal antibody (we detected TCoV antibodies in sera from day-old poults, which must have been maternally derived, indicating that the parent flocks had experienced infection with TCoV; or (c) the TCoV was out-competed by other enteric pathogens. Another contrast between the coronavirus and astrovirus was that the former was detected for long periods, including up to 18 weeks of age, whereas the astrovirus was detected much less often after six weeks of age.

The most severe instances of scouring, associated with the presence of both viruses, also involved EPEC, in agreement with reports from the USA. As there are no

vaccines for TCoV or TAstV, control of disease caused by them requires appropriate farm organisation e.g. not multi-age sites, and maintenance of high biosecurity.

ACKNOWLEDGEMENTS

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REFERENCES

Batchelor, M., Knutton, S., Caprioli. A., Huter, V., Zanial, M., Dougan, G., & Frankel, G. (1999) Development of a universal intimin antiserum and PCR primers. *Journal of Clinical Microbiology, 37*, 3822-3827.

Breslin, J.J., Smith, L.G., Fuller, F.J. & Guy, J.S. (1999a). Sequence analysis of the matrix/nucleocapsid gene region of turkey coronavirus. *Intervirology, 42*, 22-29.

Breslin, J.J., Smith, L.G., Fuller, F.J. & Guy, J.S. (1999b). Sequence analysis of the turkey coronavirus nucleocapsid gene and 3' untranslated region identifies the virus as a close relative of infectious bronchitis virus. *Virus Research, 65*, 187-198.

Breslin, J.J., Smith, L.G., Barnes, H.J. & Guy, J.S. (2000). Comparison of virus isolation, immunohistochemistry, and reverse transcriptase-polymerase chain reaction procedures for detection of turkey coronavirus. *Avian Diseases, 44*, 624-631.

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S. E., Ainsworth, H.L., Britton, P. & Gough, R.E. (2001) Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. *Avian Pathology*, *30*, 365-378.

Cavanagh, D., Mawditt, K., Welchman, D. De. B., Britton, P. & Gough, R.E. (2002) Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. *Avian Pathology*, *31*, 81-93.

Culver, F., Dziva, F., Cavanagh, D. & Stevens, M.P. (2006). Poult enteritis and mortality syndrome in turkeys in Great Britain. *The Veterinary Record*, in press.

Guy, J.S. (2000).Turkey coronavirus is more closely related to avian infectious bronchitis virus than to mammalian coronaviruses. *Avian Pathology, 29*, 206-212.

Guy, J.S. (2003). Turkey coronavirus enteritis. In: *Diseases of Poultry*. Eds. H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald & D.E. Swayne. 11th Edn. Pp. 300-307. Ames, Iowa, Iowa State Press.

Guy, J.S., Barnes, H.J., Smith, L.G., Breslin, J. (1997). Antigenic characterization of a turkey coronavirus identified in poult enteritis and mortality syndrome-affected turkeys. *Avian Diseases, 41*, 583-590.

Guy, J.S., Smith, L.G., Breslin, J.J., Vaillancourt, J.P. & Barnes, H.J. (2000). High mortality and growth depression experimentally produced in young turkeys by dual infection with enteropathogenic Escherichia coli and turkey coronavirus. *Avian Diseases, 44,* 105-113.

Guy, J.S., Smith, L.G., Breslin, J.J., Pakpinyo, S. (2002). Development of a competitive enzyme-linked immunosorbent assay for detection of turkey coronavirus antibodies. *Avian Diseases, 46*, 334-341.

Ismail, M.M., Cho, K.O, Hasoksuz, M. Saif, L.J. & Saif, Y.M. (2001). Antigenic and genomic relatedness of turkey-origin coronaviruses, bovine coronaviruses, and infectious bronchitis virus of chickens. *Avian Diseases, 45*, 978-984.

Knutton, S., Baldwin, T., Williams, P. H., & Mcneish, A. S. (1989) Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infection and Immunity, 57*, 1290-1298.

Koci, M., & Schulz-Cherry, S. (2002) Avian astroviruses. A Review. *Avian Pathology, 31*, 213-227.

McNulty, M.S., Curran, W.L. & McFerran, J.B. (1980) Detection of astroviruses in turkey faeces by direct electron microscopy. *The Veterinary Record, 106*, 561.

Pakpinyo, S., Ley, D. H., Barnes, H. J., Vaillancourt, J. P., & Guy, J. S. (2002) Prevalence of enteropathogenic *Escherichia coli* in naturally occurring cases of poult enteritis-mortality syndrome. *Avian Diseases, 46*, 360-369.

Stevens, M. P., Roe, A. J., Vlisidou, I., Van Diemen, P. M., La Ragione, R. M., Best, A., Woodward, M. J., Gally, D. L., & Wallis, T. S. (2004) Mutation of *toxB* and a truncated version of the *efa-1* gene in *Escherichia coli* O157:H7 influences the expression and secretion of locus of enterocyte effacement-encoded proteins but not intestinal colonization in calves or sheep. *Infection Immunity, 72*, 5402-5411.

Weisman, Y., Aronovici, A. & Malkinson, M. (1987). Prevalence of IBV antibodies in turkey breeding flocks in Israel. *Veterinary Record, 120*, 94.

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF NOVEL CORONAVIRUSES INFECTING WILD BIRD SPECIES

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SUMMARY

In light of the finding of a previously unknown coronavirus as the aetiology of the Severe Acute Respiratory Syndrome (SARS), it is probable that other coronaviruses than those recognised to date, are circulating in animal populations. We present here the results of a screening of birds for coronavirus from 2003 to 2005, using a universal coronavirus RT-PCR. The wild bird species sampled were graylag goose (*Anser anser*), feral pigeon (*Columbia livia*) and the duck species; mallard (*Anas platyrhynchos*), wigeon (*Anas penelope*), teal (*Anas crecca*), and common scoter (*Melanitta nigra*). Coronaviruses were detected in cloacal swab samples from most investigated bird species, with rather high prevalence in the graylag goose, mallard, teal and wigeon, indicating that anserifomres are prone to coronavirus infection. In the graylag goose, 103 of the 319 sampled birds were coronavirus positive, whereas 40 of 141 sampled mallards, 4 of 24 sampled wigeons, 3 of 13 sampled teals, none of 4 sampled common scoters, and two of 100 sampled pigeons tested positive. The infected graylag geese showed lower body weights compared to virus negative birds, suggesting clinical significance of the infection. Phylogenetic analyses performed on the replicase gene and nucleocapsid protein sequences, indicated that the novel coronaviruses all branch off from group III coronaviruses, but are clearly distinct from IBV and TcoV, and from each other. As opposed to avian influenza virus, where similar subtypes are found in duck and goose populations, the coronaviruses isolated from ducks were phylogenetically different from those found in the goose samples, suggesting that these viruses have a narrower host range than the influenza viruses, and while several animal species, including pigs, bats, dogs and humans, have been found to harbour different coronaviruses, from serogroup I and II, all identified wild bird coronaviruses that were sequenced were closely related to each other within the same species.
All the novel avian coronaviruses harboured the conserved s2m RNA structure in their 3' untranslated region, like all other previously described group III coronaviruses, and like the SARS coronavirus. Sequencing of the complete

nucleocapsid gene and downstream regions of goose and pigeon coronaviruses, evidenced the presence of two additional open reading frames for the goose coronavirus with no sequence similarity to known proteins, but with predicted transmembrane domains for one of the encoded proteins, and one additional open reading frame for the pigeon coronavirus, with a predicted transmembrane domain, downstream of the nucleocapsid gene.

INTRODUCTION

The coronaviruses comprise three serologically and genetically distinct groups. Coronaviruses from group I and II have been found to infect several mammalian species, including humans, pigs, cows, dogs, horses, cats and rodents. Group III coronaviruses have until now only been found to infect poultry, and include the chicken infectious bronchitis virus (IBV), the turkey coronavirus (TCoV) and the pheasant coronavirus (Enjuanes et al., 2000).
After the finding of a previously unknown coronavirus as the aetiology of the Severe Acute Respiratory Syndrome (SARS), new coronaviruses infecting humans have been identified, one of group I (NL63) and one of group II (HKU1). The SARS coronavirus has been postulated to be an early split-off from the group II coronaviruses, but it has been argued that there are evidences for recombination events in the evolution of this virus, involving both mammalian (group I and II) and, in the 3'-end, avian coronaviruses (III). An interesting feature of the SARS coronavirus genome is that it harbours a short motif (s2m) at its 3'-end, also found in group III coronaviruses, but not in other coronavirus (Marra et al., 2003). S2m is also present in all but one of the characterised astroviruses, and in one picornavirus (equine rhinitis virus B), and seems to be an element that is readily transferred between RNA virus genomes (Jonassen et al. 1998). The emergence of the SARS coronavirus has made it important to further characterise the coronavirus family to better understand which viruses are circulating in animal populations as potential zoonotic agents.
In 2003, a Norwegian survey, originally aimed at investigating the presence of enteropathogenic bacteria as well as influenza A virus and Newcastle disease virus, was carried out. The samples investigated included fresh droppings, cloacal and tracheal swabs, as well as a limited number of organs, from three bird species; the graylag geese (*Anser anser*), feral pigeons (*Columbia livia*) and mallards (*Anas platyrhynchos*)**.** The same samples, as well as additional cloacal swabs sampled from graylag geese in August 2004 and 2005, and from different duck species, including mallards, wigeons (*Anas penelope*), teals (*Anas crecca*), and common scoters (*Melanitta nigra*), from September to December 2005, were investigated for the presence of coronaviruses, using a pan-coronavirus reverse transcription and polymerase chain reaction (RT-PCR). We report here the detection and characterisation of novel coronaviruses infecting geese, pigeons, and ducks.

MATERIALS AND METHODS

Cloacal swab samples were collected from a total of 349 graylag geese, 100 feral pigeons, 141 mallards, 13 teals, 24 wigeons and 4 common scoters. The swabs were placed in tubes containing virus transport medium, and all samples were kept on ice, or frozen, until they reached the laboratory. For some of the birds, organ samples

were taken as well. Information concerning the age, sex and body weights was recorded for the graylag goose population.
RNA was extracted using the QIAamp Viral RNA mini kit (Qiagen), and a pan-coronavirus RT-PCR was performed on the samples, using primers designed to amplify 250 bp of the replicase gene of all groups of coronaviruses (Stephensen et al., 1999), and later on, as sequence information became available, a modified version of these primers (Jonassen et al. 2005). From 2005, the screening PCR was performed by real-time amplification using SybrGreen detection. The Tm of the coronavirus amplification product from the anseriformes tested was of about 79-80ºC. The sequences in the 3'-end of some of the coronavirus samples were determined, making use of the probable presence of the highly conserved s2m RNA structure as a handle, and of a subsequent 5'-RACE/primer walking strategy (Jonassen et. al, 1998; Jonassen et al. 2005).

RESULTS

Coronavirus could be found in cloacal swab samples from most bird species. In the graylag goose, 107 of the 349 sampled birds were coronavirus positive, whereas 40 of 141 sampled mallards, 4 of 24 sampled wigeons, 3 of 13 sampled teals, none of 4 sampled common scoters, and two of 100 sampled pigeons tested positive (Table 1). The infected graylag geese showed lower body weights compared to virus negative birds. Phylogenetic analyses performed on the polymerase gene and nucleocapsid protein sequences, indicated that the novel avian coronaviruses all branch off from group III coronaviruses, but are clearly distinct from IBV and TcoV (Fig. 1). They harboured the conserved s2m RNA structure in their 3' non-coding region, like other previously described group III coronaviruses, and like the SARS coronavirus. Sequencing of the complete nucleocapsid gene and downstream regions of goose and pigeon coronaviruses, evidenced the presence of two additional open reading frames for the goose coronavirus with no sequence similarity to known proteins, but with predicted transmembrane domains for one of the encoded proteins, and one additional open reading frame for the pigeon coronavirus, with a predicted transmembrane domain, downstream of the nucleocapsid gene. These small ORFs display some nucleotide sequence similarity with the highly variable region (HVR) of the 3' –UTR of some strains of IBV, as shown in Fig.2, and this could suggest that the HVR in IBV and TCoV is a truncated, presumably non-functional, remnant of a longer gene of a common ancestor of bird coronaviruses (Jonassen et al., 2005).

DISCUSSION

The prevalence of coronavirus infection in the sampled species was highest in graylag geese, reaching 40% both in 2004 and 2005, but was, as well, high in duck species, suggesting that anseriformes are prone to coronavirus infections. Even if all goose and duck samples were obtained from live birds shot during hunting season, the weight and age data collected in the case of the goose population, showed that infected graylag geese showed lower body weights compared to virus negative birds, suggesting clinical significance of the infection.
All samples were investigated for avian influenza virus as well, and none of the goose samples were found to harbour influenza virus, while the prevalence of

influenza virus in the different duck species was similar to that of coronavirus (Table 1). Of the 40 coronavirus infected mallards, 12 were also infected with an influenza virus. A probability estimation showed however, that the occurrence of coronavirus was not conditional on influenza virus being present, given the overall high prevalence of both viruses in mallards (Fig. 3).

As opposed to avian influenza virus, where similar subtypes are found in duck and goose populations, the coronaviruses isolated from ducks were phylogenetically different from those found in the goose samples, suggesting that these viruses have a narrower host range than the influenza viruses, and while several animal species, including pigs, bats, dogs and humans, have been found to harbour different coronaviruses, from serogroup I and II, all identified wild bird coronaviruses that were sequenced were closely related to each other within the same species. Sequences obtained from wigeon and mallard coronavirus were very closely related (data not shown), indicating that the different duck species harbour similar coronaviruses.

REFERENCES

Enjuanes, L., Brian, D., Cavanagh, D. & other authors (2000) *Coronaviridae*. In *Virus taxonomy. Classification and nomenclature of viruses.*, Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carsten, M. K. Estes, S. M. Lemon, D. J. McGeoch, J. Maniloff, M. A. Mayo, C. R. Pringle & R. B. Wickner, San Diego: Academic Press.

Jonassen,C.M., Jonassen,T.O., & Grinde,B. (1998) A common RNA motif in the 3' end of the genomes of astroviruses, avian infectious bronchitis virus and an equine rhinovirus. *J Gen Virol* 79 (Pt 4**)**, 715-718.

Jonassen, CM., Kofstad T, Larsen IL, Løvland A, Handeland K., Follestad,A. & Lillehaug,A. Molecular identification and characterization of novel coronaviruses infecting graylag geese (*Anser anser*), feral pigeons (*Columbia livia*) and mallards (*Anas platyrhynchos*)*J Gen Virol* 86, 1597-1607 (2005).

Marra,M.A., Jones,S.J., Astell,C.R.& other authors (2003) The Genome sequence of the SARS-associated coronavirus. *Science* 300, 1399-1404.

Stephensen,C.B., Casebolt,D.B., & Gangopadhyay,N.N. (1999) Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. *Virus Res* 60, 181-189.

Table 1: Results of testing for both coronavirus and avian influenza virus by RT-PCR on samples from mallards, wigeons, teals, common scoters, graylag geese, and feral pigeons in Norway 2003-2005

	Total coronavirus		Total avian influenza virus		Number of positive for both coronavirus and AIV
	Number of positive/analysed samples	Positive rate %	Number of positive/analysed samples	Positive rate %	
Mallards					
2003	1/5	25.0	1/5	25	1
2005	39/140	27.9	33/140	23.6	11
Wigeons					
2005	4/24	16.7	2/24	8.3	0
Teals					
2005	3/13	23.1	5/13	38.5	2
Common scoters 2005	0/4	0	1/4	25.0	0
Graylag geese					
2003	16/100	16.0	0/100	0	0
2004	24/63	38.1	0/63	0	0
2005	67/186	36.0	0/186	0	0
droppings 2003	3/100		0/100		0
Feral pigeons					
2003	2/100	2	0/100	0	0

Figure 1. Phylogenetic analysis on part of the replicase gene of the coronavirus family. The tree was constructed by the Fitch method using the F84 model of nucleotide substitution in the PHYLIP package

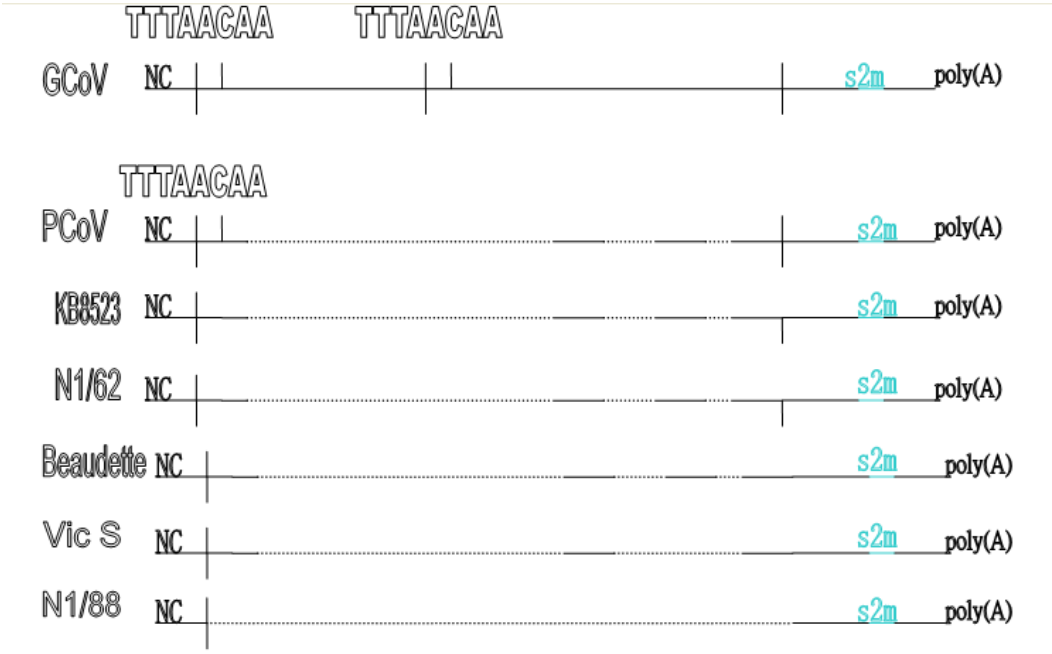

Figure 2. Schematic representation of the sequence alignment in the 3'-end of the bird coronaviruses, showing the additional ORFs of goose and pigeon coronavirus, and their sequence homology to the hypervariable region of the 3'UTR of infectious bronchitis virus (IBV). Stop codons are shown as long vertical bars, and start codons as vertical bars above the sequence line. The putative transcription regulatory sequences upstream of the ORFs are shown above the sequence line. Hypothetic stop codons of the predicted ORF in two IBV isolates (KB8523 and N162) are shown as vertical bars below the sequence line. The alignment starts at the 3'-end of the nucleocapsid (NC) gene.

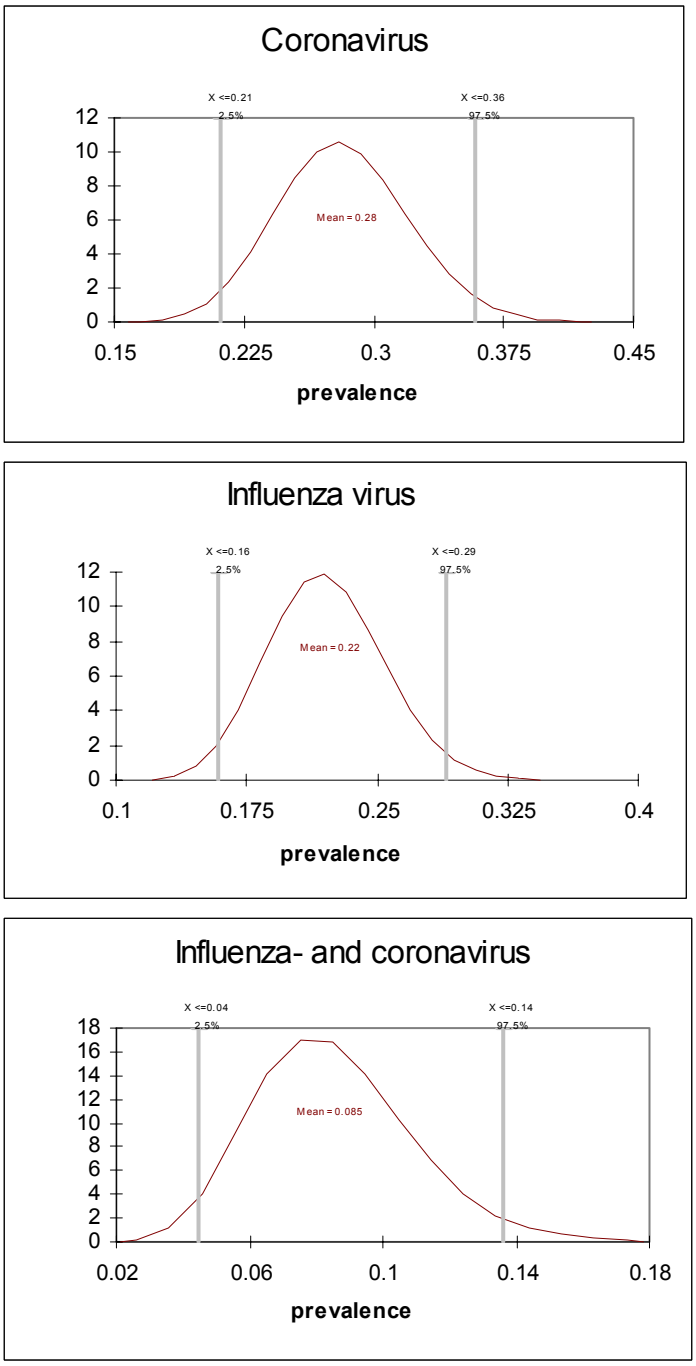

Figure 3. Distribution of prevalence for coronavirus, influenza virus, and both viruses being present, in mallards (@Risk-beta-distribution)

ISOLATION OF A CORONAVIRUS FROM A GREEN-CHEEKED AMAZON PARROT (*AMAZON VIRIDIGENALIS CASSIN*) WITH SUSPECTED PROVENTRICULAR DILATATION DISEASE (PDD)

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SUMMARY

During an investigation into the etiological role of viruses in psittacine proventricular dilatation disease (PDD), samples were received from a male green-cheeked amazon parrot (*Amazon viridigenalis Cassin*). A virus (AV71/99) was isolated by propagation and passage in both primary embryo liver cells derived from blue and yellow macaw (*Ara ararauna*) embryos and chicken embryo liver cells. Electron microscopic examination of cytopathic agents derived from both types of cell cultures suggested that it was a coronavirus. Following two-way neutralisation tests and one-way HI tests with AV71/99 and a variety of avian coronavirus strains and antiserum, no relationship with other coronaviruses was demonstrated. The identity of the virus was confirmed using a pan-coronavirus reverse transcriptase polymerase chain reaction (RT-PCR) that amplified part of gene 1 that encodes 66 amino acids of the RNA-dependent RNA polymerase. Within existing coronavirus Groups (1, 2 and 3), coronavirus species have approximately 85% or more amino acid identity in this region. Sequence comparisons between species of one Group with those of another Group reveal identities in the range 60 to 70%. The parrot virus 66 amino acids had 66 to 74% amino acid identity with the corresponding sequence of coronaviruses in Groups 1, 2 and 3. Whilst this suggests that the parrot coronavirus might not be closely related to viruses of Groups 1, 2 and 3, the extent of our sequence data is too little to make conclusions on this point. Several other oligonucleotide primer pairs that give PCR products corresponding to genes 3, 5, N and the 3' untranslated region of infectious bronchitis virus, turkey coronavirus and pheasant coronavirus (all in Group 3) failed to do so with RNA from the parrot coronavirus. This further suggests that the parrot coronavirus might not be closely related to known Group 3 coronaviruses. This is the first demonstration of a coronavirus in a psittacine species.

INTRODUCTION

During an investigation into the etiological role of viruses in psittacine proventricular dilatation disease (PDD), formerly known as Macaw wasting disease, samples were received from a male green-cheeked amazon parrot (*Amazon viridigenalis Cassin*). The bird had died in a poor condition following a history of anorexia, regurgitation and passing undigested food. At postmortem examination the main feature was a thin-walled grossly dilated proventriculus/ventriculus. Following histopathology examination of sections of crop and proventriculus, lesions similar but not conclusive of PDD were found. In proven cases of PDD a non-suppurative encephalomyelitis and ganglioneuritis is seen in the brain and spinal cord (Gerlach, 1994). Unfortunately these organs were not available for examination. We subsequently attempted virus isolation from tissues that were available, which has led to the discovery and isolation of a coronavirus, the first one reported for a psittacine species. We have recently reported our findings in detail (Gough *et al.,* 2006).

MATERIALS AND METHODS

Virus isolation

Samples of proventriculus, heart, liver, spleen, pancreas, kidneys and intestinal contents were processed and inoculated onto cell cultures as described (Gough *et al.* 1988). Primary cell cultures were prepared from the livers of 15-day-old specific pathogen free (SPF) chicken embryos and 18-day-old blue and yellow macaw (*Ara ararauna*) embryos. The samples were also inoculated into embryonated SPF chicken eggs by both allantoic cavity and yolk-sac routes.

Electron microscopy

Inoculated cultures of liver cells from blue and yellow macaw and chicken embryos were frozen after 2 to 3 days and cell lysates prepared by two cycles of freezing and thawing. After low speed clarification the clarified supernatant was centrifuged at 32 000 x g for 1 h at 4^0 C. The pellet was resuspended in deionised water and examined by negative contrast electron microscopy. Uninoculated control cultures of macaw and chicken embryo liver cells received three blind passages and were similarly examined by electron microscopy.

Serology

As electron microscopy had revealed the presence of coronavirus-like particles in inoculated cell cultures, monospecific antiserum against the agent, designated AV71/99, was prepared in six SPF chickens. Briefly, each bird was inoculated firstly by the intranasal route, then intravenously and finally intramuscularly (Gough et al., 2006). The sera were used in haemagglutination inhibition (HI), virus neutralisation (VN) and agar gel precipitation (AGP) tests as described by Gough *et al.* (1996). For the HI tests, antigens were prepared from several infectious bronchitis virus (IBV) reference strains from chickens (M41, 793B, D274 and D1466), and two pheasant coronavirus isolates (ph/UK/438/94 and ph/UK/602/95; Cavanagh et al, 2002), and used in one-way HI tests.

A total of 53 sera were examined for neutralising antibodies to the coronavirus-like agent from the following psittacine birds; various species of macaws (42),

budgerigars (*Melopsittacus undulatus*) (3), sulphur-crested cockatoos (*Cacatua galerita*) (2), a Patagonian conure (*Cyanoliseus patagonus*) (1) and unspecified parrots (5)(Gough et al., 2006).

Sequence analysis

Viral RNA was extracted from a pool of clarified CEL cell lysate and culture supernatant, followed by RT-PCR (Gough et al., 2006). The pan-coronavirus, degenerate oligonucleotide primers 8p, 1Ap and 2Bp (genome sense) and 4Bm and 7m (antigenome sense) described by Stephensen *et al.* (1999) were initially used. They correspond to sequences within the highly conserved (throughout the coronaviruses) RNA-dependent RNA polymerase (RdRp)-encoding region of gene 1b. Subsequently several other primer pairs were used, designed to amplify genes 3 and 5, and the 3' untranslated region (UTR; Cavanagh *et al.* 2001, 2002), and gene N (Sellers *et al.*, 2004) of avian (Group 3) coronaviruses. RT–PCR conditions, electrophoresis, and sequencing were as described by Cavanagh *et al.* (2001). The partial sequence of the RdRp region of gene 1 of the parrot coronavirus has been submitted to GenBank, accession number DQ233651.

RESULTS

Virus isolation and electron microscopy

Following inoculation of primary cell cultures, a widespread cytopathic effect (CPE) was seen in the macaw embryo liver (MEL) cultures inoculated with samples of liver, spleen and kidney. The CPE was characterised by the appearance of rounded, highly refractile cells that detached from the monolayer two to three days after inoculation. A similar CPE was noted in the CEL cultures, although two passages were required before the CPE was observed. Due to unavailability of macaw embryos all subsequent propagation of the agent was undertaken in CEL cultures. No cytopathic agents were detected in the uninoculated control cultures following three blind passages. The inoculated SPF chicken embryos remained normal following three passages in the allantoic cavity and yolk sac.

Negative contrast electron microscopy of material recovered from both the MEL and CEL cultures following ultracentrifugation revealed spherical and pleomorphic virus particles, which in size and morphology resembled coronaviruses (Figure 1).

RT-PCR and sequence analysis

We used the pan-coronavirus, degenerate oligonucleotide primers, corresponding to part of the highly conserved RNA-dependent RNA polymerase-encoding region of gene 1b, of Stephensen et al. (1999; Figure 1). Primer combinations 8p+/7m- and 1Ap+/7m- did not yield a PCR product with the parrot virus RNA. However, primer pair 2Bp+/4Bm- did yield a 251 bp product, the size expected if the parrot virus was a coronavirus. A continuous sequence of 66 amino acids was deduced. BLAST analysis against the EBI database revealed identity with only one protein, namely the RdRp of coronaviruses. Comparison of the deduced amino acid sequence with that of coronaviruses representative of Groups 1, 2, 3 and SARS-CoV revealed amino acid identity in the range 66 to 74% (Figure 2). Within existing Groups coronavirus species have approximately 85% or more amino acid identity in this region. Sequence comparisons between species of one Group with those of another Group

reveal identities in the range 60 to 70%. This data confirmed the deduction made after the electron microscopy observations; the virus was a coronavirus-parrot coronavirus (PaCoV)
Phylogenetic analysis of the 66 residue partial sequence of the RdRp of the PaCoV revealed that it did not coincide with any of the three existing coronavirus groups (Figure 3). Although the RdRp is one of the most conserved protein sequences amongst coronaviruses, the small size of our sequence, only 66 residues, is such as to preclude at this stage any conclusions with regard to which, if any, of the existing groups the parrot virus might belong.
Apart from the RdRp oligonucleotides, none of the other oligonucleotide pairs used in this study, based on IBV (Group 3) sequences (see Figure 1 in Cavanagh *et al.*, 2002), gave PCR products. These primer pairs had been chosen because they gave PCR products with >18 strains of IBV and also with turkey coronavirus (TcoV) and pheasant coronavirus (PhCoV) (Cavanagh *et al.*, 2001, 2002; Sellers *et al.*, 2004). This suggests a low degree of relationship between the PaCoV and the Group 3 coronaviruses of the galliform birds.

Serological analysis
Antiserum to the PaCoV, prepared in SPF chickens, did not cause inhibition of haemagglutination or neutralisation of the six serotypes of IBV investigated. No neutralisation of the PaCoV was recorded when tested in CEL cultures with six reference IBV antisera. In the IBV group- specific AGP test no lines of precipitation were detected.
In a limited serological survey, 53 psittacine serum samples from a variety of sources, including four private aviaries, a collection in a zoological garden, quarantine aviaries and individual pet birds, were examined for neutralising antibodies to the PaCoV. Antibodies to the isolate were detected in 6 macaw sera, with antibody titres ranging from 2^3 (1/8) to 2^6 (1/64). In three cases PDD had been reported in the aviaries from which positive sera had originated, but three other positive sera originated from birds in quarantine (2) and a private aviary (1) in which PDD had not been reported.

DISCUSSION

Given that IBV exists as dozens of serotypes (Lee & Jackwood, 2001; Cavanagh, 2003; Liu & Kong, 2004; Cavanagh *et al.,* 2005; Gelb *et al.,* 2005), determined by the spike (S) protein, it was not surprising that there was no cross-reaction of the PaCoV serum with the six IBV serotypes and PhCoV in HI and VN tests. However, the N and M proteins are much more conserved amongst IBV, TCoV and PhCoV, resulting in antigenic cross-reactivity (Gough *et al.*, 1996; Guy *et al*., 1997, 2002; Ismail *et al.,* 2001; Chen *et al.,* 2003). The lack of antigenic relationship between IBV and PaCoV revealed by the AGP test indicates that the virion proteins of the parrot virus have substantially different sequences from those of the IBV-like viruses of Group 3.
Our investigation clearly shows that the virus isolated from the green-cheeked Amazon parrot with suspected PDD is a “new” coronavirus. Several coronaviruses have been detected within recent years in galliform (peafowl, *Pavo cristatus*, Liu *et al.*, 2005; partridge (*Alectoris* sp.); guinea fowl, *Numida meleagris*, Ito *et al.,* 1991). It is not clear whether these are new coronaviruses or strains of IBV that had infected

these species; domestic chickens were kept nearby. The peafowl isolate of Liu et al. (2005) had >99% nucleotide identity, throughout its genome, with the H120 IB vaccine strain, making it most likely that the peafowl virus was a reisolation of H120. The same authors, in China, isolated a coronavirus from a teal duck (*Anas* sp.), which had S gene sequence similar to that of nephropathogenic IBVs in China. Indeed, the duck isolate caused kidney disease when inoculated into chickens, suggesting that it was probably a strain of IBV. Jonassen and colleagues (2005) have detected coronaviruses in greylag goose, *Anser anser*; mallard duck, *Anas platyrhynchos*; and pigeon, *Columbia livia*, Jonassen *et al*., 2005), reviewed by Cavanagh (2005). Sequencing of the nucleocapsid (N) protein gene and the 3' untranslated region revealed high identity with the Group 3 coronaviruses of the galliform birds. That said, they were clearly not strains of IBV, TCoV or PhCoV as they had one or two additional open reading frames between the N gene and the 3' UTR. The parrot coronavirus that we have described would seem to be a distant relative of the Group 3 viruses at best, and might not actually be sufficiently related to them, or to other known coronaviruses, to be assigned to any of the existing three coronavirus groups. Further analysis of the PaCoV genome is required to clarify this point.

It is unclear what the etiological role of this coronavirus is in relation to PDD. The fact that the virus was isolated from a suspected case of PDD implies a causal relationship to the disease. During the course of this study a coronavirus-like agent was also isolated in CEL cultures from a Senegal parrot (*Poicephalus senegalus*) that had died in quarantine. Although MEL cultures were unavailable for attempted virus isolation, the agent had a similar morphological appearance when examined by EM, replicated in CEL cell cultures causing a widespread CPE very similar to the PaCoV and appeared antigenically unrelated to IBV. In this case, however, there were no gross lesions of PDD and the cause of death was attributed to aspergillosis. Further transmission studies will be required to determine if the parrot coronavirus of the present study is associated with PDD.

ACKNOWLEDGEMENTS

Thanks are due to Mark Evans MRCVS for referring the suspected case of PDD to us for investigation. Also, the Parrot Society (UK), Parrot Fund International and the Mid-West Avian Research Expo (MARE) for their financial support. Francesca Culver was a graduate student supported jointly by the British Poultry Council (Turkey Sector) and Merial Animal Health. Dave Cavanagh and Paul Britton are supported by the Department of the Environment, Food and Rural Affairs (grant OD0714), and by the Biotechnology and Biological Sciences Research Council.

REFERENCES

Cavanagh, D. (2003). Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis virus. Avian Pathology, 32, 567-582.

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. Avian Pathology, 34, 439-448.

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S.E., Ainsworth, H.L., Britton, P. & Gough, R.E. (2001). Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. Avian Pathology, 30, 365-378.

Cavanagh, D., K. Mawditt, D. d. B. Welchman, Britton, P. & Gough, R.E. (2002). Coronaviruses from pheasants (Phasianus colchicus) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian Pathology, 31, 181-93. 2002.

Cavanagh, D., Picault, J-P., Gough, R.E., Hess, M., Mawditt, K. & Britton, P. (2005). Variation in the spike protein of the 793/B type of infectious bronchitis virus, in the field and during alternate passage in chickens and embryonated eggs. Avian Pathology, 34, 20-25.

Chen, H., Coote, B., Attree, S. & Hiscox, J.A. (2003). Evaluation of a nucleoprotein-based enzyme-linked immunosorbent assay for the detection of antibodies against infectious bronchitis virus**.** Avian Pathology, 32, 519-526.

Gelb, J. Jr, Weisman, Y, Ladman, B.S., & Meir, R. (2005). S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996 to 2000). Avian Pathology, 34, 194-203.

Gerlach, H. (1994). Viruses. In B. Ritchie, G. Harrison and L. Harrison (Eds), Avian Medicine: Principles and Application (pp.862-948). Lake Worth, Florida: Wingers Publishing.

Gough, R. E., Alexander, D. J., Collins, M. S., Lister, S.A. & Cox, W.J. (1988). Routine virus isolation or detection in the diagnosis of diseases of birds. Avian Pathology, 17, 893-907.

Gough, R.E., Cox, W.J., Winkler, C.E. Sharp, M.W. & Spackman, D. (1996). Isolation and identification of infectious bronchitis virus from pheasants. The Veterinary Record, 138, 208-209.

Gough, R. E, Drury, S.E., Culver, F., Britton, P. & Cavanagh, D. (2006). Isolation of a coronavirus from a green-cheeked Amazon parrot (Amazon viridigenalis Cassin). Avian Pathology, 35, 122-126.

Guy, J.S., Barnes, H.J., Smith, L.G., Breslin, J. (1997). Antigenic characterization of a turkey coronavirus identified in poult enteritis and mortality syndrome-affected turkeys. Avian Diseases, 41, 583-590.

Guy, J.S., Smith, L.G., Breslin, J.J., Pakpinyo, S. (2002). Development of a competitive enzyme-linked immunosorbent assay for detection of turkey coronavirus antibodies. Avian Diseases, 46, 334-341.

Ismail, M.M., Cho, K.O, Hasoksuz, M. Saif, L.J. & Saif, Y.M. (2001). Antigenic and genomic relatedness of turkey-origin coronaviruses, bovine coronaviruses, and infectious bronchitis virus of chickens. Avian Diseases, 45, 978-984.

Ito, N.M.K., Miyaji, C.I. & Capellaro, C.E.M.P.D.M. (1991). Studies on broiler's IBV and IB-like virus from guinea fowl. In II International Symposium on Infectious Bronchitis, pp. 302-307. E.F. Kaleta and U. Heffels-Redmann (Eds). Giessen: Justus Leibig University.

Jonassen, C.M., Kofstad, T., Larsen I-L., Lovland, A., Handeland, K., Follestad, A. & Lillehaug, A. (2005). Molecular identification and characterization of novel coronaviruses infecting greylag geese (Anser anser), feral pigeons (Columba livia) and mallards (Anas platyrhynchos). Journal of General Virology, 86, 1597-1607.

Lee, C.W. & Jackwood, M.W. (2001). Origin and evolution of Georgia 98 (GA98), a new serotype of avian infectious bronchitis virus.Virus Research, 80, 33-9.

Liu, S. & Kong, X. (2004). A new genotype of nephropathogenic infectious bronchitis virus circulating in vaccinated and non-vaccinated flocks in China. Avian Pathology, 33, 321-327.

Liu, S., Chen, J., Chen, J., Kong, X., Shao, Y., Han, Z., Feng, L., Cai, X., Gu, S. & Liu, M. (2005). Isolation of avian infectious bronchitis coronavirus from domestic peafowl (Pavo cristatus) and teal (Anas). Journal of General Virology, 86, 719-725

Sellers, H.S., Koci, M.D., Linnemann, E., Kelley, L.A., Schultz-Cherry, S. (2004). Development of a multiplex reverse transcription-polymerase chain reaction diagnostic test specific for turkey astrovirus and coronavirus. Avian Diseases, 48, 531-539.

Stephensen, C. B., Casebolt, D.B. & Gangopadhyay, N.N. (1999). Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronavirus and development of a consensus polymerase chain reaction assay. Virus Research, 60, 181-189.

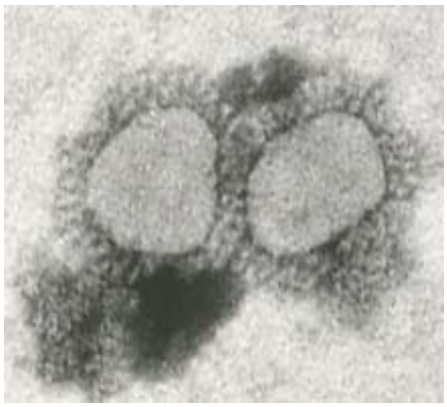

Figure 1. Electron micrograph of the parrot coronavirus grown in macaw embryo liver cultures, following inoculation with samples of liver, spleen and kidney.

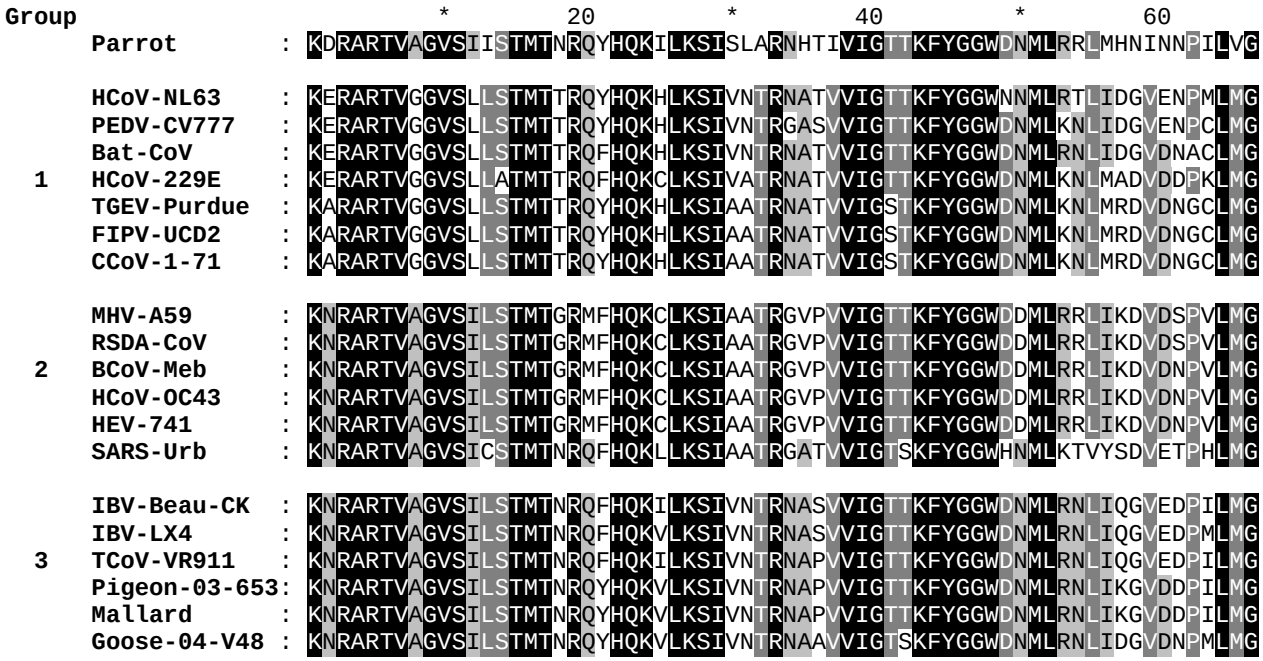

Figure 2. Comparison of the deduced amino acid sequences of the RdRp protein region corresponding to the parrot-derived sequence. The sequences were aligned using ClustalX and displayed using Genedoc. The comparison comprises selected species of coronaviruses representing each of the coronavirus groups, 1, 2 and 3. Where the sequences of more than one strain of a coronavirus species was available (e.g. MHVmurine hepatitis virus) a representative sequence, identical to that of the other strains that are not included in the figure, has been used in this figure. The accession numbers of the sequences used are reported in Gough et al. (2006).

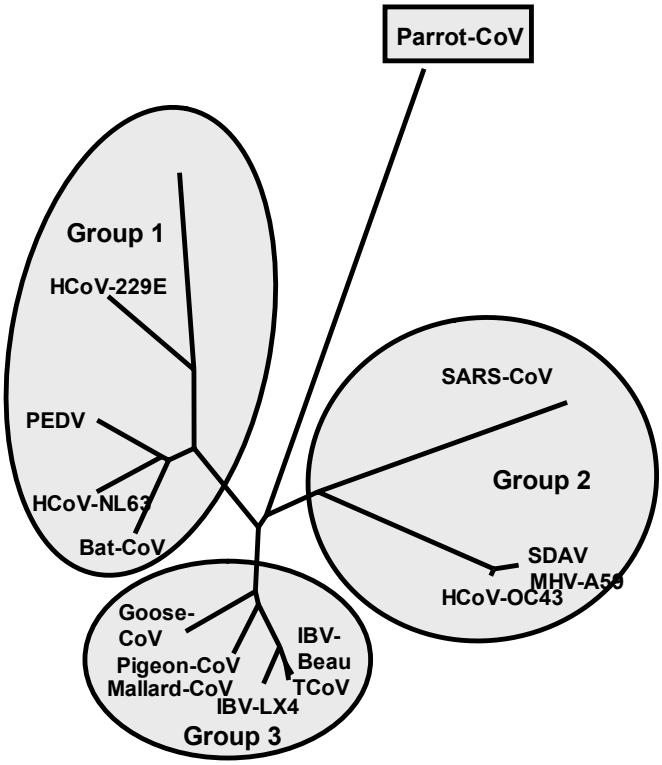

Figure 3. Phylogenetic relationship of the 66 amino acid parrot-derived RdRp protein sequences compared with the corresponding region of the RdRp protein from coronaviruses representing each of the three groups. The sequences were aligned using Clustal X and analysed using the PHYLIP programs, PROTDIST using Kimura's distance method and NEIGHBOUR using randomization of the sequences. The resulting tree is unrooted. References for the sequences are in Gough et al., 2006).

VARIATIONS IN S1 SPIKE PROTEIN OF SOME EUROPEAN INFECTIOUS BRONCHITIS VIRUSES, OBTAINED AFTER DIFFERENT LEVELS OF *IN OVO* PASSAGE

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INTRODUCTION

Avian infectious bronchitis (IB) is considered one of the most important and complex diseases of chicken, even if other species of bird may be affected. It is associated with respiratory symptoms, drop in egg laying and, sometime and with some strains, with nephritis and enteric symptoms (7,9,10,12). Moreover, it is known for many years that IB virus (IBV) exists in over 60 different serotypes or variants, which are continuously increasing (5,14). Consequently, such characteristic makes quite complex to control the disease by vaccination, particularly in long-life birds.
To better control the disease, several strains of IBV have been attenuated, as from years '50s, by serial passages in embryonated egg. The most used of them are various strains belonging to the first isolate, the Massachusetts (Mass.) serotype. Afterwards, some other serotypes have also been used, obviously previous attenuation: particularly Connecticut, Arkansas, DE-072, GA-98 in USA, D-274, D-1466, AZ-23/74, UK-4/91 in Europe, Australia B and C. More recently also some recombinant vaccines have been studied. The above mentioned and other serotypes of IBV have also been included in inactivated vaccines, to induce a wider specific immunity in layers and breeders, a few weeks before to enter in lay.
The degree of virus attenuation seemed to vary among different commercial vaccines. Both the stability of virus attenuation and efficacy appear to be a very important tool, when the vaccine has to be used in field, particularly in multi-aged farms.
The first evaluations on the variability, exhibited within the S1 spike protein genes of a IBV serotype, were done: with 8 strains of Mass. serotype, collected in about 30 years: maximum difference in amino acid sequences of about 6% (3); with 7 strains of D-274 variant over eight-years period: maximum difference in amino acids of 5%

(4); more recently, with different strains of 793B serotype: maximum difference in amino acids of 8% (6).
This paper reports on possible variation in nucleotide and, consequently, in amino acid sequence in S1 spike protein of three of the most common European IBV strains and of commercial vaccines Mass. type, undergone to low and high *in ovo* passages.

MATERIALS and METHODS

IBV isolates
AZ-23/74, 15th and 101st passage, AZ-27/98, 11th, 85th and 101st passage, AZ-40/05, 10th, 60th and 85th passage in embryonated egg, isolated respectively in 1974, 1998 and 2005(11.12.15); Mass. M41, H52 and H120, vaccinal type after 52 and 120 passages of H strain (1). The material for sequentiation is represented by allantoid fluid of infected embryonated eggs.

Molecular characterization
RT-PCR tests were performed according to the described methods, as follows: - extraction of RNA from allantoic fluids ,purification by the method of Chomezynski and Sacchi (8) and storage at -20°C;
- RNA detection by RT-PCR assay using primers: XCE1+(5' CTC TAT AAA CAC CTT ACA 3') plus XCE2– (5' CTC TAT AAA CAC YTT ACA 3') or IBP1(5' CAA TTA ATT TGG ACC TTA TCCA 3'), able to amplify respectively a fragment of 456 bp or 1100 bp of hypervariable region of S1 spike protein;
- amplified fragments analysis by 1.7 agarose gel electrophoresis, stained with ethidium bromide and observed with an ultraviolet transilluminator;
- sequencing of various strains performed on PCR product directly after gel purification with Qiaquick gel extraction kit (QUIAGEN) with primers XCE1+ and XCE2- and IBP1, by means of a Big Dye Terminator DNA Sequencing Kit on ABI Prism 3130 Genetic Analyzer (Applied Biosystem, Foster City, CA).Comparative analysis of nucleotide and deduced amino acid sequences was carried out with Clustal W Package of DNA Star (MegAlign v.5 2001; DNA Star inc., Madison, WI)
A molecular weight standard was added and used to determine the size of cDNA fragments.

RESULTS and CONSIDERATIONS

The comparative analysis of 152 amino acids (from aa 235 to aa 287, numbering referred to M41 strain) the S1 spike protein between the low (10/15) and high (85/101) egg passages of three IBV strains, isolated in Italy (AZ-23/74, AZ-27/98, AZ-40/05) demonstrated no difference between low and high passages of virus *in ovo.* Only an amino acids substitution in position 341(leucine with phenylalanine)in AZ-27/98 strain has been detected. Similar results were obtained comparing commercial vaccine prepared with Mass. H52 and H120 strains, even if it was not possible to compare them with low passage of Mass. H., but only with Mass.41. The results of the test are reported in figures 1,2,3 and 4. Therefore, it is likely that sequences outside of the S1 gene may account for the difference in pathogenicity level of a IBV strain.

A difference only in one amino acid (residue in serine-alanine in position 283) in the protein of UK-4/91, strain during passages and back passages in embryonated eggs and chicken, was also observed.
Such change was apparently not associated with pathogenicity of the virus (6). No substantial differences (0.6% in S1 protein) between pathogenic and attenuated UK-4/91 were also detected with PCR/RFLP(2). Apparently no record on other part of genome has been so far done, to differentiate wild and vaccinal IBV strains.
The non significative difference in sequences of S1 spike protein between low and high passage of virus would explain the rather good conservation of antigenic characteristics and, consequently, the respective antibody response.
These researches demonstrated that about one hundred passages *in ovo* wouldn't modify substantially the immunogenicity of IBV. In the past, non extensive passage were believed in order to avoid reduction of immunogenicity. However, a less ability to multiply in vivo could occur; that should be better verified.
Moreover, it has been pointed out that:
- attenuation of strain AZ-23/74 resulted immunologically stable after one hundred passages *in ovo* and five back passages in chicken(11);
- strain AZ-27/98 induced very few or no lesions in trachea and in kidney of SPF chicks after 50 and 75 passages *in ovo* (12; Zanella, pers. observ.) Further investigations on reported strains are ahead.
In conclusion, the attenuation and the antigenic stability of the virus appear to be very important factors in order to licence a live attenuated IBV vaccine.Moreover, also with about 100 passages, the antigenicity would seem to be sufficiently maintained. Further studies are programmed in order to better characterize other variation between wild viruses and the respective attenuated strains.

REFERENCES

1) Bijleng G., J.K.A. Cook, J. Gelb & J.J. de Wit. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathology, 33: 550-557 (2004)
2) Callison S.A.; M.W. Jackwood & D.A. Hilt. Molecular characterization of infectious bronchitis virus isolates foreign to the Unites States and comparison with United States isolates. Avian Disease, 45: 492-499 (2001)
3) Cavanagh,D., P.J. Davis & A.P.A. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Research, 11: 141-150 (1988).
4) Cavanagh,D., P. J. Davis, J.K.A. Cook, D. Li, A. Kant & G. Koch. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathology, 21: 33-23 (1992).
5) Cavanagh,D. Discussion of issue raised during the 1998 Symposium. Proc. III Int. Symposium on Infectious Bronchitis and Pneumovirus Infections in Poultry, Rauischholtzhausen 15-18 June, pp. 373-380 (1998).
6) Cavanagh,D., J. P. Picault, R. E. Gough, M. Hess, K. Mawditt & P. Britton. Variation in the spike protein of the 793/B type of infectious bronchitis virus in the field and during alternate passage in chicken and embrionated eggs. Avian Pathology, 34: 20-25 (2005).

7) Cavanagh,D. Coronaviruses in poultry and other birds. Avian Pathology, 34: 439-448 (2005).
8) Chomezynski P. & N. Sacchi. Single-step method of RNA isolation by acid guanidinum thicyanated–phenol-chloroform extraction. Anal. Biochem., 162: 156-159 (1987).
9) Cook, J. K. A., S.I. Orbell, M.A. Wood, & M.B. Huggins. A survey of the presence of a new infectious bronchitis virus designated 4/91 (793B). Vet. Rec., 138: 178-180 (1996).
10) Cook, J. K. A., Infectious bronchitis-worldwide situation. Publications on IB since 1998. Proc. IV Int. Symposium on Avian Corona and Pneumovirus Infections, Rauischholzhauses, Germany 20-23 June, pp 1-5 (2004).
11) Zanella, A., Avian infectious bronchitis: properties and application of attenuated vaccine prepared with nephropatogenic strain AZ 23/74. Proc. I Int. Symposium on Infectious Bronchitis, Rauischholzhausen, Germany, June 23-26, pp 335-342 (1981).
12) Zanella, A., A. Lavazza, R. Marchi, A. Moreno-Martin, & F. Paganelli, Avian infectious bronchitis: characterization of new isolates from Italy. Avian Diseases, 47: 180-185 (2003).
13) Zanella A. Control of residual pathogenicity and immunogenicity in IBV strain AZ-27/98 after 50 embryo passages. Proc. IV Int. Symposium on Avian Corona and Pneumovirus Infections. Rauischholzhausen, Germany, June 20-23, pp 220-224 (2004).
14) Zanella A. Ceruti R & Gavazzi L. Infectious bronchitis: an over view of European epidemiological situation. Proc. 54th Western Poultry Didease Conference, Vancouver, Canada, April 25-27, pp 94-97 (2005).
15) Zanella A., I. Barbieri, L. Gavazzi & G. Tosi. Avian Infectious Bronchitis: isolation of an umpteenth new serotype of virus in Italy. Proc. V Int. Symposium on Avian Corona- and Pneumovirus Infections. Rauischholzhausen, Germany, June 20-23, in press (2006).

Figures 1, 2, 3 and 4:
Alignment of amino acidic sequences of low and high *in ovo* passages of 4 types of IBV virus: AZ-23/74 (1); AZ-27/98 (2); AZ-40/05 (3) and Mass H (4).

1.

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                    245                 255                 265                 275
            --------------------+-------------------+-------------------+-------------------+-
AZ-23/74 15 T G N F S D G F Y P Y T N S S L V K E R F I V Y R E S S V N T T L V L T N I T F
AZ-23/74 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    285                 295                 305                 315
            --------------------+-------------------+-------------------+-------------------+-
AZ-23/74 15 S N E S N A R P N T G G V H S I V L H Q T Q T A Q S G Y Y N F N F S F L S S F R
AZ-23/74 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    325                 335                 345                 355
            --------------------+-------------------+-------------------+-------------------+-
AZ-23/74 15 Y V E S D F M Y G S Y H P K C S F R L E T I N N G L W F N S L S V S L G Y G P L
AZ-23/74 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    365                 375                 385
            --------------------+-------------------+-------------------+-----
AZ-23/74 15 Q G G C K Q S V F N N M A T C C Y A Y S Y G G P T L C K G V Y R
AZ-23/74 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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2.

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AZ-27/98 11 T G N F S D G F Y P F T N S S L V K D R F I V Y R E S S I N T T L E L T N F T F
AZ-27/98 85 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-27/98 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    285                 295                 305                 315
            --------------------+-------------------+-------------------+-------------------+-
AZ-27/98 11 T N V S N A A P N S G G V A T F Q L Y Q T H T A Q D G Y Y N F N L S F L S S F V
AZ-27/98 85 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-27/98 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    325                 335                 345                 355
            --------------------+-------------------+-------------------+-------------------+-
AZ-27/98 11 Y K S S D F M Y G S Y H P K C N F R P E N I N N G L W F N S L S V S L T Y G P I
AZ-27/98 85 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . F . . . . . . . .
AZ-27/98 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . F . . . . . . . .

                    365                 375                 385
            --------------------+-------------------+-------------------+-----
AZ-27/98 11 Q G G C K Q S V F S N R A T C C Y A Y S Y H G P T R C K G V Y R
AZ-27/98 85 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-27/98 101. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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3.

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                    245                 255                 265                 275
          ----------+-------------------+-------------------+-------------------+--
AZ-40/05 10   T G N F S D G F Y P F T N S T L V R E K F I V Y R E S S V N T T L A L T N F T F
AZ-40/05 60   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-40/05 85   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    285                 295                 305                 315
          ----------+-------------------+-------------------+-------------------+--
AZ-40/05 10   T N V S N A Q P N S G G V N T F H L Y Q T Q T A Q S G Y Y N F N L S F L S Q F V
AZ-40/05 60   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-40/05 85   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    325                 335                 345                 355
          ----------+-------------------+-------------------+-------------------+--
AZ-40/05 10   Y K A S D F M Y G S Y H P S C S F R P E T I N S G L W F N S L S V S L T Y G P L
AZ-40/05 60   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-40/05 85   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

                    365                 375                 385
          ----------+-------------------+-------------------+-----
AZ-40/05 10   Q G G C K Q S V F S G K A T C C Y A Y S Y K G P M A C K G V Y S
AZ-40/05 60   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
AZ-40/05 85   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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4.

```
               245                 255                 265                 275
     ----------+-------------------+-------------------+-------------------+--
M41  T G N F S D G F Y P F I N S S L V K Q K F I V Y R E N S V N T T F T L H N F T F
H52  . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . .
H120 . . . . . . . . . . T . . . . . . . . . . . . . . . . . . . . . . . . . . . .

               285                 295                 305                 315
     ----------+-------------------+-------------------+-------------------+--
M41  H N E T G A N P N P S G V Q N I L T Y Q T Q T A Q S G Y Y N F N F S F L S S F V
H52  . . . . . . . . . . . . . . . . Q . . . . . . . . . . . . . . . . . . . . . .
H120 . . . . . . . . . . . . . . . . Q . . . . . . . . . . . . . . . . . . . . . .

               325                 335                 345                 355
     ----------+-------------------+-------------------+-------------------+--
M41  Y K E S N F M Y G S Y H P S C N F R L E T I N N G L W F N S L S V S I A Y G P L
H52  . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
H120 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .

               365                 375                 385
     ----------+-------------------+-------------------+-----
M41  Q G G C K Q S V F S G R A T C C Y A Y S Y G G P S L C K G V Y R
H52  . . . . . . . . . . . . . . . . . . . . . . . . L . . . . . . S
H120 . . . . . . . . . . . . . . . . . . . . . . . . L . . . . . . S
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SEQUENCE CHANGES OF INFECITOUS BRONCHITIS VIRUS AFTER PASSAGES IN CHICKENS EMBRYOS

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SUMMARY

In previous work, we developed attenuated infectious bronchitis virus (IBV) vaccines. But the relationship between sequences and IBV virulence is not clear. In this study, the sequences at the 3' 7.3 kb genome amplified by reverse transcription-polymerase chain reaction (RT-PCR) before and after attenuation were compared to study the relationship between virulence and sequences of IBV. Three to 7 amino acid substitutions were found in the spike 1 (S1) subunit, and two or three amino acid substitutions were found in the S2 subunit after attenuation. No or one amino acid substitution was found in the small membrane gene, and one to three amino acid substitutions were found in the membrane gene. However, no amino acid substitution was found in the nucleocapsid (N) gene. The un-translated sequence after N of one strain was partially deleted after attenuation.

INTRODUCTION

Infectious bronchitis virus (IBV), with a large positive-sense RNA genome of 27.6 kb, is a prototype of the *Coronaviridae* family. IBV is in Group 3 of the *Coronavirus* genus, and the other two groups comprise mammalian coronaviruses that differ extensively from IBV (Cavanagh & Naqi, 2003).

IBV has been identified in many parts of the world and is an economically important disease of chickens. Chickens of all ages may be infected, and the virus replicates in many tissues. The infected chickens show signs of depression, coughing, sneezing, nasal discharge, polyuria and death. Different strains of IBV may cause distinct illness. The Gray and Holte strains cause both respiratory and nephrogenic diseases, whereas the Mass41 and Ark99 strains cause respiratory diseases (Johnson & Marquardt, 1976; Winterfield & Hitchner, 1962). Less commonly, some strains of IBV exhibit tropisms for the alimentary tract and kidneys, and the major IBVs isolated in Taiwan are mostly nephrogenic strains (Tseng *et al*., 1996). Based on restriction fragment length polymorphism, the IBVs in Taiwan may be divided to two groups,

Taiwan Group I (TW I) and Taiwan Group II (TW II), different to other genotypes in the world (Liu *et al*., 2003; Wang & Huang, 2000; Wang & Tsai, 1996). Although vaccines have generally been used in controlling the clinical disease, outbreaks of IB occur frequently due to the serotype differences (Arvidson *et al*., 1991; CooK *et al*., 2001).

The RNA genome of IBV has a unique discontinuous transcription which forms a nested set of genomic and subgenomic mRNAs. These mRNAs consist of an overlapping nested set of 3' coterminal RNAs containing a common leader RNA sequence (64 nucleotides (nt)) at the 5' end. Four major structural proteins, the glycosylated spike (S) protein, the envelope or small membrane (E) protein, the membrane (M) protein, and the phosphorylated nucleocapsid (N) protein, make up the virion of IBV. Meanwhile, four nonstructural proteins, 3a, 3b, 5a, and 5b, are also encoded (Cavanagh & Naqi, 2003). The functions of the structural proteins have been extensively studied. For example, the S protein is post-translationally cleaved into S1 and S2 subunits (Cavanagh & Naqi, 2003). The S1 subunit forms a globular structure anchored to the viral membrane by the S2 subunit (Cavanagh, 1983). The S1 subunit is known to contain regions that induce neutralizing, serotype-specific, membrane fusion, attachment, and hemagglutination-inhibiting antibodies (Cavanagh *et al*., 1986; Hodgson *et al*., 2004; Ignjatovic & Sapats, 1991; Kant *et al*., 1992; Koch *et al*., 1990; Moore *et al*., 1997). Mutations in the antigenically important spike glycoprotein S1 subunit have been shown to lead to the proliferation of variant serotypes associated with new disease outbreaks, and to be required to induce protective immunity (Cavanagh *et al*., 1997). The fusion activity can be affected by sequences at multiple sites in the S2 subunit as well as S1 (Gallagher *et al*., 1991; Routledge *et al*., 1991). In addition, the S protein is a determinant of cell tropism (Casais *et al*., 2003). The N protein plays a role in viral replication, assembly, and immunity (Lai & Holmes, 2001). An epitope within the N protein has been shown to prime cellular immune responses to IBV (Boots *et al*., 1992). Some antigenic epitopes are identified on the S, N, and M proteins by monoclonal antibodies (Ignjatovic & McWaters, 1991). Both E and M proteins are required for assembly and budding of virions (Corse & Machamer, 2003; Vennema *et al*., 1996). Whereas little is known about the functions of 3a, 3b, 5a, and 5b proteins of IBV, though the nonstructural proteins have been identified in virus-infected cells (Liu *et al*., 1991; Liu & Inglis, 1992), but their roles in the IBV life cycle are not clearly identified (Shen *et al*., 2003).

The character of high rate of evolution, including nucleotide insertions, deletions, or point mutations in IBV variants, is due to a lack of proofreading enzymes. Sequence analysis indicates that vaccine strains may be naturally involved in recombination of IBV, and that recombination occurs not limited in the S1 gene (Brooks *et al*., 2004; Cavanagh & Davis, 1988; Wang *et al*., 1993). Fortunately, no recombination is found between H120 and the Taiwanese strains in the S1 gene (Huang *et al*., 2004).

In previous research, we developed attenuated vaccines from Taiwanese IBV strains by passaging in the specific-pathogen-free (SPF) embryonated eggs (Huang & Wang, 2006). The results showed that these attenuated vaccines had potential for controlling local Taiwanese IBV infections. This study compared the genomic sequences from the 5' end of the S gene through the 3' end of the N gene of IBV between before and after passages for recognizing the effects of mutations on attenuation.

MATERIALS and METHODS

Viruses

IBV 1171/92, a TW I strain, was isolated from broilers in Taoyuan, Taiwan in January 1992. IBV 2575/98, another TW I strain, was isolated from broilers in Changhua, Taiwan in July 1998. IBV 2296/95, a TW II strain, was isolated from broilers in Taoyuan, Taiwan in September 1995. All these IBV strains were attenuated for 74-76 passages in SPF embryonated eggs (Animal Health Research Institute, Council of Agriculture, Tamsui, Taiwan). Viruses were proved to be attenuated after passages (Huang & Wang, 2006). Viruses before (at 5-7 passages) and after attenuation (at 74-76 passages) were propagated in SFP chicken embroynated eggs and frozen in -80 C for gene sequencing.

RNA extraction

Viral RNA was extracted as described previously (Huang & Wang, 2006), using TRIzol Reagent (Life Technologies, Frederick, MD), according to the protocol provided by the manufacturer.

Reverse transcriptase-polymerase chain reaction and direct sequencing

A total volume of 50 µL of reaction mixture was prepared by adding 5 µL of 10X DNA polymerase buffer, 0.5 µL of *Taq* DNA polymerase (Promega, Madison, WI), 16 µL of 1.25 mM dNTPs (Promega), 0.5 µL of 50 pmole/µL upstream primer, 0.5 µL of 50 pmole/µL downstream primer (Table 1), 0.4 µL of 40 U/µL Recombinant RNasin ribonuclease inhibitor (Promega), 0.1 µL of 10 U/µL of Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (RT) (Invitrogen, Carlsbad, California), 20 µL of viral RNA solution and 7 µL of DEPC-treated water. RT-polymerase chain reaction (PCR) was performed in one step and conducted in the GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California). Reverse transcription was performed at 40°C for 30 minutes. PCR was then performed for 35 cycles of denaturation at 94°C for 1 minute, annealing at 52°C for 1 minute and polymerization at 72°C for 1 minute and 40 seconds. The initial denaturation step was conducted at 94°C for 3 minutes, and the final polymerization step was at 72°C for 15 minutes. A volume of 10 µL amplified DNA products were analyzed by electrophoresis in 1.5% agarose gel (electrophoresis grade, Gibco BRL, Life Technologies, Grand Island, NY), stained with ethidium bromide. Sequencing was performed using a commercial service (Mission Biotech Company, Taipei, Taiwan). For the above RT-PCR products, each region was sequenced at least twice from at least two RT-PCR products to make sure that the sequences were identical.

Sequence analysis

DNA sequences were compiled and edited with Vector NTI Suite 9 software (InforMax, North Bethesda, MD) and DNAStar software (DNAStar, Inc., Madison, WI). Sequences were tested for putative glycosylation sites to evaluate possible differences among the sequences (http://bioinfo.nhri.org.tw/seqweb).

RESULTS

The IBV genomes of these three strains from S1 subunit of the spike gene through the 3' end included S1, S2, 3a, 3b, E, M, 5a, 5b, N, and an un-translated region (UTR) after N gene. The sizes of those fragments were 1620 bp (1614 bp in strain 2296/95), 1878 bp, 195 bp, 330 bp, 678 bp, 198 bp, 249 bp, 1230 bp, and 503 bp, respectively. Among them, S1, S2, E, M, and N were structural proteins and the others were non-structural proteins. The amino acid change percentages of different strains after attenuation were 0.3-1.1, 0.3-0.5, 0, 0, 0-0.9, 0.4-1.3, 0-1.5, 0-1.2, and 0% in S1, S2, 3a, 3b, E, M, 5a, 5b, and N genes. One attenuated strain showed 49 bp deletion in the UTR after the N gene. Three, 7 and 5 amino acid substitutions were found in the S1 subunit of 1171/92, 2296/95, and 2575/98, respectively (Table 2). One glycosylation site was missing at 76 passage of 1171/92 (residue 427 Ser to Asp). One glycosylation site was missing at passage 76 of 2296/95 (residue 63 Ser to Pro).

Three, 2 and 3 amino acid substitutions were found in the S2 subunit of IBV 1171/92, 2296/95, and 2575/98, respectively (Table 3).

In the E gene of 1171/92, and 2296/95, each had one amino acid substitution, whereas only 2 nucleotide mutations without amino acid substitutions in 2575/98 were found. In the E gene of 1171/92, an Arg was replaced by a stop codon. In the M gene, each of 1171/92 and 2296/95 had one amino acid substitution, and 2575/98 had 3 (Table 3). No changes were found in the N gene after attenuation.

In the four nonstructural proteins, one amino acid substitution in 5a protein (residue 7 Phe to Cys) of 2575/98, one in 5a protein (residue 32 Glu to Lys) of 2296/95, and one in 5b protein (residue 19 Ile to Val) of 1171/92.

In the UTR after N gene, a deletion of 49 bp at the 3' UTR immediately downstream from the N gene was observed in 2296/95 after attenuation.

DISCUSSION

Taiwan-isolated IBVs were grouped into Taiwan Group I and Taiwan Group II based on the C2U-C3L region of S1 gene (Wang *et al.*, 1996). The S1 gene is known to contain regions that induce neutralizing, serotype-specific, membrane fusion, attachment, and hemagglutination-inhibiting antibodies (Cavanagh *et al*., 1986; Hodgson *et al*., 2004; Ignjatovic & Sapats, 1991; Kant *et al*., 1992; Koch *et al*., 1990; Moore *et al*., 1997). It is known that many residues were determined to be correlated with antigenicity and/or neutralization inducing epitope. Kant *et al*. located the antigenic sites on the amino acid sequence of the spike sequence (Kant *et al.*, 1992), They induced residue 56 (Ser to Phe), residue 132 (His to Tyr), residue 299 (Tyr to His). Residues at 304 and 386 on the S1 gene are involved in a virus neutralization serotype specific epitope (Moore *et al.*, 1997). According to previous studies, an antigenic region of S1 gene was identified between residue 240 to 255 (Wang *et al*., 1995), 3 HVRs are located within residues of 38-67, 91-141, and 274-387 (Lee *et al*., 2003). Furthermore, additional antigenic regions were determined to those previously identified and that two regions located in the S1 and one in the S2 at residues 294-316, 532-537, and 566-584 may have a role in protection (Ignjatovic and Sapats, 2005). In this study, none of the six mutations of S1 and S2 genes of 1171/92 was within above regions. No changes in the antigenic regions indicate that the

antigenicity of attenuation still preserved, approved by previous vaccine efficacy test (Huang and Wang, 2006).
Interestingly, at the same residue, one substitution at residue 131 of 2296/95 was observed after 76 passages (His to Tyr). It suggested the important role of antigenic and/or pathogenic sites at this residue. Similarly, at residue 56, the amino acid substitution was observed in 2296/95 (Leu to Phe) after 76 passages. Whether it is related to virulence needs further evaluation.
At passage 76 of 1171/92, a stop codon was observed in the E gene at residue 104 due to the amino acid substitution (Arg to Stop), and the UTR containes six amino acids. This indicates that this region is not necessary for virus formation. The amino acid substitution at residue 188 (Thr to Ile) in the M gene in H52 and H120 was also observed in 2296/95 after attenuation (Thr to Ala).
An ORF was detected within the 3' UTR of Taiwan isolates with the potential to encode hydrophobic proteins. A similar ORF has been identified in other IBV strains, e.g. BJ/China and Cal99. In contrast with the strain Mass41, no deletion in ORF7 was observed in Taiwan strains. The porcine transmissible gastroenteritis virus (TGEV) protein 7 influences virus pathogenesis (Ortego *et al.*, 2003). The high hydrophobicity of TGEV protein 7 facilitates its insertion in membranes providing a role in virus replication (Garwes *et al.*, 1989). A relationship between gene 7 and virulence has also been observed in the FCoVs. Until now, there is little information available for the corresponding protein and its function for IBV in infected cells. Although there is no clear association between the length of the HVR downstream of the N gene and virulence (Sapats *et al*., 1996), but a 49 bp deletion (from the initiation codon of ORF 7) in the HVR was observed at passage 76 of strain 2296/95, an attenuated type, which results in the absence of ORF 7. This result suggests that the deletion may be correlated with the virulence.

REFERENCES

Arvidson, Y., Tannock, G..A. & Zerbes, M. (1991). Efficacy of Australian vaccines against recent isolates of avian infectious bronchitis viruses. *Australian Veterinary Journal, 68,* 211-212.

Boots, A.M.H., Benaissa-Trouw, B.J., Hesselink, W., Rijke, E., Schrier, C. & Hensen, E.J. (1992). Induction of anti-viral immune responses by immunization with recombinant-DNA encoded avian coronavirus nucleocapsid protein. *Vaccine, 10,* 119-124.

Brooks, J.E., Rainer, A.C., Parr, R.L., Woolcock, P., Hoerr, Fred. & Collisson, E.W. (2004). Comparisons of envelope through 5B sequences of infectious bronchitis coronaviruses indicates recombination occurs in the envelope and membrane genes. *Virus Research, 100,* 191-198.

Casais, R., Dove, B., Cavanagh, D. & Britton, P. (2003). Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *Journal of Virology, 77,* 9084-9089.

Cavanagh, D. (1983). Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. *The Journal of General Virology, 64,* 1787-1791.

Cavanagh, D. & Davis, P.J. (1988). Evolution of avian coronavirus IBV: sequence of the matrix glycoprotein gene and intergenic region of several serotypes. *The Journal of General Virology, 69,* 621-629.

Cavanagh, D, Davis, P.J., Darbyshire, J.H. & Peters, R.W. (1986). Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralising or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *The Journal of General Virology, 67,* 1435-1442.

Cavanagh, D., Ellis, M.M. & Cook, J.K.A. (1997). Relationship between variation in the S1 spike protein of infectious bronchitis virus and the extent of cross-protection. *Avian Pathology, 26,* 63-74.

Cavanagh, D. & Naqi, S. A. (2003). Infectious bronchitis. In Y.M. Saif, A. M. Fadly, L.R. McDougald & D.E. Swayne (Eds.), *Diseases of poultry* 11th edn (pp. 101-119). Ames: Iowa State Press.

Cook, J.K.A., Chesher, J., Baxendale, W., Greenwood, N., Huggins, M.B. & Orbell, S.J. (2001). Protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus. *Avian pathology, 30,* 423-26.

Corse, E. & Machamer, C.E. (2003). The cytoplasmic tails of infectious bronchitis virus E and M proteins mediate their interaction. *Virology, 312,* 25-34.

Gallagher, T.M., Escarmis, C. & Buchmeier, M.J. (1991). Alteration of the pH dependence of coronavirus-induced cell fusion: Effect of mutation in the spike glycoptrotein. *Journal of Virology, 65,* 1916-1928.

Garwes, D.J., Stewart, F. & Britton, P. (1989). The polypeptide of Mr 14000 of porcine transmissible gastroenteritis virus: gene assignment and intracellular location. *The Journal of General Virology, 70,* 2495-2499.

Hodgson, T., Casais, R., Dove, B., Britton, P. & Cavanagh, D. (2004). Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. *Journal of Virology, 78,* 13804-13811.

Huang, Y.P., Lee, H.C., Cheng, M.C. & Wang, C.H. (2004). S1 and N Gene Analysis of avian infectious bronchitis viruses in Taiwan. *Avian Diseases, 48,* 581-589.

Huang, Y.P. & Wang, C.H. (2006). Development of attenuated vaccines from Taiwanese infectious bronchitis virus strains. *Vaccine, 24,* 785-791.

Ignjatovic, J. & McWaters, P.G. (1991). Monoclonal antibodies to three structural proteins of avian infectious bronchitis: characterization of epitopes and antigenic differentiation of Australian strains. *The Journal of General Virology, 72,* 2915-2922.

Ignjatovic, J. & Sapats, S. (2005). Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. *Archives of Virology, 150,* 1813-1831.

Johnson, R.B. & Marquardt, W.W. (1976). Strains of infectious bronchitis virus on the Delmarva Peninsula and in Arkansas. *Avian Diseases, 20,* 382-386.

Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A.J. & van der Zeijst, B.A.M. (1992). Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. *The Journal of General Virology, 73,* 591-596.

Koch, G., Hartog, L., Kant, A. & van Roozelaar, D.J. (1990). Antigenic domains on the peplomer protein of avian infectious bronchitis virus; correlation with biological functions. *The Journal of General Virology, 71,* 1929-1935.

Lai, M.M. & Holmes, K.V. (2001). Coronaviridae: the virus and their replication. In: D.M. Knipe & P.M. Howley (Eds.), *Field Virology* 4th edn (pp. 1163-1185), Linppincott Williams & Wilkins Publisher, Philadelphia, PA.

Lee, C.W., Hilt, D.A. & Jackwood, M.W. (2003). Typing of field isolates of infectious bronchitis virus based on the sequence of the hypervariable region in the S1 gene. *Journal of Veterinary Diagnostic Investigation, 15,* 344-348.

Liu, D.X., Cavanagh, D., Green, P. & Inglis, S.C. (1991). A polycistronic mRNA specified by the coronavirus infectious bronchitis virus. *Virology, 184,* 531–544.

Liu, D.X. & Inglis, S.C. (1992). Identification of two new polypeptides encoded by mRNA5 of the coronavirus infectious bronchitis virus. *Virology, 186,* 342–347.

Liu, H.J., Lee, L.H., Shih, W.L., Lin, M.Y. & Liao, M.H. (2003). Detection of infectious bronchitis virus by multiplex polymerase chain reaction and sequence analysis. *Journal of Virological Methods, 109,* 31-37.

Moore, K.M., Jackwood, M.W. & Hilt, D.A. (1997). Identification of amino acids involved in a serotype and neutralization specific epitope within the s1 subunit of avian infectious bronchitis virus. *Archives of Virology, 142,* 2249-2256.

Ortego, J., Sola, I., Almazán, F., Ceriani, J.E., Riquelme, C., Balasch, M., Olana, J. & Enjuanes, L. (2003). Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. *Virology, 308,* 13-22.

Routledge, E., Stauber, R., Pfleiderer, M. & Siddell, S.G. (1991). Analysis of murine coronavirus surface glycoprotein functions by using monoclonal antibodies. *Journal of Virology, 65,* 254-262.

Sapats, S.I., Ashton, F., Wright, P.J. & Ignjatovic, J. (1996). Novel variation in the N protein of avian infectious bronchitis virus. *Virology, 226,* 412-417.

Shen, S., Wen, Z.L. & Liu, D.X. (2003). Emergence of a coronavirus infectious bronchitis virus mutant with a truncated 3b gene: functional characterization of the 3b protein in pathogenesis and replication. *Virology, 311,* 16-27.

Tseng, C.C., Li, N.Z., Yao, C.H. & Wang, C.H. (1996). Isolation and adaptation of infectious bronchitis virus in Taiwan from 1993 to 1995. *Journal of the Chinese Society of Veterinary Science, 22,* 113-120.

Vennema, H., Godeke, G.J., Rossen, J.W., Voorhout, W.F., Horzinek, M.C., Opstelten, D.J. & Rottier, P.J. (1996). Nucleocapsid-independent assembly of coronavirus-like particles by co-expression of viral envelope protein genes. *The EMBO Journal, 15,* 2020-2028.

Wang ,C.H. & Huang, Y.C. (2000). Relationship between serotypes and genotypes based on the hypervariable region of the S1 gene of infectious bronchitis virus. *Archives of Virology, 145,* 291-300.

Wang, C.H. & Tsai, C.T. (1996). Genetic grouping for the isolates of infectious bronchitis virus in Taiwan. *Archives of Virology, 141,* 1677-1688.

Wang, L., Junker, D. & Collisson, E.W. (1993). Evidence of natural recombination within the S1 gene of infectious bronchitis virus. *Virology, 192,* 710-716.

Wang, L., Parr, R.L. & Collisson, E.W. (1995). A highly conserved epitope on the spike protein of infectious bronchitis virus. *Archives of Virology, 140,* 2201-2213.

Winterfield, R.W. & Hitchner, S.B. (1962). Etiology of an infectious nephritis-nephrosis syndrome of chickens. *American Journal of Veterinary Research, 23,* 1273-1279.

Table 1**.** Primers used for spike 1,spike 2, 3a, 3b, envelope, membrane, 5a, 5b, and nucleocapsid gene.

Target segment	Primer	Primer sequence	Sense	Expected product
Spike 1	rC2U (20481F)[a]	5'- TGGTT GGCAY TTACA HGG-3'	Forward	229 bp
	rC3L (20709R)[a]	5'- RCAAT GTGTA ACAAA YACT-3'	Reverse	
	Oligo5 (20305F)	5'- AAACT GAACA AAAGA CAGAC TTAG-3'	Forward	1719 bp
	IBVc2 (22023R)[b]	5'- GCCAT AACTA ACATA TGGAC AAC -3'	Reverse	
Spike 2	Beau-21890F[b]	5'- TTCTG ACTTC TCGTA ATGA-3'	Forward	907 bp
	Beau-22796R	5'- CCTTC CTGCA TATGA CCAA-3'	Reverse	
	Beau-22459F	5'- TTTAC AAGTG TTGAA ACAGT TG-3'	Forward	804 bp
	Beau-23262R	5'- TGCAT ACTGA STAGC ATTAG-3'	Reverse	
	Beau-23110F	5'- TTTTG TGGTA GTGGA AGACA-3'	Forward	727 bp
	Beau-23836R	5'- TGTAT TGTTC AGTTA CCACA T-3'	Reverse	
3a, 3b, and Envelope	Beau-23626F	5'- AAAAC TTATA TTAAG TGGCC TTGG-3'	Forward	1065 bp
	Beau-24690R	5'- GTTAA GGGGC CAAAA GCACC -3'	Reverse	
Membrane	Beau-24444F	5'- GTTAT TGTTA ACGAG TTTCC WA-3'	Forward	1110 bp
	Beau-25553R	5'- TTGAG TTAAT AGTAG GGATT T-3'	Reverse	
5a and 5b	Beau-24960F	5'- TTGTG AGGGT CAGTG GCTTG C-3'	Forward	1151 bp
	Beau-26110R	5'- CTGGC TTAWA CCTGG CTTGG-3'	Reverse	
Nucleocapsid	Beau-25751F	5'- GGATT AGATT GTGTT TACTT -3'	Forward	724 bp
	Beau-26474R	5'- GCTGC ACGAG CAATA AGA -3'	Reverse	
	Beau-26601F	5'- CGTAC TAAAG GTAAG CAGG -3'	Forward	1007 bp
	Beau-27607R	5'- GCTCT AACTC TATAC TAGCC T-3'	Reverse	
	NP1 (26030F)[c]	5'- GGTAG YGGYG TTCCT GATAA -3'	Forward	618 bp
	NP2 (26647R)[c]	5'- TCATC TTGTC RTCAC CAAAA -3'	Reverse	
	NP3 (26554F)	5'- TGTAA GCGCA CTATA CCA -3'	Forward	

[a]Huang *et al*., 2004, [b]Wang *et al*., 1994, [c]Huang *et al*., 2004

Table 2. The amino acid sequence substitutions of spike 1 gene of IBV strains 1171/92, 2296/95, and 2575/98 during attenuation.

Strain	Passage number	Position						
		10			410		427	
1171/92	7	Ala			Phe		Ser	
	76	Thr			Ser		Asn	
Strain	Passage number	Position						
		43	47	56	63	117	131	161
2296/95	7	His	Asn	Leu	Ser	Ser	His	Ser
	76	His	Tyr	Phe	Pro	Ala	Tyr	Cys
Strain	Passage number	Position						
		47		56	94	269		387
2575/98	5	Tyr		Pro	Ala	Thr		Val
	74	Tyr		Thr	Ser	Thr		Val

Table 3. The amino acid sequence substitutions of spike 2, envelope, membrane, and nucleocapsid genes of IBV strains 1171/92, 2296/95, and 2575/98 during attenuation.

Strain	Passage number	Position							
		S2			E	M		N	
		98	148	596	104	193		No change	
1171/92	7	Ile	Ser	Leu	Arg	Gly			
1171/92	76	Val	Gly	Ile	stop	Val			
		S2			E	M		N	
		346	495		58	188		No change	
2296/95	4	Val	Glu		Ala	Thr			
2296/95	76	Ala	Gly		Ser	Ala			
		S2			E	M			N
		45	55	72	Only nt. changes	4	11	182	No change
2575/98	5	Glu	Leu	Asn		Glu	Ser	Arg	
2575/98	74	Gly	Phe	Ile		Val	Phe	Cys	

RANDOM RECOMBINATION WITHIN THE SPIKE PROTEIN GENE OF INFECTIOUS BRONCHITIS VIRUS DOES LEAD TO THE GENERATION OF VIABLE RECOMBINANT IBVS

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SUMMARY

We have tested by experiment the notion that recombination between the spike glycoprotein gene of different strains of IBV would lead to the production of viable viruses with chimaeric S genes. We have used our infectious clone system to generate the recombinant IBVs. Briefly, we permitted recombination between (a) the S gene of the full-length cDNA of the Beaudette strain that we had previously cloned within the genome of vaccinia virus, and (b) the S gene of the nephropathogenic B1648 strain, which we had cloned in a plasmid. Thirteen recombinant vaccinia viruses were recovered in which the IBV S gene was a chimaera – with sequences of both Beaudette and B1648. Recovery of viable recombinant IBVs (rIBVs), with chimaeric S genes, was attempted from seven of these, and was successful with four of them. Thus random recombination had resulted in viable IBVs in over 50% of cases. Three of the rIBVs had the *in* vitro growth characteristics of the B1648 strain, and one replicated very poorly. Our results show that recombination within the S gene can indeed result in viable chimaeric viruses, at quite a high frequency.

INTRODUCTION

When cells are infected with two strains of a given coronavirus, recombination can occur, resulting sometimes in viable progeny. Some years ago we demonstrated recombination between the Beaudette and M41 strains of IBV, though we did not have a means of isolating recombinant virus (Kottier et al., 1995). Comparison of gene sequences, including of the spike (S) glycoprotein gene, of many strains of infectious bronchitis virus (IBV) has led to the conclusion that many – perhaps all – strains of IBV have had recombination as part of their evolution. We sought to test this notion by experiment, to make an estimate of the frequency at which viable virus might emerge from the recombination process, and to assess the replicative ability of the chimaeric viruses.

We have previously made a full length cDNA of the IBV Beaudette genome, which we cloned in the genome of vaccinia virus (Casais et al., 2001). In order to replace the S protein gene with that of the M41 strain we first deleted the S gene from the cDNA within the vaccinia virus genome. When recombination took place between this cDNA and the M41 gene within a plasmid, the entire M41 S gene was recombined into the cDNA copy of the Beaudette genome, from which we recovered a viable recombinant virus (Casais et al., 2003; Britton et al., 2005). The reason that we removed the S gene from the full-length cDNA was that otherwise recombination would have taken place randomly between the S genes of Beaudette and M41, resulting in chimaeras – which at that time we did not want. For the current study we did want recombination to take place within the Beaudette S gene. Therefore we used our original vaccine virus containing the full-length Beaudette genome, complete with S gene. For recombination we used a plasmid containing the S gene of the Belgian BE/B1648/84 neprhopathogenic strain of IBV (Shaw et al., 1996).

MATERIALS and METHODS

Viruses

The Beaudette strain of IBV grows very well in chick kidney (CK) cell cultures and Vero cells, whilst the B1648 strain replicates very little in CK cells and not at all in Vero cells. The cloning of the Beaudette genome within vaccinia virus has been described (Casais et al., 2001) as has the cloning of the S gene of B1648 in a plasmid (Shaw et al., 1996).

Recombination and rescue of rIBVs

The procedure for making recombinant IBVs was essentially as described for complete S gene replacement (Casais et al., 2003), and for alteration of other IBV genes (Casais et al., 2005; Hodgson et al., 2006). By virtue of the mechanism by which recombinant vaccinia viruses were recovered, the IBV cDNA within recovered vaccinia viruses would comprise a mixture: some would have chimaeric S genes, others would have intact Beaudette S genes. Sequencing from the end of gene 1 through the S gene (gene 2) and into gene 3 was done to identify which vaccinia viruses contained IBV sequences with chimaeric S genes. Rescue of rIBVs was attempted from seven of the latter. Recombinant IBVs were rescued by passage in 10-day-old embryonated chicken eggs, rather than in chick kidney (CK) cells as used previously. Otherwise the recovery procedure was as previously described.

Characterization of recombinant IBVs

Recombinant IBVs and the Beaudette Beau-R strain (recovered from our cloned Beaudette strain; Casais et al., 2001) were titrated in tracheal organ cultures (TOCs). The growth characteristics of the viruses in embryonated eggs, and in cultures of CK cells and Vero cells were assessed by inoculation with 2.4 log10 ciliostatic dose 50 (CD50) of each virus, followed by harvesting at 24-hour intervals, and titration of the progeny in TOCs. In one experiment the progeny in the 24-hour samples were also titrated in embryonated eggs, to give an egg infectious dose 50 titre (EID50).

RESULTS AND DISCUSSION

The results of our experiments will be described in full elsewhere.

Vaccinia viruses containing chimaeric S protein genes

Thirteen recombinant vaccinia viruses containing the Beaudette cDNA with chimaeric S genes were obtained. Seven of these were selected for characterization (Figure 1). We also included a rIBV (#7), recovered during the same recombination process, which had an unchanged Beaudette S protein gene.

Characterization of the rIBVs

We first assessed the replication of the rIBVs in embryonated eggs, the progeny being titrated in TOCs. rIBV #7 replicated in parallel with Beau-R, as expected. The B1648 strain replicated to titres ~2 log10 lower than #7 and Beau-R, when titrated in TOCs. rIBVs #9, 11 and 12 replicated to titres about 1 log10 lower that of B1648, and 3 log10 lower than that of Beaudette.

When the 24 hour samples from the embryo growth curves were titrated in embryos, the EID50 titres of B1648 and rIBVs 9, 11 and 12 were almost the same as that of Beau-R. This showed that these recombinants, and the parental B1648, had essentially the same replicative ability in embryos as the other parental strain, Beau-R. The data also shows that B1648 is intrinsically less ciliostatic than Beau-R, and that rIBVs which had S genes with most of the sequence derived from the B1648 parent shared this property with B1648. In other words, the phenotype of the rIBVs was determined by the S protein.

rIBV #23 replicated to titres ~2 log10 less than that of the other rIBVs in embryonated eggs, whether titrated in TOCs or embryos. Given that both parental viruses replicated equally well in embryonated eggs i.e. both had receptor binding domains able to recognise receptors in embryonated eggs, this suggests that the tertiary and/or quaternary structure of the S protein of #23 was suboptimal for replication.

In CK cells rIBVs #9, 11 and 12 replicated very poorly (~5 log10 less than Beau-R and rIBV #7), as predicted, given that the B1648 strain had not been adapted to growth in CK cells. We presume that the rIBVs replicated very poorly because they could not use CK cell surface proteins as receptors.

A similar result was obtained with Vero cells, as expected. Thus the phenotype of the rIBVs was again determined by the S protein. Interestingly rIBVs #9, 11 and 12 did replicate slightly, whereas B1648 did not. These rIBVs had carboxy-terminal S sequences from Beaudette, sequences which may be involved in the virus-cell fusion process. We hypothesize that whilst the rIBVs probably could not bind efficiently to Vero cells, some virus might have entered the cells. Possession of Beaudette terminal sequences might have been advantageous e.g. in the fusion process.

rIBV #23 replicated to titres even lower than those of rIBVs #9, 11 and 12, again indicative that the structure of the S protein of #23 was sub-optimal.

In conclusion, random recombination between the S protein genes of two strains of IBV resulted in viable virus in ~50% of cases, though in some cases the resultant virus replicated poorer than both parents. Our results strongly support the view that recombination in the field, involving S protein genes, is a highly likely occurrence, some of the progeny being able to replicate well.

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REFERENCES

Britton, P. Evans, S., Dove, B., Davies, M., Casais, R. & Cavanagh, D. (2005). Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. *Journal of Virological Methods, 123*, 203-211.

Casais, R., Thiel, V., Siddell, S., Cavanagh, D. & Britton, P. (2001). A reverse genetics system for the avian coronavirus infectious bronchitis virus. *Journal of Virology*, 75, 12359-12369.

Casais, R., Dove, B., Cavanagh, D. & Britton, P. (2003). A recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. *Journal of Virology*, *77,* 9084-9089.

Casais, R., Davies, M., Cavanagh, D. & Britton, P. (2005). Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. *Journal of Virology*, 79, 8065-8078.

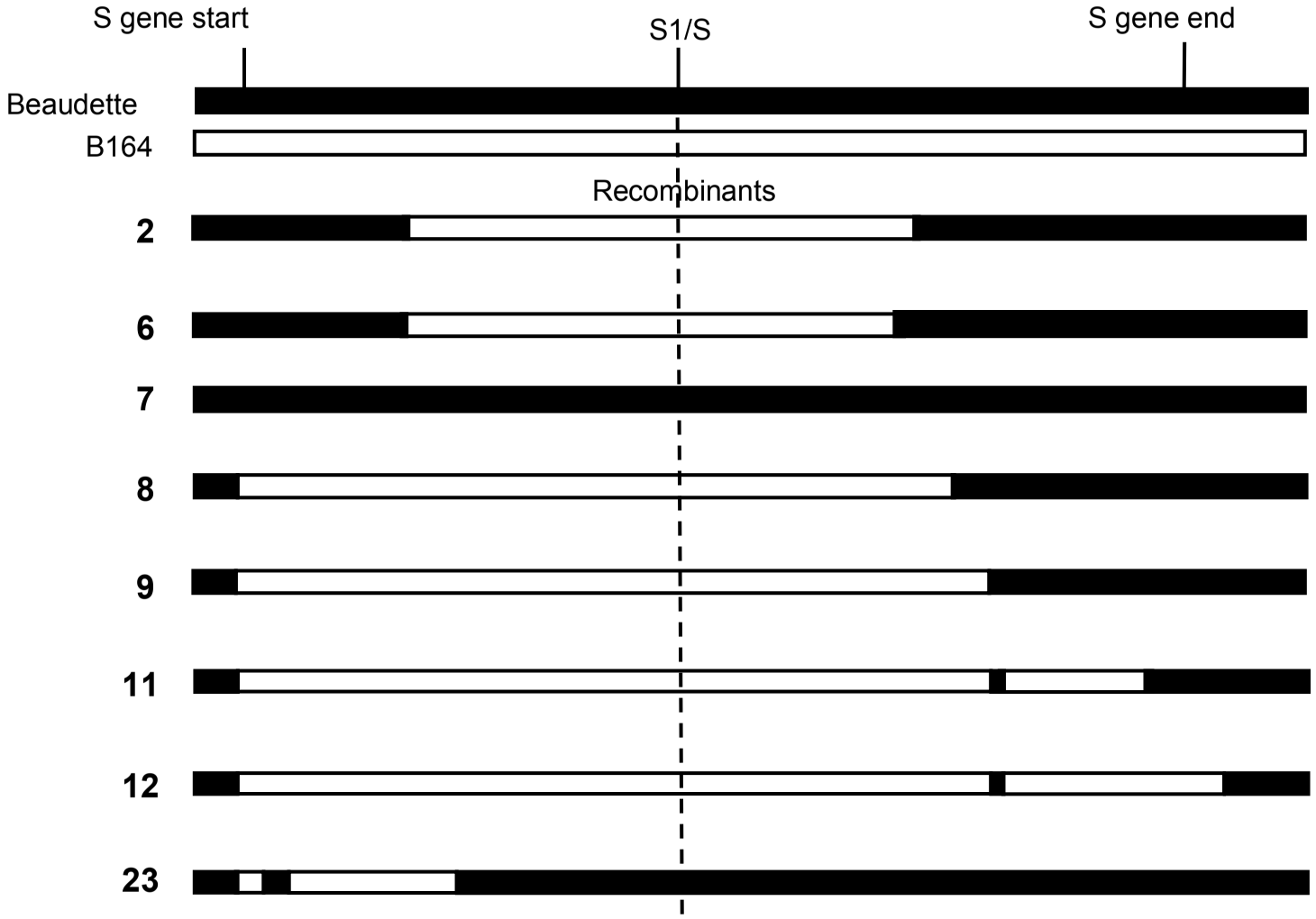
Hodgson, T., Casais, R, Britton, P. & Cavanagh, D. (2004). Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces good protective immunity. *Journal of Virology*, *78*, 13804-13811.

Hodgson, T., Britton, P. & Cavanagh, D. (2006). Neither the RNA nor the proteins of open reading frames 3a and 3b of Infectious bronchitis virus are essential for replication. *Journal of Virology*, *80*, 296-305.

Kottier, S.A., Cavanagh, D. and Britton, P. (1995). Experimental evidence of recombination in coronavirus infectious bronchitis virus. *Virology*, 213, 569-580

Shaw, K., Britton, P. and Cavanagh, D. (1996). Sequence of the spike protein of the Belgian B1648 isolate of nephropathogenic infectious bronchitis virus. *Avian Pathology*, 25, 607-611.

Figure 1. Recombinant vaccinia viruses used to attempt rescue of rIBVs. The horizontal bars represent the S protein gene within the full-length cDNA of the IBV Beau-R genome within the genome of vaccinia virus. Beau-R and B1648 sequences are indicated by black and open rectangles, respectively. The end of the S1 sequences and beginning of the S2 sequences are indicated by 'S1/S2'.



MOLECULAR AND BIOLOGICAL PROPERTIES OF AN AVIAN CORONAVIRUS CLOSELY RELATED TO GROUP II CORONAVIRUSES

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SUMMARY

Avian coronaviruses are classified in group III of the *Coronavirus* genus and cause several host specific diseases of economic importance. This paper describes the identification of a group II coronavirus recovered from intestinal contents and stools samples of broilers, laying hens and breeders and its relationship with other known coronaviruses by phylogeny based on the RNA-polymerase RNA-dependent gene and the 3'UTR and the gross lesions, tissue alterations and clinical features after the experimental inoculation in SPF chickens.

INTRODUCTION

The *Coronaviridae* is a large family of RNA viruses that infect a variety of mammalian and avian species (Saif, 1990). Coronavirus is an enveloped round-shaped virus, with 100-150 nm in diameter, composed by six or five structural proteins (N,M, sM, HE, S, and I), depending on the viral species. (Risco et al., 1996), divided into three different antigenic groups (I, II and III).

Avian coronaviruses, IBV and TCoV, classified in group III, cause several host specific diseases of economic importance. Infectious Bronchitis virus is the cause of an acute, highly contagious respiratory disease in chickens with potential involvement in kidney and reproductive tract (Cavanagh & Naqi, 1997).

Turkey Coronavirus (TCoV) was characterized as an emerging disease of Minnesota turkeys in 1951 (Pomeroy & Nagaraja, 1991), this virus causes a watery diarrhea in turkeys between 1 a 6 weeks old and circulating disorders that mediate the so-called bluecomb disease (Möstl, 1990). Also, a coronavirus was isolated from pheasants with serological identity with infectious bronchitis virus (Spackman & Cameron, 1983; Gough et al., 1996; Pennycott, 2000).

Interestlingly, a coronavirus isolated during an investigation on a disease affecting the seabird *Puffinus puffinus*, named puffinosis, on islands off the southwest coast of

Wales was characterized as a member of Group II (Nuttal & Harrap, 1982; Klausegger et al., 1999)
This paper describes the identification of a group II coronavirus recovered from intestinal contents and stools samples of broilers, laying hens and breeders and its relationship with other known coronaviruses by phylogeny based on the RNA-polymerase RNA-dependent gene and the 3'UTR and to evaluate the gross lesions, tissue alterations and clinical features after the experimental inoculation in SPF chickens.

MATERIALS and METHODS

1. Case history and sampling

A total of 119 samples of intestinal contents was collected, being 94 from broilers, 5 from laying hens and 20 from female breeders from 9 brazilian States/locations (Estados de São Paulo, Rio Grande do Sul, Minas Gerais, Paraná, Rio de Janeiro, Pará, Ceará, Distrito Federal e Santa Catarina). Each sample was made by a pool of the enteric contents of 5 birds collected under necropsy and stored at -20ºC.
These samples were prepared was 20% (w/v) in PBS 0,01 M pH 7,2 and clarified by centrifugation at 12,000 x g/ 30 minutes at 4 ºC, the supernatant stored at -80 ºC for analysis.

2. Detection of group III coronaviruses

Group III avian coronaviruses were screened with an RT-PCR targeted to the 3'UTR with primers and reaction conditions as described by Cavanagh et al. (2002).

3. Group II coronaviruses detection

A group-II-specific RT-PCR assay targeted to the ORF1b/RdRp gene was used for the screening of group II coronaviruses, with a predicted product of 136bp (Brandão et al., 2005).

4. Embryonated eggs and cell culture isolation

One sample positive for group II coronaviruses, as determined by the RT-PCR described above, was prepared as a 20% (w/v) suspension in PBS, clarified by centrifugation at 12,000 x g/ 30 minutes at 4ºC and the supernatant added 0.1mg/mL gentamicin and filtered through 0.22 membranes.
One mL of the flowthrough was inoculated in HRT-18 cells (25cm^2 flasks) and allowed to adsorb for 1 hour at 37ºC, then 6 mL of MEM plus 5% bovine fetal sera were added and the flasks were incubated form up to 15 days and up to 6 serial passages.
Besides, 8-day-old chicken embryonated eggs were innoculatd with 0.2mL of the same samples prepared as described above via the allantoic route and incubated for 7 days and up to 4 passages.
Each cell culture and egg passage was monitored for groups II and III coronaviruses by the respective PCR assays described above.

5. Characterization of group II coronaviruses

5.1 RT- PCR for the detection of the gene of the Hemagglutinin-esterase gene (HE) of group II coronaviruses

A universal RT-PCR assay was developed to detect the HE gene, exclusive of group II coronaviruses, with a predicted amplicon size ranging of 796 bp for HCoV-OC43, BCoV, HEV and HCoV-4408 and 826 bp for SDAV, MHV and puffinosis virus.

This RT-PCR was applied to the group II strain isolated in HRT-18 cells accoerding to the following protocol:

Seven microliters of total RNA extracted with TRIzol (Invitrogem™) were added to cDNA-synthesis mix with 1 x First Strand Buffer (Invitrogen™), 1mM dNTPs, 10mM DTT, 1µM of each primers (sense primer CHES 5'TMTTTGGYGACAGTCGTTC3' and antisense primer CHEA5'TTATCMGAMTGCYTRGCATT3') and 200U M-MLV Reverse Transcriptase (Invitrogen™) in a 20µL final reaction and incubated at 42ºC/60 minutes.

Next, 5µL of RT product were added to a PCR mix 1 x *PCR Buffer* (Invitrogen™), 0,2mM dNTPs, 0,5 µM of each primer (CHES and CHEA), 1.5mM MgCl2, 25.25 µL sterile ultra-pure water and e 1.25U *Taq DNA* polymerase (Invitrogen™) for a 50µL reaction. The reaction was submitted to 35 cyclos of 94ºC/1 min, 58,5 ºC/1,5 min. and 72ºC/1 min., followed by 72ºC/10 min. for final extension.

5.2 RT-PCRs for the spike (S) protein gene

The group II coronavirus isolated in HRT-18 cells was submitted to RT-PCRs assays aimed to detect IBV *S* gene according to Keeler et al. (1998) and Ziegler et al. (2002) with expected products of 573-601bp and 706bp, respectively.

5.3 RT-PCRs for genes 5 and 3 of avian coronaviruses

The existence of genes 3 and 5 of avian coronaviruses was searched in the group II coronavirus isolated in cell culture following the protocols described by Cavanagh et al. (2001).

5.4 Phylogenetic studies

All coronavirus-related RT-PCR products were submitted to bi-direcional DNA sequencing with DYEnamic ET Dye Terminator Kit (GE Healthcare™) in a MegaBACE™ 1000 automatic DNA sequencer (GE Healthcare™).

The sequences obtained were aligned with homologous sequences from GenBank with the CLUSTAL/W method with Bioedit v. 5.0.9 (Hall, 1999) and distance Neighbor - Joining trees were generated with Mega 3.1 (Kumar et al., 2004) with the K-2-P model and 1000 bootstrap replicates.

6. Immunoelectronmicroscopy

Strain CECoV/BR-03/USP-01 was tested by immunoelctronmicroscopy with anti-CECoV hiperimmune serum produced by intramuscular inoculation of this strain in SPF hens.

7. Experimental inoculation

Fifty-nine specific-pathogen free (SPF) white leghorn chickens aged 5 days (42 birds) and 21 days (17 birds) were obtained from Biovet Laboratories. The source of the

chickens was certified as negative to coronaviruses by testing embryos following the PCR protocol described above.
Prior to the inoculation, stool samples were collected from the birds and tested by PCR.
Birds were divided in three experimental groups: group 1 included 17 5-day old birds given 0.5 mL of the inoculum orally, group 2 included 17 21-day old birds given 200 µL of the inoculum by eye drop and group 3 with non-inoculated 21 birds kept as control group. Birds were housed in separated 1.70 m^2 boxes and received a commercial broiler diet and water *ad libitum*.
The groups were examined for nine weeks for diarrhea, depigmentation, delayed growth and respiratory signs. At days 1 and 3 post-inoculation, stool samples were taken from groups 1 and 2.
Birds from groups 1 and 2 were slaughtered and autopsied at days 7 (3 birds/group) and days 21, 34 and 60 (6 birds/group). Birds from group 3 (control) were not slaughtered until day 60, when 5 sample birds were autopsied .
At the necropsy, birds were searched for gross lesions and tissue samples for both PCR and histopathological examination were taken from rectum, small intestine, pancreas and lungs. Furthermore, the intestinal content of each bird was collected to PCR examination.
Group II coronavirus in the collected tissues and stool and intestinal contents samples was searched according to the PCR procedure described in item 3.

RESULTS

1. Groups III and II coronaviruses detection

The results of the virological survey are shown in Table 1. The group II coronavirus detected was named Chicken enteric coronavirus (CECoV).

2. Embryonated eggs and cell culture isolation

By the second passage in HRT-18 cells, a syncytial effect was noticed and, in the fifth passage, syncytia appeared at 3 days post-inoculation. The supernatant of passages 2 to 7 were all positives to group II coronaviruses (RdRp gene) and group III coronaviruses (3'UTR) after tested by the respective RT-PCRs described above. This CECoV isolate was named CECoV/BR-03/USP-01.
After 4 serial passages in chicken embryonated eggs, no alterations in the embryos were found. The alantoic liquid collected of each passage resulted negative to groups II and III coronaviruses as tested by RT-PCRs.
Strain CECoVBR03USP-01 isolated in HRT-18 cells resulted negative to the RT-PCRs targeted to genes S, 3, 5 and HE.

3. Characterization of group II coronaviruses

Besides strain CECoV/BR-05/USP-01, for which RdRp sequences were obtained both from directly from the fecal speciemns and isolated vírus, five other samples resulted in RdRp sequenes, the strains named CECoVBR05USP-02 to 06 (GenBank accession numbers DQ401279 a DQ401285). The sequence of the 3'UTR from strain CECoV/BR-05/USP-01 isolated in HRT-18 cells was assigned Genbank accesion number DQ401286

The phylogenetic tree for the RdRp sequences reveald that these segregated inside the Group II of coronaviruses, close to bovine coronavirus (Figure 1), in an unique cluster, while the tree for the 3'UTR from strain CECoV/BR-05/USP-01 isolated in HRT-18 showed that this strain grouped with group III avian coronaviruses (Figure 1)
.

4 Immunoelectronmicroscopy

Highly pleomorphic and spiked particles were seen after immunoelectronmicroscopy with anti-CECoV serum (Figure 2), compatible with coronavirus.

5. Experimental inoculation

Diarrhea was identified from day 3 throughout the experiment, in groups 1 and 2. Depression, depigmentation of beaks, combs and legs was also observed in experimental groups. Growth delay was inferred from the different sizes of birds in a same group both in groups 1 and 2.

At postmortem examination, experimental groups showed dilation and hyperemia of the rectum, hyperemia of the small intestine, hypertrophy and hyperemia of the pancreas, bursa and spleen with hypertrophy and, some cases, atrophy. Lungs, trachea, liver, cecum had no gross lesions. Birds from control group showed no alterations at the necropsy. These findings were constant throughout the experiment.

Polymerase chain reaction revealed that the inoculated group II coronavirus had spreaded to rectum, pancreas, small intestine and, some cases, lungs, as well as intestinal contents for days 7, 21, 34 and 60 PI (table 2). Stool samples collected from day 1 PI were negative, while those from day 3 were positive to group II. The same organs from group 3 birds were found negative at the end of the experiment (day 60 PI). One positive sample per grou-p was submitted to DNA sequence and the identity with the inoculated virus was confirmed (data not shown).

Lesions were most consistent at days 21 and 34 post inoculation. Histologically, there was evidence of enteric damage, characterized by epithelial necrosis, *lamina propria* infiltrates. Small gut had exposition of the *lamina própria* due to necrosis and cell desquamation

DISCUSSION

A coronavirus was found in the intestinal; contents of birds which simultaneously harbours homology of group II coronaviruses, according to the *RdRp* gene, and group III coronaviruses, according to the 3'UTR. The virus was named Chicken enteric coronavirus (CECoV).

Although coronaviruses have already been found in intestinal contents of domestic birds, all of them belong to group III, as infectious bronchitis coronavirus, pheasant coronavirus and turkey coronavirus (Dhinakar & Jones, 1997; Cavanagh et al., 2001; Cavanagh et al., 2002). Besides, the only known report of a group II coronavirus in birds relates to puffinosis virus, a coronavirus found in seabirds (*Puffinus puffinus*) in the UK (Nuttal & Harrap, 1982; Klausegger et al., 1999).

The virus was shown to have a broad tissue tropism, once it was found in enteric tissues as rectum and sections of the small intestine, pancreas and, in some cases, lungs, tissues in which the virus persisted for at least 60 days. The virological examination is in agreement with the sites where gross and histopathological lesions were found, i. e., enteritis in the rectum and small intestine and pancreatitis.

The most probable hyphothesis for the emergence of CECoV is a recombination evento between groups II and III coronaviruses, as RT-PCRs directed to genes HE, S, 3 and 5 could not be identified, what rules out the possibility that a co-infection rather than a recombinant virus was detected.
Furthermore, as CECoV was found spread although the Brazilian territory, the virus here described is not an odd event, but rather a consistent finding.

REFERENCES

Brandão, P. E.; Gregorl, F.; Villarreal, L. Y. B.; Rodriguez, C. A. R; Soares, R M; Jerez, J A. A nested polymerase chain reaction to bovine coronavirus diagnosis. Virus Rev. Res., v. 10, n.1, p. 45-49, 2005

Cavanagh, D., Naqi, S., 1997. Infectious bronchitis. in: Calnek, B. W. ; Barnes, H. J.; Beard, C. W.; Reid, W. M.; Yoder, H. W. (Eds), Diseases of Poultry 10th ed. Iowa State University Press, Ames, pp. 511-526

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S.E., Ainsworth, H.L., Britton, P., Gough, R.E., 2001. Detection of a Coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. Avian Pathol. 30, 355-368.

Cavanagh, D., Mawditt, K., Welchman, D.de B., Britton, P., Gough, R.E., 2002. Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian Pathol. 31, 81-93.

Dhinakar, G.; Jones, R. C. Infectious Bronchitis virus: immunopathogenesis of infection in the chicken. Avian Pathol., v. 26, p. 677-706. 1997.

Gough, R.E., Cox, W.J., Winkler, C.E., Sharp, M.W. and Spackman, D., 1996. Isolation and identification of infectious bronchitis virus from pheasants. Vet. Rec. 138, 208-209.

Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser., v. 41, p. 95-98. 1999

Keeler, C. L.; Reed, K. L.; Nix, W. A.; Gelb, J. Jr. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Dis., v. 42, p. 275-284. 1998.

Klausegger, A., Strobl, B., Regl, G., Kaser, A., Luytjes, W., Vlasak, R., 1999. Identification of a coronavirus hemagglutinin – esterase with a substrate specificity different from those of influenza c virus and bovine coronavirus. J. Virol. 73, 3737–3743.

Kumar, S.; Tamura, K; Nei, M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Brief Bioinform, v. 5, p.150-163. 2004

Möstl, K., 1990. Coronaviridae, pathogenic and clinical aspects: an update. Comp. Immunol. Microbiol. Infect. Dis. 13, 169-180.

Nuttal, P. A., Harrap, K. A., 1982. Isolation of a coronavirus during studies on puffinosis, a disease of the Manx shearwater *(Puffinus puffinus*). Arch. Virol. 73, 1-13.

Pennycott, T.W., 2000. Causes of mortality and culling in adult pheasants. Vet. Rec. 146, 273 – 278.

Pomeroy, B. S., Nagaraja, K. V., 1991. Coronaviral enteritis of turkeys (bluecomb disease). in: Calnek, B. W., Barnes, J., Beard, C. W., Reid, W. M., Yoder, H. W. Diseases of Poultry 9th ed. Iowa State University Press, Ames.

Risco, C., Anton, I. M., Enjuanes, L., Carrascosa, J. L., 1996. The transmissible gastroenteritis coronavirus contains a spherical core shell consisting of M and N proteins. J. Virol. 70, 4773-4777.

Saif, Y. M., Saif, L. J., Hofacre, C. L., Hayhow, C., Swayne, D. E., Dearth, R. N., 1990. A small round virus associated with enteritis in turkey poults. Avian Dis. 34, 762-764.

Spackman, D. and Cameron, I.R.D., 1983. Isolation of infectious bronchitis virus from pheasants. Vet. Rec. 113, 354–355.

Ziegler, A.F.; Ladman, B.S.; Dunn, P.A.; Schneider, A.; Davison, S.; Miller, P.G.; Lu, H.; Weinstock, D.; Salem, M.; Eckroade, R.J.; Gelb, J.Jr. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Diseases, 46: 847-858. 2002.

Table 1 - Occurrence of Group III coronaviruses and CECoV (chicken enteric coronavirus) in the enteric contents of the three different types of birds surveyed in the present study.

Type of bird	Total	Group III coronaviruses	CECoV
Broiler	94	64 (68%)	19 (20,21%)
Laying hens	5	3 (60%)	1 (20%)
Breeders	20	9 (45%)	5 (25%)
TOTAL	119	78	25

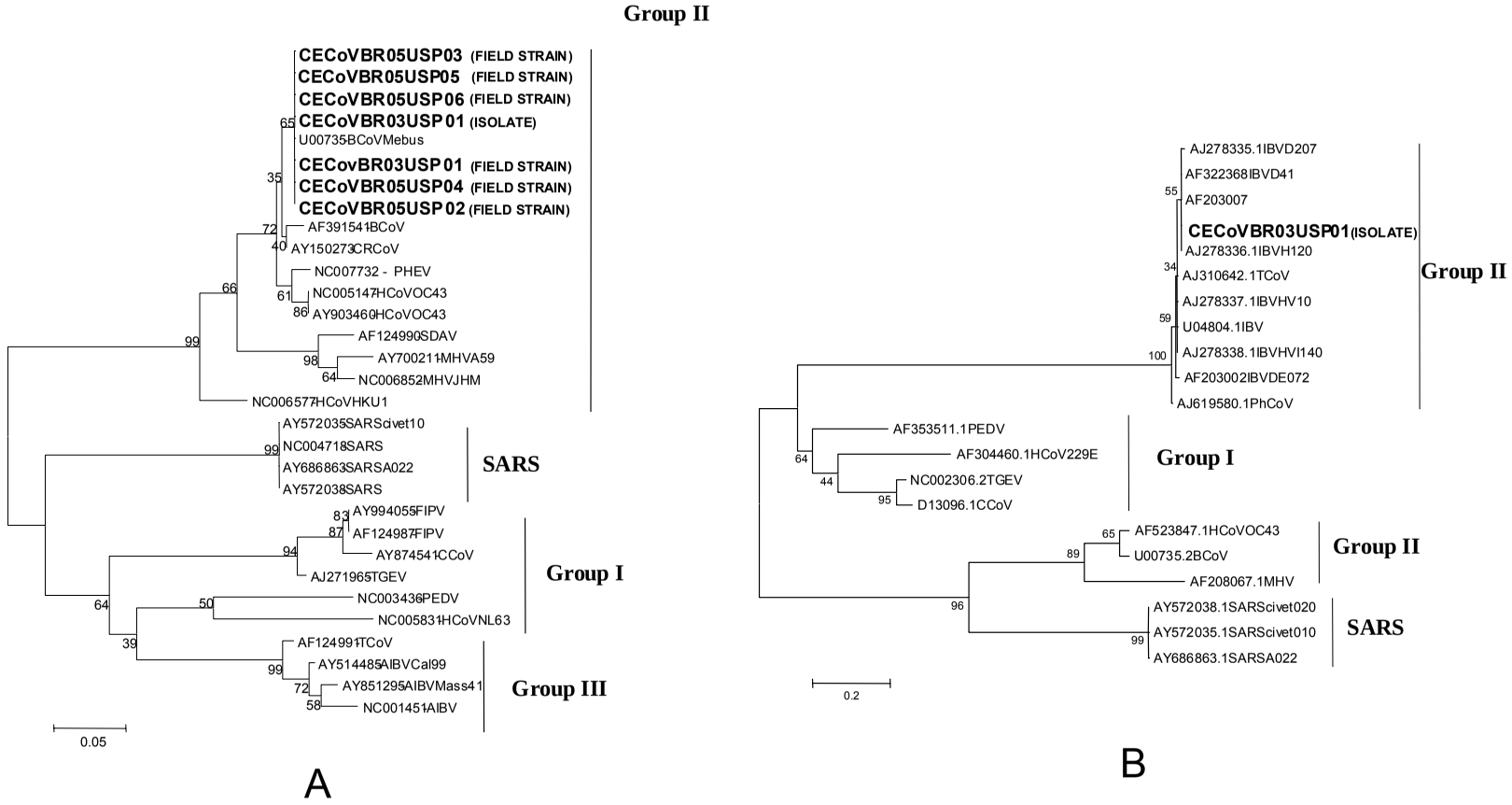

Figure 1 – Neighbor-joining phylogenetic trees for the *Coronavirus* and CECoV (Chicken enteric coronavirus) to A: RdRp gene and B: 3'UTR. Numbers at each node are 1000 replicates bootstrap values; the bar indicates the number of nucleotide substitution per site.

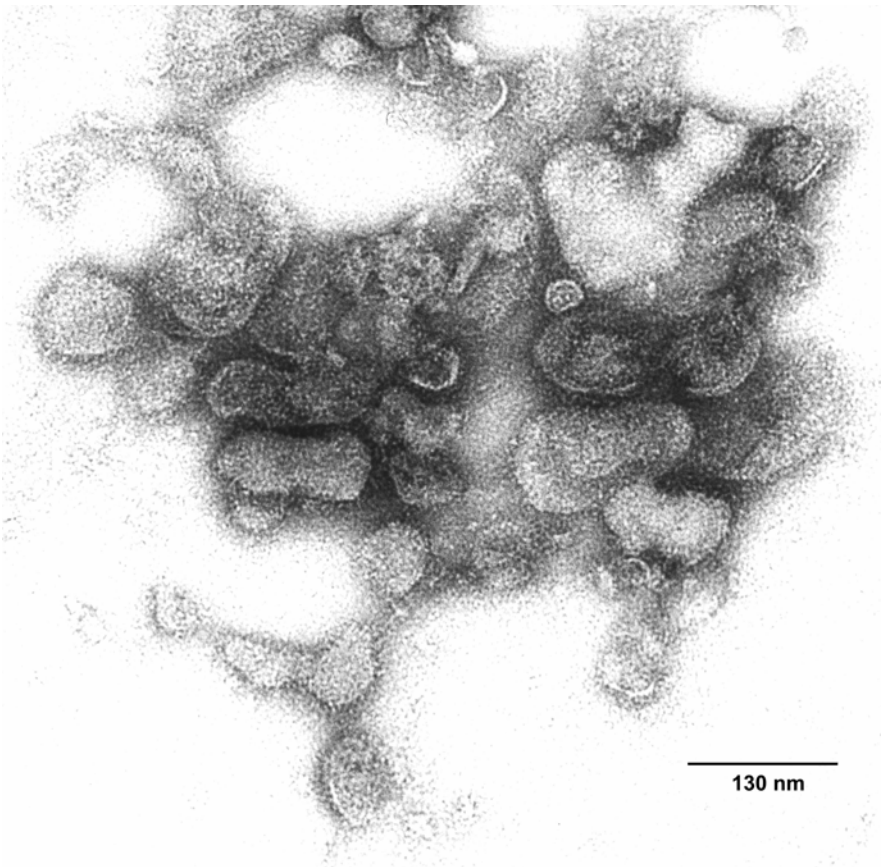

Figure 2 – Immunoelectronmicroscopy of CECoV (Chicken enteric coronavirus) strain CECoV/BR-05/USP-01 isolated in HRT-18 cells.

COMPARISON OF GENE SEQUENCES OF PHEASANT CORONAVIRUS WITH THOSE OF INFECTIOUS BRONCHITIS VIRUS

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SUMMARY

We have previously shown that pheasants (*Phasianus colchicus*) are commonly infected with a coronavirus, named pheasant coronavirus (PhCoV), that is genetically very similar to IBV, although we had only sequenced small parts of the genome. We have now sequenced the ~8 kb of the genome that encodes all genes downstream of gene 1, and compared this with the corresponding sequences of infectious bronchitis virus of the domestic fowl (chicken), to better understand the relationship between these viruses. The percentage amino acid identities of the four structural proteins were: spike (S) glycoprotein (87), small envelope (E) protein (80), integral membrane (M) glycoprotein (92) and nucleocapsid (N) protein (92). The four non-structural proteins encoded by this part of the genome had identities of 84% (3a protein), 80% (3b), 85% (5a) and 79% (5b). The degree of relatedness between PhCoV and IBV is very similar to that exhibited amongst serotypes of IBV, and between IBV and turkey coronavirus, except that the S proteins of IBV and TCoV have only ~34% identity. The results are discussed in the light of the host range of these viruses, and the presence of genetically IBV-like viruses in other galliform and non-galliform birds.

INTRODUCTION

Electron microscopy and some antigenic analysis had indicated that pheasants were infected by a coronavirus (PhCoV), sometimes associated with respiratory disease, and sometimes associated with kidney disease (Spackman & Cameron, 1983; Lister *et al*., 1985; Gough *et al.*, 1996; Pennycott, 2000). Sequencing of genes 3 and 5, and the 3' untranslated region (UTR), subsequently confirmed that it was coronavirus (Cavanagh *et al.*, 2002). In the field mortalities, associated with the presence of PhCoV, have been high. For example, 15% of breeding pheasants died rapidly, the only clinical sign being sneezing. In another group of pheasants there were no respiratory signs but 45% had died by 10 weeks of age. Specific lesions that have been described are visceral gout

and urolithiasis, with gross swelling and pallor of the kidneys. Moderately severe interstitial nephrosis was revealed by histological analysis.
PhCoV has since been detected in many pheasants, collected from many pheasant ranges that were experiencing respiratory disease (Welchman *et al.*, 2002). The degree of genetic relatedness to infectious bronchitis virus (IBV) in genes 3 and 5, and the 3' UTR, was very similar to that between serotypes of IBV, and when IBV was compared with turkey coronavirus (TCoV). There were differences in gene sequences amongst the pheasant coronaviruses, similar to those that exist amongst serotypes of IBV. To clarify further the relationship between PhCoV and IBV, we have sequenced the remaining part (~ 8 kb) of the genome downstream from gene 1, i.e. to include genes 2 (encoding the large spike glycoprotein), 3, 4 (integral membrane protein, M), 5 and 6 (nucleocapsid protein, N).

MATERIALS and METHODS

Procedures will be described more fully elsewhere.

Virus
PhCoV strain ph/UK/602/95 was propagated in 10-day-old embryonated SPF eggs (Gough *et al.*, 1996; Cavanagh et al., 2002).

RNA extraction, RT-PCR and sequencing
RNA was extracted from allantoic fluid using an RNeasy mini kit (Qiagen). Amplification of the RNA was done by RT-PCR using Ready-to-go one-step RT-PCR beads (Amersham Biosciences) according to manufacturer's instructions. Primer, and sequencing, oligonucleotides were selected from amongst those that we had previously developed for use with IBV. RT–PCR conditions and sequencing were as previously described (Cavanagh et al., 2001, 2002).

RESULTS

The results of our study will be described in full elsewhere.

Comparison of the amino acid sequences of PhCoV with those of IBV
These are shown in Table 1.
The degree of relatedness between PhCoV and IBV is very similar to that exhibited amongst serotypes of IBV, and between IBV and turkey coronavirus, except that the S proteins of IBV and TCoV have only ~34% identity (Lin et al., 2004).

DISCUSSION

Although the protein sequences of PhCoV are very similar to those of IBV, and both replicate readily in domestic fowl eggs, it is reasonable to conclude that they are separate virus species. Athough PhCoV does replicate in chickens, as evidenced by antibody production, it does so asymptomatically, (Gough *et al*., 1996). Also, we have observed that PhCoV is poorly ciliostatic in chicken tracheal organ cultures. Similarly, TCoV replicates asymptomatically in chickens (Ismail *et al*., 2003). Recent research in China suggests IBV can replicate in domestic peafowl (*Pavo cristatus*; (Liu *et al*.,

2005)), partridge (*Alectoris* sp.), guinea fowl (*Numida meleagris*) and teal (*Anas* sp. f duck; Liu *et al.,* 2005) (reviewed by Cavanagh (2005)). These, like the domestic fowl, turkey and pheasant, are gallinaceous birds ('fowl-like', order Galliformes).

Sequencing of the entire genome of the viruses isolated from these species revealed that they were IBV-like with respect to their genome organisation and with the sequences of the genes encoded by their genes.

Details of the events which led to these sequences have only been documented in respect of the peafowl and teal reported by (Liu *et al*., 2005). In both cases these two species were being maintained as domestic animals in the vicinity of chickens. The proteins of the peafowl virus (strain pf/China/LKQ3/03) had >99% identity with the widely used IBV H120 live vaccine, making it almost certain that it was the H120 vaccine strain that had been recovered from the peafowl. The complete genome sequence of another coronavirus isolated from peafowl (pe/China/GD-KQ6/03) has been submitted to the databanks (J.D. Fu, M. Liao, T. Ren, G.H. Zhang, C.A. Xin and J.D. Chen, 2004: accession number AY641576). This had genes with ~ 95% identity with those of IBV strains.

The isolate from a teal (te/China/LDT3/03) had a spike protein that had ~90% identity with some known IBV strains, including a nephropathogenic one ((Liu *et al*., 2005)). When this isolate was inoculated into chickens, it caused disease, including kidney involvement. This suggests that the teal isolate was actually an IBV strain that had spread to the teal from nearby chickens. Assuming that these findings did not result from cross-contamination in the laboratory, it would appear that IBV can replicate not only in the chicken and other gallinaceous birds, but also in teal, a non-gallincaceous bird. It is possible that the virus was a natural pathogen of the teal that was able to infect and cause disease in chickens.

Infection of guinea fowl with virus antigenically related to IBV had been reported earlier, in Brazil (Ito *et al*., 1991). The guinea fowl had been suffering high mortality and low feed consumption, and enteritis was reported. When the isolate was inoculated intranasally into chickens and into guinea fowl, both species exhibited respiratory distress and aqueous faeces. Whether the guinea fowl virus was a 'genuine' guinea fowl coronavirus i.e. a separate species, or an IBV that had spread from nearby chickens to the guinea fowl, is not known. Nevertheless, the field observations and the laboratory infections indicate that the virus from the guinea fowl had a host range of more than one bird species.

The S protein of TCoV is more distantly related to that of IBV than is that of IBV with PhCoV or the other coronaviruses described above. This makes it easier to consider TCoV as a distinct species of coronavirus. This concept is more difficult in respect of IBV, PhCoV and the virus from guinea fowl, where the viruses from the different species of bird are no more different from each other than are one serotype of IBV from another. Although they can, and probably will, continue to be regarded as distinct species, it is also possible, and instructive, to consider them as host range variants of a common coronavirus (Cavanagh, 2001).

The situation is similar to that of SARS-coronavirus, where strains with very similar genome sequence have been isolated from mammals such as humans, the palm civet cat (*Paguma larvata)* and racoon dog (*Nyctereutes procyonoides*) and, most recently observed, in bats (Poon et al., 2005). SARS-coronavirus has also been shown to replicate in cynomolgus macaques (*Macaca fascicularis*), ferrets (*Mustela furo*),

domestic cats (*Felis domesticus*) and mice (*Mus musculus*) (references in Cavanagh, 2005)

An older example of host range overlaps amongst coronaviruses is that of some of the Group 1 coronaviruses. Porcine transmissible gastroenteritis virus (TGEV) is a Group 1 coronavirus that causes severe enteritis in young pigs. Over 20 years ago Woods and colleagues had demonstrated that the Group 1 canine coronavirus (CCoV) and feline coronavirus (FCoV) can replicate in pigs (Woods *et al.*, 1981; 1992). Moreover, inoculation of pigs with a virulent FCoV resulted in clinical signs typical of a virulent TGEV infection (Woods *et al.*, 1981). Tissue-culture adapted FCoV and CCoV did not give clinical signs. Thus, FCoV and CCoV are able to replicate in pigs, and some, but not all, strains are able to cause disease. The type II strains of these two viruses and TGEV have integral membrane (M) and nucleocapsid (N) proteins with very similar (>90%) protein sequences (Gonzalez *et al.*, 2003). Their surface spike (S) proteins, that are responsible for the attachment of the virus to cells, have >80% identity overall, including >94% identity from amino acid residues 275–1447. We know now that these three viruses can use the same cell surface molecule for attaching to cells *in vitro*. For example, feline amino peptidase N, which is a receptor for FCoV, also acts as a receptor for TGEV and for human coronavirus 229E, another group 1 coronavirus (Kolb *et al.*, 1997).

An isolate of a coronavirus apparently from a child experiencing diarrhoea had proteins with 99% identity with those of bovine enteric coronavirus (BCoV; Zhang *et al.*, 1994). Assuming that there had not been cross-contamination in the laboratory then this indicates that bovines and humans can be infected with the same coronavirus.

The recently discovered canine respiratory coronavirus has 95% identity with the proteins of bovine coronavirus (Erles *et al.*, 2003). Might each of these two virus species infect both dogs and cattle?

These observations make it clear that we should not assume that a given coronavirus species is limited to replication in – or to causing disease in – a single host. With regard to the genetically-IBV like Group 3 coronaviruses, there is mounting evidence that IBV can replicate in other species. The experimental infection of chickens with coronaviruses from guinea fowl and teal, resulting in clinical signs, can be interpreted either as IBV having spread to these species in the field, or that coronaviruses of these two very different avian species can replicate and cause disease in chickens. One can consider the genetically IBV-like viruses as a genetic cloud comprising viruses with generally very similar gene sequences, able to infect a number of avian species but with unpredictable outcome with respect to causing disease.

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REFERENCES

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. Avian Pathol. *34*, 439-448.

Cavanagh, D., Mawditt, K., Sharma, M., Drury, S.E., Ainsworth, H.L., Britton, P. & Gough, R.E. (2001). Detection of a coronavirus from turkey poults in Europe genetically related to infectious bronchitis virus of chickens. *Avian Pathology, 30*, 365-378.

Cavanagh, D., Mawditt, K., Welchman, D. De. B., Britton, P. & Gough, R.E. (2002) Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. *Avian Pathology*, *31*, 81-93.

Erles, K., Toomey, C., Brooks, H.W. & Brownlie, J. (2003). Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. *Virology, 310*, 216-223

Gonzalez, J. M., P. Gomez-Puertas, D. Cavanagh, A. E. Gorbalenya, and L. Enjuanes. 2003. A comparative sequence analysis to revise the current taxonomy of the family Coronaviridae. *Archives of Virology*, 148, 2207-35.

Gough, R.E., Cox, W.J., Winkler, C.E., Sharp, M.W. & Spackman, D. (1996). Isolation and identification of infectious bronchitis virus from pheasants. *Veterinary Record, 138*, 208-209.

Ismail, M. M., Tang, A.Y. and Y. M. Saif. (2003). Pathogenicity of turkey coronavirus in turkeys and chickens. *Avian Diseases, 47*, 515-522.

Ito, N.M.K., Miyaji, C.I. & Capellaro, C.E.M.P.D.M. (1991). Studies on broiler's IBV and IB-like virus from guinea fowl. In *II International Symposium on Infectious Bronchitis*, pp. 302-307. E.F. Kaleta and U. Heffels-Redmann (Eds). Giessen: Justus Leibig University.

Lister, S.A., Beer, J.V., Gough, R.E., Holmes, R.G., Jones, J.M.W. and Orton, R.G. (1985). Outbreaks of nephritis in pheasants (*Phasianus colchicus*) with a possible coronavirus aetiology. *Veterinary Record*, *117*, 612-613.

Lin, T.L., Loa, C.C. & Wu, C.C. (2004). Complete sequences of 3' end coding region for structural protein genes of turkey coronavirus. *Virus Research, 106,* 61-70.

Liu, S., Chen, J., Chen, J., Kong, X., Shao, Y., Han, Z., Feng, L., Cai, X., Gu, S. & Liu, M. (2005). Isolation of avian infectious bronchitis coronavirus from domestic peafowl (Pavo cristatus) and teal (Anas). *Journal of General Virology, 86,* 719-725

Kolb, A.F., Hegyi, A. & Siddell, S.G. (1997). Identification of residues critical for the human coronavirus 229E receptor function of human aminopeptidase N. *Journal of General Virology, 78,* 2795-2802.

Pennycott, T.W. (2000). Causes of mortality and culling in adult pheasants. *Veterinary Record*, *146*, 273-278.

Poon, L.L., Chu, D.K., Chan, K.H., Wong, O.K., Ellis, T.M., Leung, Y.H., Lau, S.K., Woo, P.C., Suen, K.Y., Yuen, K.Y., Guan, Y. & Peiris, J.S. (2005). Identification of a novel coronavirus in bats. *Journal of Virology, 79*, 2001-2009.

Spackman, D. & Cameron, I.R.D. (1983). Isolation of infectious bronchitis virus from pheasants. *Veterinary Record, 113*, 354-355.

Welchman Dde B, Bradbury J.M, Cavanagh D, Aebischer N.J. (2002). Infectious agents associated with respiratory disease in pheasants. *Veterinary Record, 150*, *658-664.*

Woods, R.D., Cheville, N.F. and Gallagher, J.E. (1981). Lesions in the small intestine of newborn pigs inoculated with porcine, feline and canine coronaviruses. *American Journal of Veterinary Research, 42*, 1163-1169.

Woods, R. D., and R. D. Wesley. (1992). Seroconversion of pigs in contact with dogs exposed to canine coronavirus. *Canadian Journal of Veterinary Research, 56*, 78-80.

Zhang, X.M., Herbst, W., Kousoulas, K.G. & Storz, J. (1994). Biological and genetic characterization of a hemagglutinating coronavirus isolated from a diarrhoeic child. *Journal of Medical Virology, 44*, 152-161.

Table 1. Amino acid identities of proteins of PhCoV UK/602/95 compared with those of IBV M41

Protein	Amino acid identity (%)
S	87
3a	84
3b	80
E	80
M	92
5a	85
5b	79
N	92

IMPORTANCE OF SIALIC ACID FOR THE INFFECTION OF IBV

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SUMMARY

We have analysed the importance of sialic acid for infection by avian infectious bronchitis virus (IBV). Neuraminidase treatment rendered Vero, baby hamster kidney, Chinese hamster ovary and primary chicken kidney cells resistant to infection by IBV Baudette. Sialic acid-dependent infection was also observed with M41 on primary chicken kidney cells. The binding of IBV to sialic acid may be a primary attachment to the cell surface. More tight binding and fusion between the viral and the cellular membrane may require the interaction with a second receptor.

INTRODUCTION

Avian infectious bronchitis virus (IBV) belongs to group 3 of coronaviruses. For IBV no protein receptor on cells of its natural host has been identified yet. IBV has a sialic acid binding activity as evidenced by the ability of several strains to agglutinate erythrocytes (Bingham et al., 1975) which is based on the capability of the virus to bind to α2,3-linked sialic acids on the surface to red blood cells (Schultze et al., 1992). We could show the significance of this binding activity for virus infection.
Cells become resistant to IBV infection after treatment with neuraminidase (Winter et al. 2006). This effect was observed with two strains (Beaudette and M41) and two different cell types (Vero cells and chicken kidney cells). Our results suggest that sialic acid serves as a receptor determinant for primary attachment of IBV to host cells.

MATERIALS and METHODS

Cells
Vero were grown in Dulbecco´s modified Eagle medium containing 5 % fetal calf serum. BHK 21 were incubated in Eagle minimal essential medium supplemented with 3 % fetal calf serum and 1% non essential amini acids. Primary chicken kidney cells were prepared from 19-20 day old embryos. The kidney cells were distributed on plastic tissue culture flasks and incubated at 37°C and 5 % CO2 in M199.

Viruses
Stock virus of the M41 strain of IBV was obtained by inoculating embryonated SPF chicken eggs. Following incuabtion at 37°C, the allantoic fluid was collected, clarified by low speed centrifugation and stored at –80°C. Strain Beaudette of IBV was propagated in Vero cells. Virus containing supernatants of infected cell cultures were harvested, clarified by low speed centrifugation and strored at –80°C.

Neuraminidase treatment and virus infection of cells.
Cells grown on coverslips and were washed twice with PBS and incubated with neuraminidase from *Vibrio cholerae* or *Streptococcus pneumoniae* using MES buffer as a diluant. If not otherwise indicated cells were incubated with 50 mU neuraminidase per coverslip. After gentle shaking at 37°C for 1 hour, the cells were washed three times with PBS and infected by IBV-Beaudette (10^5 $TCID_{50}$/ml) or any of the other viruses.

Immunofluorescence analysis
Cells grown on coverslips were infected by the virus indicated. At 24 h.p.i., the cells were fixed with 3% paraformaldehyde for 20 min at room temperature For detection of viral antigen, the samples were incubated with either of the following antibodies: an antiserum raised in SPF rabbits against IBV-Beaudette; monoclonal antibodies A38 and 124 directed against the S and M protein of IBV M41, respectively,

RESULTS

To analyse the importance of sialic acid for the initiation of an IBV infection, Vero cells were treated with neuraminidase from *Vibrio cholerae* prior to infection by IBV Beaudette. Incubation of the cells with increasing amounts of enzyme resulted in a decrease of the yield of infectious virus released into the supernatant. Treatment of the cells with 1 mU of neuraminidase was sufficient to reduce the virus titre by 80%. Incubation with 20 mU of enzyme caused a reduction of the infectivity titre by 99%. This result suggests that sialic acids are involved in the initial stage of an IBV infection.
To find out whether the dependence of the IBV infection on sialic acid is restricted to Vero cells, we included other cells in the analysis. BHK and chicken kidney cells are sensitive to infection by IBV-Beaudette. Both cell types became resistant to infection after pretreatment with neuraminidase. Therefore, the Beaudette strain requires the presence of surface-bound sialic acid not only for infection of heterologous cell cultures but also for infection of cells from its natural host.
We were also interested to know whether other strains of IBV are dependent on sialic acid for initiation of infection. We included the M41 strain in our analysis which is able to infect chicken kidney cells. Pretreatment with neuraminidase rendered these cells resistant to infection also by IBV-M41. These results indicate that the sialic acid dependence of the IBV infection is a general feature and not restricted to a certain cell type or virus strain.

DISCUSSION

Coronaviruses with Sialic acid binding activity of the spike protein can be found in all three groups of coronaviruses. Viruses such as bovine coronavirus (group 2) recognise 9-O-acetylated sialic acids and contain an acetylesterase as receptor-destroying enzyme (Herrler et al., 1985; Schultze et al., 1991a; Schultze et al., 1991b; Vlasak et al., 1988). This enzyme releases the 9-O-acetyl group from sialic acid and thus abolishes virus binding to the respective sialoglycoconjugate. Group 1 and 3 coronaviruses don´t have such an enzyme. Among group 1 coronaviruses, a sialic acid binding activity has been demonstrated for porcine transmissible gastroenteritis virus (TGEV) (Schultze et al., 1996). In addition to sialic acid, TGEV is able to bind to porcine aminopeptidase N. This surface protein functions as a cellular receptor for TGEV (Delmas et al., 1992). Binding to sialic acid increases the efficiency of binding, but it is not an absolute requirement for infection of cultured cells (Schwegmann-Wessels et al., 2002). Mutants or variants of TGEV that have lost the sialic acid binding activity can be propagated in cultured cells to high titres (Krempl et al., 2000). The interaction with sialic acids seems to be required for the intestinal infection by TGEV. Mutants or variants of TGEV that have lost the sialic acid binding activitiy by a single point mutation in the S protein also have lost the enteropathogenicity (Krempl et al., 1997).
IBV is a group 3 coronavirus and takes an intermediate position between group1 and group 2 coronaviruses regarding receptor interaction. Like TGEV, IBV lacks a receptor-destroying enzyme; on the other hand, it infects cultured cells in a sialic acid-dependent manner, thus resembling BCoV. Because of the low affinity binding to sialic acid (Winter et al., 2006), it is possible that IBV uses sialoglycoconjugates for primary attachment to cells. Successful infection may need the interaction with another receptor molecule for a stronger binding and the initiation of the fusion process.

REFERENCES

Bingham, R. W., Madge, M. H., and Tyrrell, D. A. (1975). Haemagglutination by avian infectious bronchitis virus-a coronavirus *J.Gen.Virol.* 28, 381-390.

Delmas, B., Gelfi, J., L'Haridon, R., Vogel, L. K., Sjostrom, H., Noren, O., and Laude, H. (1992). Aminopeptidase N is a major receptor for the entero-pathogenic coronavirus TGEV *Nature* 357, 417-420.

Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K., and Schauer, R. (1985). The receptor-destroying enzyme of influenza C virus is neuraminate-O-acetylesterase *EMBO J.* 4, 1503-1506.

Krempl, C., Ballesteros, M. L., Zimmer, G., Enjuanes, L., Klenk, H. D., and Herrler, G. (2000). Characterization of the sialic acid binding activity of transmissible gastroenteritis coronavirus by analysis of haemagglutination-deficient mutants *J.Gen.Virol.* 81, 489-496.

Krempl, C., Schultze, B., Laude, H., and Herrler, G. (1997). Point mutations in the S protein connect the sialic acid binding activity with the enteropathogenicity of transmissible gastroenteritis coronavirus *J.Virol.* 71, 3285-3287.

Schultze, B., Cavanagh, D., and Herrler, G. (1992). Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes *Virology* 189, 792-794.

Schultze, B., Krempl, C., Ballesteros, M. L., Shaw, L., Schauer, R., Enjuanes, L., and Herrler, G. (1996). Transmissible gastroenteritis coronavirus, but not the related porcine respiratory coronavirus, has a sialic acid (N-glycolylneuraminic acid) binding activity *J.Virol.* 70, 5634-5637.

Schultze, B., Wahn, K., Klenk, H. D., and Herrler, G. (1991a). Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity *Virology* 180, 221-228.

Schultze, B., Wahn, K., Klenk, H. D., and Herrler, G. (1991b). Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity *Virology* 180, 221-228.

Schwegmann-Wessels, C., Zimmer, G., Laude, H., Enjuanes, L., and Herrler, G. (2002). Binding of transmissible gastroenteritis coronavirus to cell surface sialoglycoproteins *J.Virol.* 76, 6037-6043.

Vlasak, R., Luytjes, W., Leider, J., Spaan, W., and Palese, P. (1988). The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetylesterase activity *J.Virol.* 62, 4686-4690.

Winter, C., Schwegmann-Weßels, C., Cavanagh, D., Neumann, U., and Herrler, G. (2006). Sialic acid is a receptor determinant for infection of cells by avian Infectious bronchitis virus *J.Gen.Virol*. 87, 1209-1216.

SOME OBSERVATIONS ON THE DETECTION OF AVIAN METAPNEUMOVIRUS AND INFECTIOUS BRONCHITIS VIRUS IN CLINICAL MATERIAL

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SUMMARY

For detection of avian pneumovirus (APV) in clinical material, the traditional method has been isolation in tracheal organ culture (TOC). As for infectious bronchitis virus (IBV) detection, samples are inoculated into embryonated chicken eggs or TOC. After virus propagation in these systems, the identity of the viruses needs to be confirmed by immunofluorescence or serum neutralisation. However, with the introduction of reverse-transcriptase polymerase chain reaction (RT-PCR), both viruses can be rapidly detected in clinical material with or without prior propagation in TOCs or eggs. This paper presents some observations from our experiences on aspects of detection of these viruses, including relative sensitivity of the methods, effects of storage and testing of pooled and single swabs.

INTRODUCTION

Diagnosis of APV relies on isolation of the virus using tracheal organ culture (TOC) followed by identification by RT-PCR. Commonly, for diagnosis, 10 oropharyngeal (OP) swabs are collected from suspected flocks and these swabs are pooled together and treated as single sample for RT-PCR (Cavanagh et al., 1999). Whilst it is economic to do so, the sensitivity of such procedure has not been investigated before. The first part of this paper provides some preliminary findings on such an approach. The second part of the APV studies, reports on detection of avirulent or virulent APV in cloacal swabs. This finding highlights the potential implication for immunopathogenesis, epidemiology, control and prevention of APV infection in chickens. Hess et al. (2004a,b) reported on isolation and detection of APV from cloacal swabs of APV vaccinated and challenged chickens.

Diagnosis of IB is confirmed by isolation of the virus using either chicken embryonated eggs (ECE) or tracheal organ culture (TOC), and detection by RT-PCR (Cavanagh and Naqi, 2003). However, no reports are available to indicate time limits, beyond which virus detection is no longer detectable. This paper compares

three different IBV detection methods in confirming the presence of the pathogen in tissues collected from cold-stored carcasses.

MATERIALS and METHODS

Avian pneumovirus

Detection on pooled and single swabs:
RT-PCR was carried out on oropharyngeal swabs that were pooled or kept separate. The swabs were obtained from two experiments, in each, day-old SPF chicks received subtype B APV vaccine via the oculo-oral route. Twenty-one days following the vaccination, unvaccinated and the vaccinated chicks were challenged with a virulent subtype B APV virus. Following the challenge, in Experiment 1, OP swabs were collected from unvaccinated and vaccinated chicks at 3, 6 and 9 days post challenge (Table 1). In experiment 2, OP swabs were collected only at 5 days post challenge (Table 1). For the detection of APV, swabs were processed either singly or pooled together.

APV detection in cloacal swabs
Cloacal swabs were obtained from two experiments. In Experiment 1, two groups of broiler chicks were given APV vaccine via the oculo-oral route and cloacal swabs were collected at 2, 5, 10, 16, 24 and 37 days post vaccination. In Experiment 2, two groups of day-old specific pathogen free chicks were vaccinated with APV by oculo-oral route, and 21 days later were challenged with a virulent subtype B virus by eye drop. Following the challenge, cloacal swabs were collected at 3, 6, and 9 days from unvaccinated and the two vaccinated groups.

OP and cloacal swabs were examined for APV by RT-PCR, as described previously (Cavanagh et al., 1999; Ganapathy et al., 2005a).

Infectious bronchitis virus

Seven-week old specific pathogen chicks were administered with a virulent Mass 41 IBV by the oculo-nasal route. After 10 days of observation, all chickens were humanely killed and carcasses were immediately stored in a cold room (4-6 °C). At one, 3, 6, 9, 12 and 24 hours post storage, 4 carcasses were randomly removed and necropsy was carried out. Samples of trachea, lungs, kidney and rectum were collected aseptically and placed individually in plastic begs. All tissues were store at 70 °C until used.
Tissues were processed (Ganapathy et al., 2005a) and the resulting supernatant was used for isolation of virus via embryonated chicken eggs (ECE) (Gelb and Jackwood, 1998) or tracheal organ culture (Cook et al., 1976). From the homogenized samples, individual swabs were collected and they were pooled according to tissues and sampling intervals. RT-PCR (Cavanagh *et al*., 1999) was carried out on these pooled samples.

RESULTS

Avian pneumovirus

Detection on pooled and single swabs
Table 1 shows that when more than 2 of 10 swabs were positive, the pooled samples likely to be positive. In Experiment 2, it appears that when one sample is positive, the pooled sample can be positive or negative (data not shown).

APV detection in cloacal swabs
In Experiment 1, APV was detected at 5 days post vaccination in both APV-vaccinated groups (data not shown). In Experiment 2, APV was detected only at 6 and 9 days post challenge in the unvaccinated-challenged chickens (data not shown). No APV was detected at any other samples intervals.

Infectious bronchitis virus

IBV was detected for up to 24 hours of storage but there were discrepancies between the three detection methods. For TOC method, in trachea, virus was isolated at 3 and 9 hours storage only and in lungs between 2 and 12 hours but never more than two out of four chickens (Table 2). At 1, 3 and 12 hours of storage, virus was isolated from the kidney and was consistently detected in rectum. When eggs were used, virus was consistently detected at all sampling points except at 1 and 24 hours in lungs. In total, rectum provided 100% recovery, followed by trachea and kidney (63%), and lungs (58%). With RT-PCR, IBV was detected at all sampling intervals in all 4 organs, except at 1, 6 and 9 hours for kidney and at 9 hours storage for rectum (Table 2). All control tissues remained negative for virus.

DISCUSSION

For APV, the ability to detect virus in pooled samples where most of them are negative will depend on the amount of virus or their genome present in the small number of positive swabs. Hence pooling, although economical, some times may cause positives to be missed. As such, laboratories need to undertake their own standardization to ascertain the number of swabs that can be pooled and this may vary according to the sensitivity of RT-PCR used.
For regular detection of APV, oropharyngeal, tracheal swabs or respiratory tissues are preferred (Cook & Cavanagh, 2002) and intestinal samples are rarely examined. However, Hess *et al.* (2004a) reported virus isolation and RT-PCR detection of APV in cloacal swabs taken from APV-challenged chickens. The viral genome was detected from 7 to 28 days post challenge in birds either challenged intravenously or oculonasally but those birds challenged by intravenous route were also positive by virus isolation. In another study, Hess *et al*. (2004b) administered APV vaccine virus by the *in ovo* method and found positive APV (by RT-PCR and isolation) in the cloacal swabs. In the present study, using RT-PCR, APV vaccine virus was detected in cloacal swabs at 5 days post vaccination. In another study, the a virulent APV challenge virus was detected in cloacal swabs collected at 6 and 9 days post challenge. These findings show that both virulent and avirulent APV viruses can

sometimes be excreted via the cloaca. However, no report exists confirming the replication of APV in the gut and the mechanism to explain the early detections reported here are unknown. These findings raise further questions on the pathogenesis, epidemiology, control and prevention of APV in chickens and suggest that study is needed.

For IBV, it appears that irrespective of the IBV detection method, the virus was found in all tissues examined for up to 24 hours of storage. This suggests that in difficult circumstances, necropsy examination and tissue collection of carcasses kept at 4 - 6 ºC could be safely delayed up to 24 hours post-killing. However, it is interesting to notice discrepancies between the three methods used, and possible explanations for them are discussed below.

For re-isolation of IBV, ECE provided a consistent and high number of isolation compare to TOC. The reason for this is not known. It have been reported that both ECE and TOC are equally good for isolation of IBV (Cook et al., 1976; Darbyshire et al., 1975; De Wit, 2000) but for TOC, the virus should not be egg-adapted (Sawaguchi et al., 1985). Cook et al. (1976) used IBV isolates that had undergone four passages in chickens and Sawaguchi et al. (1985) used field samples. Variation in our findings could be due to the low titre of virus in the tissues, as samples were collected 10 days post infection compare to 3 (Cook et al., 1976) and 3-5 (Sawaguchi et al., 1985) days post-infection in other studies. The other possibility is that the virus used in this study had undergone four subsequent passages in ECE and may have adapted to this culture system. In addition, probably use of ECE may have allowed better amplification of the likely egg-adapted IBV, which may have resulted in better isolation. Interestingly, irrespective of the method of isolation, consistently higher numbers of recoveries were obtained from rectum. In addition, other than at 9 hs, it was positive by RT-PCR, indicating that rectum could be an important tissue for diagnosis of IBV.

REFERENCES

Cavanagh, D. ,Naqi, S., 2003. Infectious bronchitis. In: Saif, Y.M., Barnes H.J., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. Editors, 2003. *Disease of Poultry* (11th ed.), Iowa State University Press, Ames, Iowa, pp. 511–526.

Cook, J.K.A. & Cavanagh, D. (2002). Detection and differentiation of avian pneumoviruses (metapneumoviruses). *Avian Pathol.*, *31*, 117-132.

Cook, J.K.A., Darbyshire, J.H., Peters, R.W., 1976. The use of chicken tracheal organ cultures for the isolation and assay of avian infectious bronchitis virus. Arch. Virol. 50, 109-118.

Darbyshire, J.H., Cook, J.K.A., Peters, R.W., 1975. Comparative growth kinetic studies on avian infectious bronchitis virus in different systems. J. Comp. Pathol. 85, 623-630.

De Wit, J.J., 2000. Detection of infectious bronchitis. Avian Pathol. 29, 71-93.

Dhinakar Raj, G., Jones, R.C., 1997. Infectious bronchitis virus: immunopathogenesis of infectious in chicken. Avian Pathol. 26, 667-706.

Ganapathy, K., Cargill, P. & Jones, R. C. (2005a). Effects of cold storage on detection of avian infectious bronchitis virus in chicken carcasses and local antibodies in tracheal washes. J.I Virol. Methods, 126, 87-90.

Ganapathy, K., Cargill, P., Montiel, E. & Jones, R.C. (2005b). Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. Avian Pathol., *34*, 297-302.

Gelb., J.Jr., Jackwood, M.W., 1998. Infectious bronchitis. In: Swayne, D.E., Glisson, J.R., Jackwood, M.W., Pearson, J.E., Reed, W.M., Editors, 1998. *A Laboratory Manual for the Isolation and Identification of Avian Pathogens* (4th ed.), American Association of Avian Pathologists, Kennett Square, PA, pp. 169–174.

Hess, M., Huggins, M.B., Mudzamiri, R. & Heincz, U. (2004a). Avian metapneumovirus excretion in vaccinated and non-vaccinated specified pathogen free laying chickens. Avian Pathol., 33, 35-40.

Hess, M., Huggins, M.B. & Heincz, U. (2004b). Hatchability, serology and virus excretion following *in ovo* vaccination of chickens with an avian metapneumovirus vaccine. Avian Pathol., 33, 576-580.

Sawaguchi, K., Yachida, S., Aoyoma, S., Takahashi, N., Iritani, Y. Hayashi, Y. (1985). Comparative use of direct organ cultures of infected chicken tracheas in isolating avian infectious bronchitis virus. Avian Dis. 29, 546 – 551.

Table 1: Detection of APV by RT-PCR on single or pooled oropharyngeal swabs.

Experiment 1 (Days post challenge)	Group	Single Positive / 8	Pool of 8
3	Unvaccinated	8	+
	Vaccinated APV1	7	+
	Vaccinated APV2	8	+
6	Unvaccinated	7	+
	Vaccinated APV1	7	+
	Vaccinated APV2	8	+
9	Unvaccinated	8	+
	Vaccinated APV1	4	+
	Vaccinated APV2	3	+
Experiment 2 (Days post challenge)	Group	Single Positive / 10	Pool of 10
5	Unvaccinated	10	+
	Vaccinated APV1	1	-
	Vaccinated APV2	1	+

Table 2: Detection of IBV by RT-PCR or isolation in TOC or ECE.

Site		Hours post-storage					
	Method	1	3	6	9	12	24
Trachea	RT-PCR	+ [a]	+	+	+	+	+
	TOC	0 [b]	1	0	2	0	0
	ECE	3 [b]	4	2	2	3	1
Lungs	RT-PCR	+	+	+	+	+	+
	TOC	0	2	1	1	1	0
	ECE	0	4	4	3	3	0
Kidney	RT-PCR	-	+	-	-	+	+
	TOC	1	2	0	0	2	0
	ECE	1	2	3	4	2	3
Rectum	RT-PCR	+	+	+	-	+	+
	TOC	3	1	1	1	3	2
	ECE	4	4	4	4	4	4

[a] Positive or negative by RT-PCR (pool of 4).
[b] Number positive isolation by TOC or ECE (of 4).

LECTIN-CAPTURE RT-PCR (LC-RT-PCR) FOR THE DETECTION OF AVIAN INFECTIOUS BRONCHITIS VIRUS

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SUMMARY

Rapid, sensitive and specific laboratory diagnostic methods are necessary to confirm outbreaks of infectious bronchitis. Reverse transcription followed by polymerase chain reaction amplification (RT-PCR) is now used commonly to detect the presence of avian respiratory RNA viruses in clinical samples. The detection of avian infectious bronchitis virus (IBV) can be achieved by virus isolation on embryonated chicken eggs, antigen detection, or molecular methods, such as RT-PCR. Aiming to reduce the time and the number of steps in the diagnostic procedure of IBV, a sensitive and rapid detection method based on viral capture by a lectin (Concanavalin A) and genomic amplification by RT-PCR was developed. This assay combines the rapidity of ELISA test, because lectin-capture and the RT reaction were carried out in the same microplate, and the sensitivity of PCR. Allantoic fluid and tissue samples from IBV experimentally infected embrionated eggs or chickens were analysed. The overall results showed that LC-RT-PCR was efficient in detecting the presence of classical and variant IBV strains in infected allantoic and chicken tissue samples and allows the processing of large number of samples. In addition, this assay was compared with a Sandwich-ELISA for IBV detection and showed a higher sensitivity. The analytical specificity of the LC-RT-PCR was assessed with regard to other avian viral pathogens and Gumboro disease virus, pneumovirus and Newcastle disease virus were not detected by this technique. Thus, the LC-RT-PCR technique, applied here for the first time for viral detection, demonstrated a great potential to be a useful alternative for the rapid and specific diagnosis of IBV.

INTRODUCTION

Infectious bronchitis virus (IBV) is one of the major causes of economic losses in the poultry industry and can be involved in respiratory disease, nephritis, and poor egg production and quality. However, these signs are not specific to IBV. The disease is

worldwide distributed and extremely difficult to control. Therefore, a fast, sensitive and specific virus detection techniques are of great interest to the poultry to identify IBV infections in relation to a clinical problem in the field. This may also include typing of the isolate involved in order to enable the choice of a vaccination programme with the best chance of achieving sufficient protection against an IBV infection in next flock (De Witt, 2000).

IBV is an important pathogen characterized by a worldwide distribution and many different variants appear continuously, despite the use of vaccines. It is a coronavirus that causes a highly contagious disease in chickens and belongs to group III of the genus *Coronavirus* of the *Coronaviridae* family (Cavanagh & Naqi, 1997). The IBV contains a genome constituted by a single stranded RNA of positive polarity, which consists of approximately 27 kb and codes for three main structural proteins: the spike glycoprotein (S), the membrane glycoprotein (M) and the nucleocapside protein (N) (Lai & Cavanagh, 1997).

The conventional diagnosis of the IBV is based on virus isolation in embryonating eggs, followed by immunological identification of isolates using immuno-fluorescence or immuno-enzymatic assays. Since two or three blind passages are often required for successful primary isolation of IBV, this procedure could be time consuming. Alternatively, IBV may be isolated by inoculation in chicken tracheal organ cultures. This method is sensitive (Cook et al, 1976) but is too laborious. In addition to this, IBV may be detected directly in tissue of infected birds by means of immunohistochemistry (IHC) (Yagyu & Ohta, 1990, Nakamura et al., 1991, Chen eta al., 1996, Dhinakar Raj & Jones, 1996) or by in situ hybridization (Collison et al., 1990). The reverse transcription-polymerase chain reaction (RT-PCR) has proved useful in the detection of a number of RNA viruses (Cavanagh, 1993). However, sample preparation and nucleic acid extraction steps prior to RT-PCR were time-consuming and did not allow the processing of large numbers of samples.

Immunocapture-RT-PCR is a procedure based on the PCR amplification of cDNA synthesized from the RNA of antibody-captured viruses. This approach was first reported for hepatitis A virus detection (Jansen et al., 1990) and successfully used for other RNA plant virus families (Wetzel et al., 1992, Nolasco et al., 1993, Chevalier et al.,1995, Lunello et al., 2004).

Adsorption solid-phase methods using lectins to capture viral antigens have been developed for the diagnosis of some diseases, as pancreatic cancer, human immunodeficiency virus and feline immunodeficiency virus. Lectins are proteins or glycoproteins derived from plants, animals or microorganisms, which are characterized to interact with carbohydrate moieties present on the surface molecules of humans, animal and plant cells in addition to bacteria and viruses. More recently, a Concanavalin A was successfully applied as capture-reagent in a Sandwich-ELISA to detect IBV, or virus specific antibodies (Bronzoni et al., 2005).

In this study, a lectin-capture-RT-PCR, based on the specific interaction of Con A with terminal mannose groups of oligo-saccharides presented by S and M glycoproteins of IBV, was developed for the molecular diagnosis of this virus.

MATERIALS and METHODS

Virus Samples

Two reference strains (H120, M41) and 4 field isolate(s) of IBV were propagated in 9 to 10-day-old specific pathogen free (SPF) embryonated chicken eggs. The eggs were inoculated by allantoic sac route, as described Owen et al. (1991). Virus strains were kindly provided by Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA (Concórdia-SC, Brazil).

LECTIN-CAPTURE-RT-PCR (LC-RT-PCR)

Polystyrene microtitre plates (96-well plates) were coated with 200μl of 0.5 mg/ml of Concanavalin A (Sigma Chemical Co., St. Louis, MO), diluted in PBS pH 7.4 and incubated overnight at 4ºC. One well out of four was used, in order to avoid cross-contamination. The wells were washed four times with PBS. After washing, 200μl of virus samples were added and the reaction was incubated for 1h at 37ºC. The wells were washed six times with PBS, taking care to avoid any cross-contamination. The reverse transcription (RT) was carried out in the same plate, by adding to each well 20μl of the RT mixture containing 200 U of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) and 25 pmol of random hexamers as primer (Invitrogen, Carlsbad, CA). The plate was incubated for 1h at 42ºC. The whole volume of each well containing cDNA solution, was transferred to 0.2ml polypropylene tubes and stored at -20ºC. Two set of primers were used for PCR; one (primer set 1) is specific for the amplification of the 5'- part of S1 gene (Yu et al., 2001) and other was specific for the amplification of the entire S1 gene (Kwon et al., 1993). The reaction was performed in volumes of 50 μl, containing 5μl of the Cdna, 20 pmol of each specific primer and 2.0 U Taq DNA polymerase (Invitrogen, Carlsbad, CA). Cycling conditions for the amplification of the first 5'-part of S1 gene were those recommended by Yu et al. (2001), or alternatively for the amplification of the entire S1 gene, the reaction was conducted as described Kwon et al. (1993).

Analytical specificity

The analytical specificity of the LC-RT-PCR was assessed using different antigen suspensions of M41 strain of IBV, IBV Brazilian field isolates (IBVPR01, IBVPR03, IBVPR06, IBVSC02, IBVSP02), as well as non-related viruses such as Newcastle Disease Virus (NDV / La Sota vaccine strain), avian pneumovirus (AVP PL-21 vaccine strain) and infectious bursal disease virus (IBDV / Lukert vaccine strain).

Analytical sensitivity

A serial ten-fold dilutions (10^0 to 10^{-7}) of a stock IBV strain (M41), where the virus infectivity determined in embryonated chicken eggs corresponded to $10^{-7.4}$/ml embryo 50% infectious doses (EID_{50}/ml), were tested on the LC-RT-PCR, as described above. The analytical sensitivity corresponded to the highest dilution that gave a specific amplified product.

Experimental Infection and Evaluation of diagnostic sensitivity and specificity of the LC-RT-PCR

Two groups of 12 commercial (SPF) White Leghorn chickens each were maintained in positive pressure isolators units. At four weeks of age, one group was inoculated

with 10^6 EID_{50} of M41 strain of IBV, while other group, taken as negative control, remained non-infected in other isolator unit. Tracheal samples were collected from each group at 5 days post-infection. Tissue samples were tested for virus isolation (VI) and passed thrice in embryonated chicken eggs. Samples were considered negative or positive on VI based on the absence or the presence of typical embryonic lesions induced by IBV, such as stunting, curling or embryo death (Gough et al., 1988) and IBV-antigen detection by Con A-S-ELISA. These tissue specimens were also submitted to LC-RT-PCR and to Con A-S-ELISA.

Concanavalin A – Sandwich – ELISA
The Con A-Sandwich ELISA was carried out according to Bronzoni et al. (2005) for the detection of IBV antigen in allantoic fluid or tissue samples.

RFLP
The amplified products of the entire S1 gene with the predict size of 1.7 kb were cut from the gel, after electrophoresis on 2% of agarose, and purified using GFX columns kit (Amersham Biosciences) according to the manufacturer's instructions. The purified DNA were digested with *Hae* III (Invitrogen, Carlsbad, CA) following the recommendations of the manufacturer. The restriction fragment pattern was analyzed following electrophoresis on a 2% agarose gel at a constant voltage of 100V.

RESULTS

The LC-RT-PCR proved to be more rapid and easier to perform than the conventional RT-PCR method, which depends on a previous RNA extraction step. Furthermore, the LC-RT-PCR allowed the manipulation of a high number of AF or tissue samples per microtitre plate in 1 day.
Agarose gel analysis of the amplification products revealed the presence of the predicted sizes of 452 bp or 1.7 kb bands in the IBV positive samples (Fig. 1), corresponding, respectively, to the amplimers obtained with the pair of primers 1, described by Yu et al. (2001), or the pair the primers 2, described by Kwon et al. (1993).
The specificity of the LC-RT-PCR was also demonstrated, either for the primer set 1, or the primer set 2. Amplified products with the expected sizes were only observed for samples infected with the two reference strains M41 AND H120), or the four field isolates of IBV (Fig. 1). None of the other heterologous and IBV non-related avian viruses tested here showed any amplified products. This indicated that the LC-RT-PCR is a specific method, probably, because the two sets of primers were not cross-reacting with other common avian viruses, such as Newcastle disease virus, avian pneumovirus, or infectious bursal disease virus. Therefore, these pair of primers were highly specific to amplify sequences of S1 gene from IBV strains (Fig. 1).
The analytical sensitivity of the LC-RT-PCR was assessed for ten-fold dilutions of AF infected with M41 strain of IBV, using the primer sets 1 or 2 and the results are showed in the Figure 2. After gel electrophoresis a 452 bp band, for primer set 1, was detected until the dilution of 10^{-4} ($10^{3.4}$ DIE_{50}), and a 1.7 kb band, for primer set 2, was visible until the dilution of 10^{-3} ($10^{4.4}$ DIE_{50}). The Con A – S – ELISA exhibited a lower analytical sensitivity and it was able to detect IBV down to a dilution of 1:10.

The analysis of RFLP products obtained by digestion of S1 gene with *Hae* III enzyme showed three bands of 950, 500 and 380 bp, for the IBV reference strains tested (2 H120 vaccine strains and 1 M41 strain) (Figure 3). Thus, the amplimer of 1.7 kb of the gene S1, obtained from LC-RT-PCR, after digested by *Hae* III enzyme gave a profile of bands which is consistent with the Mass strain pattern.
The LC-RT-PCR, using SYu-primers, was able to amplify the specific IBV S1 fragment in 8 out of 12 tissue samples collected in acute phase of IBV experimentally infected chickens, while the Con A-S-ELISA detected only 3 positives among such set of samples. The virus was isolated, after 3 passages in embryonated chicken eggs and confirmed as IBV by Con A – S – ELISA in all these samples. The detection rate of LC-RT-PCR for the acute phase of IBV infection was 8/12 (67%). IBV was not detected by LC-RT-PCR, nor virus was isolated in tissue samples collected from the uninfected birds.

DISCUSSION

IBV diagnosis is quite complex and labor-intensive, because several passages in embryonated chicken eggs are usually required, before field isolates of this virus can produce typical lesions in the embryos (Cavanagh & Naqi, 2003).
To overcome these difficulties, an alternative molecular biology method that combines a virus capture to a solid phase mediated by the lectin concanavalin A and RT-PCR was developed for the detection of IBV in fluids or tissue samples collected from experimentally infected eggs or chickens. This method is designed so that the whole procedure, including the RT step, can be carried out in the same microplate. Nolasco et al. (1993) and Le Gaull-Recullé et al. (2001) applied a similar procedure to detect plant viruses or rabbit haemorrhagic disease virus and European brown hare syndrome virus, respectively. It does not require any previous handling of samples for RNA extraction, or even a thermal disruption of trapped virions to release the genomic RNA. Under these conditions the LC-RT-PCR was more sensitive than the Con A-S-ELISA, either for IBV detection in the AF laboratory viral suspension or in the experimentally infected tissue samples.
The sensitivity of LC-RT-PCR found is in the range of those reported for other conventional molecular biology methods involving RNA extraction and RT-PCR used for viral detection (Rodriguez et al., 1994, Kho et al., 2000). In comparison with ELISA, this represents a large improvement, as recorded others authors studying plant viruses (Nolasco et al., 1993, Wetzel et al., 1992), or animal viruses, such as foot-and-mouth disease virus and European brown hare syndrome virus (Rodriguez et al., 1994, Le Gaull-Recullé et al., 2001).
Six IB viruses, including one reference strain (M41) and 5 field isolates, one homologous to M41 strain and four heterologous variants, were effectively detected by the LC-RT-PCR, indicating that the mannoside oligosaccharides present in S and M glycoproteins of M41 strain are well conserved among different IBV strains, since these carbohydrate motifs are critical for the binding of these viruses to concanavalin A. However, the specificity of this method was high and it depended directly of the pair of primers used in the PCR. Thus, heterologous and IBV non-related viruses, such as avian pneumovirus, Newcastle disease virus and infectious bursal disease virus were not detected by the LC-RT-PCR, although the interactions of concanavalin A with molecular components of these viruses can not rulled out.

Further experiments revealed that uncoated microplates were able to capture IBV, permitting the amplification of this virus by RT-PCR, although the sensitivity of this procedure was lower than that observed for LC-RT-PCR, leading to some inconsistent results (data not shown), as occurred in the detection of Hepatitis A virus in sewage sludge by antigen capture polymerase chain reaction (Graff et al., 1993).
Additionally, the LC-RT-PCR can be combined to genetic typing methods such as RFLP. Thus, the amplicons containing the entire S1 sequence, approximately 1.7 kb, that were generated by LC-RT-PCR proved to be appropriated for digestion with *HaeIII* followed by electrophoresis and RFLP characterization, demonstrating the feasibility of performing further molecular analysis of amplicons from this alternative methodology.
In summary, the LC-RT-PCR could be a potentially important step toward the reduction of PCR methodology to IBV diagnostic usage and may contribute significantly with the studies of molecular epidemiology of a virus which is evolving rapidly in nature.

ACKNOWLEDGEMENTS

The authors are grateful to Fundação de Amparo à Pesquisa no Estado de São Paulo (FAPESP – Proc. Nº 01/14/950-3), Conselho nacional de Desenvolvimento Científico e Tecnológico (CNPq Proc. Nº 477140/2003-3) and MERIAL for financial support. M.F.S. Montassier was granted a PHD scholarship from FAPESP.

REFERENCES

Bronzoni, R.R.V.M., Montassier, M.F.S., PERREIRA, G.T., GAMA, N.N.S.Q., SAKAI, V., MONTASSIER, H.J. Detection of infectious bronchitis virus and specific anti-viral antibodies using a Concanavalin A- Sandwich-ELISA. Viral Immunology, 2005, 18, 269-578.

Cavanagh D. Advances in avian diagnostic technology. In: X World Veterinary Poultry Association Congress; 1993; p 57-70.

Cavanagh D, Mawditt K, Shaw K, Britton P, Naylor C. Towards the routine application of nucleic acid technology for avian disease diagnosis. Acta Veterinaria Hungarica 1997; 45: 281-298.

Cavanagh D, Naqi S. Infectious Bronchitis. In: Calnek BW, Barnes HJ, Beard CW. Diseases of poultry; 2003. 11 ed. Ames: Iowa State University Press; p 101-119.

Chen BY, Hosi S, Nunoya T, Itakura C. Histopathology and immunohistochemistry of renal lesions due to infectious bronchitis virus in chicks. Avian Pathology 1996; 25 (2): 269-283.

Chevalier, S., Grief, C., Clauzel,J.-M., Walter, B., Fritsch, C. Use of an immunocapture-polymerase chain reaction procedure for the detection of Grapevine virusA in Kober stem grooving infected grapevines. Journal Phytopathology, 1995, 143, 369-373.

Collisson EW, Li J, Sneed LW, Peters ML, Wang GL. Detection of avian infectious bronchitis using in situ hybridization and recombinant DNA. Veterinary Microbiology 1990; 24: 261-271.

Cook JKH, Darbyshire JH, Peters RW. The use of chicken tracheal organ cultures for isolation and assay of avian bronchitis. Archives of Virology 1976; 50: 109-118.

Dhinakar Raj, G, Jones RC. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. Avian Pathology 1997; 26: 677-706.

Dhinakar Raj, G, Jones RC. Immunopathogenesis of infection in SPF chicks and commercial broiler chickens of variant infectious bronchitis virus of economic importance. Avian Pathology 1996; 25: 481-501.

Gough RE, Alexander DJ, Collins AS, Cox WJ. Routine virus isolation or detection in the diagnosis of diseases in birds. Avian Pathology 1988; 17: 893-906.

Graff, J., Ticehurst, J., Flehmig, B. Detection of hepatitis A virus in sewage sludge by antigen capture polymerase chain reaction. Applied Enviromentla Microbiology, 1993; 59, 3165-3170.

Jansen, RW., Siegl, G., Lemon, S. M. Molecular epidemiology of human hepatitis A virus defined by an antigen-capture polymerase chain reaction method. Proceedings of National. Academy of Science, 1990; 87, 2867-2871.

Kho, C.L., Mohd-Azmi, M.L., Arshad, S.S., Yusoff, K. Performance of an RT-nested PCR ELISA for the detection of Newcastle disease virus. Journal of Virological Methods, 2001; 86, 71-83.

Kwon HM, Jackwood MW, Gelb Jr JF. Differentiation of infectious bronchitis virus serotypes using polymerase chain reac tion and restriction fragment length polymorphism analysis. Avian Diseases 1993; 37: 194-202.

Lai MMC, Cavanagh D. The molecular biology of coronaviruses. Advances in Virus Research 1997; 48: 71-77.

Le Gall-Reculé, G., Zwingestein, F., Portejoie, Y., Le Gall, G. Immunocapture –RT-PCR assay for detection and molecular epidemiology studeis of Rabbit Haemorrhagic Disease and European Brown Hare Síndrome viruses. Journal of Virological Methods, 2001; 97, 49-57.

Lunello, P., Mansilla, C, Conci, V., Ponz, F. Ultra-sensitive detection of two garlic potyvirus using a real-time fluorescent (Taíman®) RT-PCR assay. Journal of Virological Methods, 2004; 118, 15-21.

Nakamura K, Cook JKH, Oktsuki K, Huggins MB, Frazier JA. Comparative study of respiratory lesions in two chicken lines of different susceptibility infected with infectious bronchitis virus: histology, ultrastructure and immunohistochemistry. Avian Pathology 1991; 20: 241-257.

Nolasco, G., de Blas, C, Torres, V., Ponz, F. A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pahogens. Journal of Virological Methods, 1993; 45, 201-218.

Owen RL. Detection of viral antigen following exposure of one-day-old chicken to the Holland-52-strain of IBV. Avian Patholoy 1991; 20: 663-673.

Rodriguez, A., Nuñez, J.I., Nolasco, G., Ponz, F., Sobrino, F., de Blas, C. direct PCR detection of foot-and-mouth disease virus. Journal of Virological Methods, 1994; 47, 345-349.

Wetzel, T., Candresse, T., Macquaire, G., Ravelonandro, M., Dunez, J. A highly sensitive immunocapture polymerase chain reaction method for plum pox potyvirus detection. Journal of Virological Methods, 1992; 39, 27-37.

Yagyu K, Ohta S. Detection of infectious bronchitis virus antigen from experimentally infected chickens by indirect immunofluorescent assay with monoclonal antibody. Avian Diseases 1990; 37: 157-169.

Yu L. Molecular epidemiology of infectious bronchitis virus isolates from China and Southeast Asia. Avian Diseases 2001; 45: 205-209.

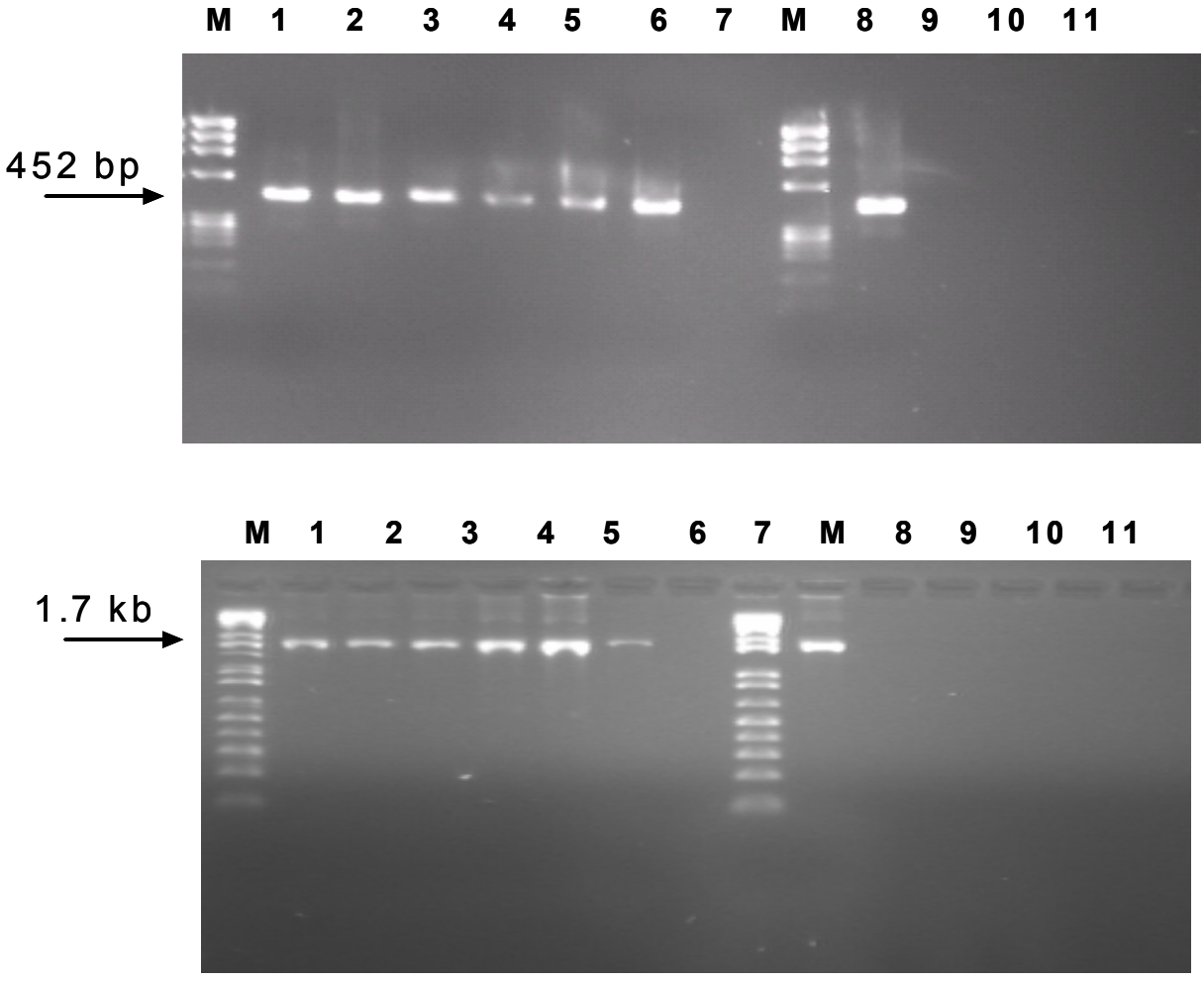

Fig. 1 Specificity analysis of LC-RT-PCR, using SYU – primers (amplicon of 452 bp) or the Oligo-S1- primers (amplicon of 1.7 kb). M – DNA marker 1 kb plus, Lane 1 – IBV-M41, Lane 2 – IBVPR01, Lane 3 -IBVPR03, lane 4 - IBVSC02, Lane 5 - IBVPR06, Lane 6 - IBVSP02 , Lane 7 – negative control, Lane 8 – IBVM41, Lane 9 – Avian Pneumvirus, Lane 10 – Newcastle disease virus, Lane 11 – Infectious bursal disease virus, Lane 12 – Negative control .

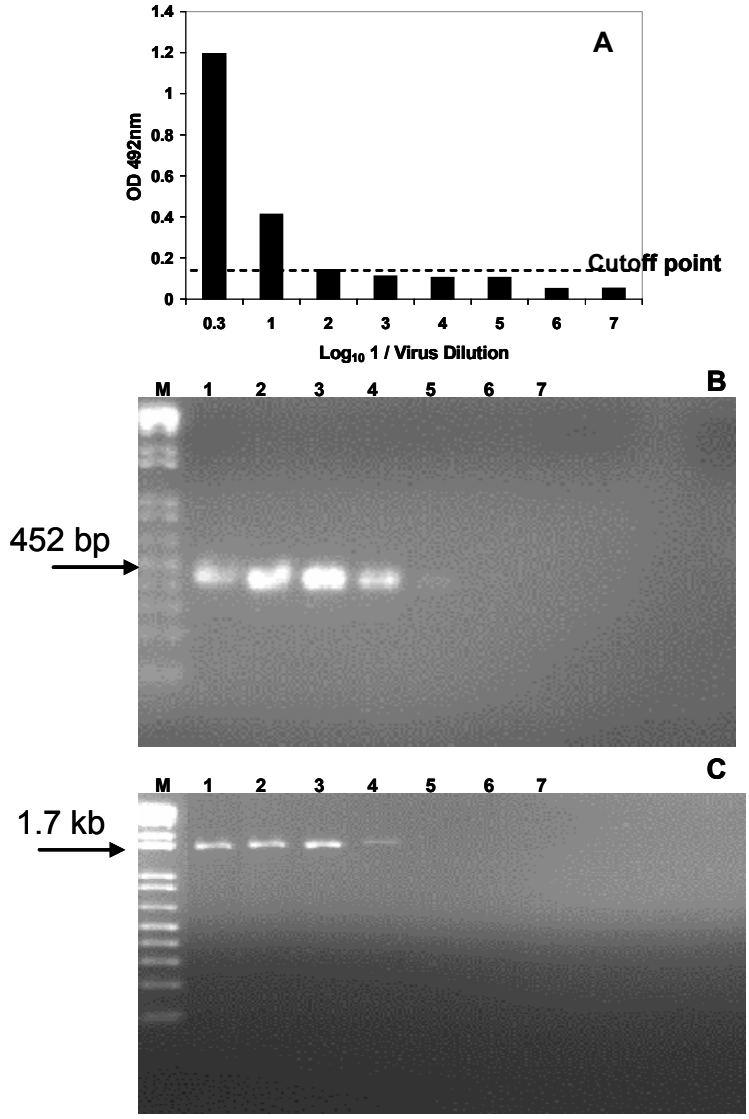

Fig. 2 Sensitivity of Con A – S – ELISA and LC-RT-PCR for the detection of IBV (M41 strain) present in AF suspension. Panel A: Con A – S – ELISA optical densitiy values. Panel B: Agarose gel electrophoresis analysis of RT-PCR (SYU – primers) products of 5'-part of S1 gene of IBV (M41 strain). Panel C: Agarose gel electrophoresis analysis of RT-PCR (S-Oligo-1 – primers) products of entire S1 gene of IBV (M41 strain). M- DNA marker 1kb (Invitrogen). Lane 1 – undiluted IBV suspension, Lane 2 to 6 – ten-fold dilutions of IBV suspension, starting 10^{-1} through 10^{-6} and Lane 7 – Negative virus suspension

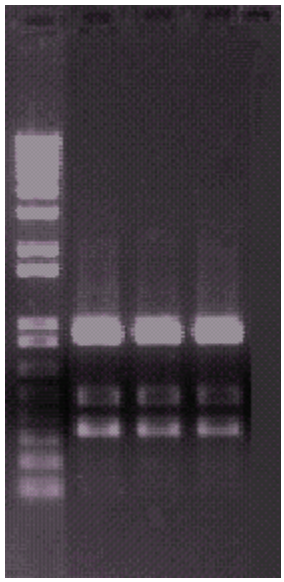

Fig. 3 - Electrophoretic profile of S1 gene product of S1 gene, amplified by LC-RT-PCR with the oligonucleotides S1-OLIGO-5' and S1-OLIGO-3' and digested with endonucleases *HaeIII*. Lane M – 1kb plus DNA marker, Lane 1 – M41 , Lane 2 – H120 strain (Vac. 1), Lane 3 – H120 strain (Vac. 2),

SERUM FREE MEDIUM APPLIED FOR INFECTIOUS BRONCHITIS VIRUS PROPAGATION

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SUMMARY

A chicken embryo kidney cell (CKC) was maintained in serum-free medium to be used for assays of infectious bronchitis virus (IBV) infection. The medium 199 was prepared by addition of insulin growth factor (IGF-1) at different concentrations and the cells were observed. The cells supported at 10 consecutives passages, and were still available to IBV infection. A cytopathic effect (CPE) was observed clearly in CKC cells infected with M_{41} strain of IBV at 72 h post-infection. By using the CPE induced by M_{41} infection, the serum neutralization (SN) test was applied in 109 chicken sera, previously titrated by conventional SN assay performed on CKC on traditional medium, and the correlation found was r= 0.97. The plaque assay performed produced visible plaques, and has showed to be suitable in conducting quantitative investigations. The described use of serum-free culture would be a new perspective for cell culture, where bovine serum is not necessary and this procedure respect the “Good Cell Culture Practice” Refine, Replace and Reduce.

INTRODUCTION

Infectious bronchitis virus (IBV) infects respiratory tract, kidneys and oviduct of chickens of all ages, causing retarded growth, mortality, reduced egg production and inferior egg shell quality (1). For the control of virus infection, broilers are usually vaccinated at one day of age with live attenuated vaccines (2). In addition, breeders and egg layers are also vaccinated at approximately 8-week intervals with live attenuated vaccines, and with inactivated vaccines after they start laying eggs (3).

Acute infections are generally diagnosed by the immunofluorescence test, ELISA capture assays, virus isolation or serological approach. However, IBV infections can also be diagnosed by detection of viral RNA, which turn the diagnostic fast and also reliable (4,5,6). In many cases virus isolation is attempted, usually performed in specific pathogen free embryonated eggs and/or primary cell culture derived from chicks. Most commonly, the serological profile of vaccinated and non-vaccinated flocks is the procedure usually applied in the field (7).
Nowadays, the production of IBV vaccines for veterinary use is based upon the use of infected chicken specific pathogen-free embryos by chorionalantoic route. Regulatory authorities in Europe (EMEA) and in the United States (Food and Drug Administration-FDA) have encourage biological manufactures to reduce or eliminate the use of animal origin in their manufacturing process (8,9,10,11). Moreover, serum-containing culture systems are becoming undesirable for the large-scale production of vaccines. There are a number of disadvantages of serum supplementation including batch-to-batch variation, in composition, the high protein content that hinders product purification and the potential for viral, mycoplasma, or prion contamination. Furthermore, the recent threat to human health caused by the undefined agents of bovine spongiform encephalopathy (BSE) is likely to limit the continued use of bovine serum in culture process used for the synthesis of health-care products such as viral vaccines (9).
Cells secrete proteins, including fibronectin (FN), laminin, vitronectin, and collagen, which form an extracellular matrix (ECM). The secreted ECM can vary in composition depending on a number of factors including cell type and extracellular signals controlling cell behavior. In vivo, the ECM can form a three-dimensional scaffold to which cells bind. In culture, ECM proteins can interact with the substrate surface. In addition, cells attach to the ECM through the binding of plasma membrane receptor proteins known as integrins to specific ECM proteins. Integrins act as contact points connecting cytoskeletal structures such as stress fibers inside cells to the surrounding ECM. The quality of cell-ECM contact depends on a number of factors such as stability, organization, and composition of ECM. Finally, in cell culture different surfaces can affect the properties of bound ECM and the respective cell behavior (12).
It has been demonstrated recently that CKC cells are the most sensible culture to IBV M_{41} infection. This fact was proven by showing the ability of CKC express the α2,3-linked sialic acid receptor on its the surface (13). In this paper, we established a serum free media M199 plus insulin growth factor (IGF-1), which is useful for assays of infectious bronchitis virus, as serum neutralization and plaque assay on CKC cultures.

MATERIAL and METHODS

Cells and virus

Chicken kidney cells (CKC) were chosen and prepared from 19-to 20-day-old embryos. The kidneys were collected, washed with PBS and digested with trypsin/EDTA. The reaction was stopped by add Medium 199 plus 5% bovine serum albumin (BSA). After centrifugation at 1000 *g* for 10 min, the kidney cells were resuspended in the same medium and filtrated through sterile gauze. The kidney cells in the filtrated were distributed on plastic tissue culture flasks and incubated at

37C with 5 % CO_2. The Massachusetts serotype strain M_{41} was used in this study gently supplied by Merial Laboratories.

Culture media
The CKC cells were cultured in Medium 199 plus different concentration of IGF-1- 10µg/ml, 5µg/ml, 2,5µg/ml and 1,75µg/ml (SIGMA cat # I 3769) supplemented with 2 mM glutamine, 1 X antibiotic/antimycotic and antimycotic solution (GIBCO-BRL) The cells were seeded in 75 cm^2 cultured flasks (TPP, Switzerland; cat # 9075) and incubated overnight at 37 °C in humidified 5% CO_2 atmosphere and cultured every 2 days as described before (14).

Cells and coverslips
Equal numbers of CKC cells were placed onto glass coversplips after grown on different IGF-1 concentration. For immunofluorescent localization of fibronectin, cells were washed twice with PBS, pH 7.4, fixed with freshly prepared 3.7% paraformaldehyde in PBS for 10 min, followed with a 3 min incubation in 0.1% Triton X-100 in PBS. Following 3 washes with PBS, the cells were blocked with 3% BSA in PBS for 30 min. After, the cells were incubated for 45 min with a monoclonal antibody anti-fibronectin (SIGMA- cat # F 7387). The coverslips were washed three times and incubated with the second antibody (goat anti-mouse antibody) for 45 min. The cells were washed and the coverslips mounted and observed by indirect Immunfluoresence.

Virus titration and neutralization test
Confluent CKC monolayers cultured in 25 cm^2 cell culture flasks were infected by M_{41} serotype of IBV at a cell concentration of 4 x 10^6 cells/ml with a multiplicity of infection (MOI) of 0.1, when 200µl of the virus inoculum was added to each flask. The flasks were incubated at 37 °C in 5% CO_2 atmosphere for 90 min to allow for virus adsorption. Each flask then received 7 ml of media. Incubation of 48-118 h at 37 °C in a CO_2 incubator resulted in virus-induced destruction of nearly 90% of the monolayer. Each virus-infected flask was frozen and thawed twice, and the contents centrifuged at 1500 *g* for 15 min to remove cell debris. The virus-containing supernatant was dispensed in cryogenic vials 0.5 ml (Corning, cat # 430487) volumes and stored at –80 °C until needed. For the virus titration, diluted virus in serum-free culture medium and a suspension of CKC cells (containing 5 x 10^4 cells) was added to each well at the same time. The plate was incubated for 7 days at 37° C in a 5% CO_2 incubator, and then the virus titer was measured by the appearance of cytopathic effect (CPE) described before. For the neutralization test, diluted sera (n = 109) and virus suspension were mixed and incubated for 1 h at 37° C. Then the mixture and the CKC cell suspension were inoculated into a 96-well microplate at the same time and the plate was incubated in a 5% CO_2 incubator for 7 days. The neutralizing antibody titer was measured by using the disappearance of CPE.

Plaque assay method
Plaque assays were undertaken using monolayers in 6-well culture plates (TPP, Switzerland; cat. # 9035) (15). Each well received 4 ml of the each cell suspension, 4 x 10^6 cells/ml in all growth medium and holding the plates 24 h at 37 °C in humidified CO_2 atmosphere. The growth medium was aspirated from each well, and 0.5 ml of

virus suspension was added to at least three wells. The dilution was performed using PBS as diluents. The plates were incubated for 90 min at 37 °C in humidified atmosphere. To redistribute the inoculum evenly over each monolayer, the plates were gently rocked immediately after virus inoculation and once more about half way through the incubation period. The overlay medium, which consisted of equal volumes of 2 x media and 2 x of 4% sephadex G-100 concentration, was prepared as described before (16) during the virus adsorption period. The 2 x media were kept in a water bath at 43° C until needed.
The sephadex solution was prepared by dissolving the powder at 4% (w/v) in 100 ml deionized distilled water and was sterilized by autoclaving for 45 min at 121.1 °C. The solutions were also held in a water bath at 43 °C.
The final overlay medium was prepared by mixing equal volumes of the 2 x of media and the 2 x of sephadex solution at 4% concentration, and at the end of the virus incubation period, each monolayer received 4 ml for 6-well plates of this mixture. The plates were left at room temperature for 20 min, and then held at 37 °C for 72-96 h in a humidified 5% CO_2 atmosphere. The monolayers were the fixed for a minimum of 4 h by adding 2 ml per well of a 3.7 % solution of formaldehyde (MERK, Brazil; cat. # 4567) in normal saline. The formaldehyde was discarded safely; the respective sephadex overlays plugs were removed by holding the cell culture plate at a 45 angle under a gentle stream of cold running tap water. The running water aids in the gentle lifting of the plugs without damaging the monolayer. The cell culture plate is then taped gently upside down onto a paper towel to remove the plugs and drain as much water as possible. The fixed cell monolayers were stained with 0.1% (w/v) crystal violet solution for 20 min. Plaques were counted, characterized and the virus infectivity titre expressed as plaquing forming units (PFU) per ml (16, 17).

Statistical analysis
The statistical significance of the differences among groups was determined by the Mann-Whitney (two-tailed) test. *P* test values less than 5% were considered to be statistically significant. The results are reported as mean ± s.d. (standard deviation).

RESULTS AND DISCUSSION

A CKC cells growth in serum free medium, replaced by 10µg/ml of IGF-1, which is useful for the assay of IBV, was established. This newly established CKC cells shows a clear CPE after infection with IBV M_{41} strain (Figure 1A, B, C and D). Virus titration and neutralization methods were also established by using the CPE as an infectious marker. The fibronectin expression was also observed by the use of different IGF-1 concentration (Figure 2A, B, C and D). Furthermore, these methods with CER show a significant correlation (r = 0.97) with the usual methods. Moreover, the sera samples (n = 109) were previously titrated by serum neutralization gold method, performed in CKC supplemented with 10% FBS.
In the case of serum-free culture, IBV grow more slowly, more cultivation time is needed for complete growth and cytopathogenicity at the virus end point. Viral antigen can be detected by immunofluorescence staining method as small focuses at the virus end point after 4 days cultivation, but not enough for direct CPE method (data not shown). CKC cell density played an important role in producing a unique monolayer. For this reason, these cells grew in serum free media, demonstrated to

be hardy and have a higher proliferation, able to produce clear zones of distinguishable plaques (Figure 3).
In spite of the necessity of more cultivation time, these serum-free culture systems have great benefits. The serum-free assay methods are simple, reliable and stable. It is the greatest advantage to prevent the contamination of the exogenous virus and BSE from foetal calf serum. In the near future, the establishment of serum-free culture cell lines will be necessary for not only basic studies on this virus, but also for bovine vaccine production and epidemiological surveillance.

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REFERENCES

1.Fabricant J (1998). The early history of Infectious Bronchitis. *Avian Diseases*, 42: 648-650.
2.Cavanagh D and Naqi, SA (1997). Infectious Bronchitis. In B.W.Calnek, H.J. Barnes, C W. Beard, L.R. McDougald & Y.M.Saif (eds) *Diseases of Poultry*, 10th edn (pp 511-526). Ames, IA:Iowa State University Press.
3.Cook JKA, Orbell SJ, Woods MA & Huggins MB (1999). Breadth of protection of respiratory tract provided by different live-attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. *Avian Pathology*, 28: 477-485.
4.Capua I, Minta Z, Karpinska E, Mawditt K, Britton P, Cavanagh D, Gough RE (1999). Co-circulation of four types of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). *Avian Pathology,* 28: 587-593.
5.Cavanagh, D (2001). Innovation and discovery: the application of nucleic-based technology to avian virus detection and characterization. *Avian Pathology,* 30: 581-598.
6.Cavanagh D, Mawditt K, Welchman DB, Britton P, Gough RE (2002). Coronavirus from pheasants (*Phasianus colcicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. *Avian Pathology,* 31: 81-93.
7.Witt JJ, Mekkes DR., Kouwenhoven B & Verheijden JHM (1997). Sensitivity and specificity of serological tests for infectious bronchitis virus antibodies in broilers. *Avian Pathology,* 26: 105-118.
8.Sakoda Y, Hikawa M, Tamura T, Fukusho A (1998). Establishment of a serum-free culture cell line, CPK-NS, which is useful for assays of classical swine fever virus. *Journal Virological Methods,* 75: 59-68.
9.Butler M, Burgener A, Patrick M, Berry M, Moffatt D, Huzel N, Barnabé N, Coombs K (2000). Application of a serum-free medium for the growth of vero cells and the production of Reovirus. *Biotechnology Progress,* 16: 854-858.
10.Frazzati-Gallina NM, Paoli RL, Mourão-Fuches RM, Soraia ACJ, Pereira CA (2001). Higher production of rabies virus in serum-free medium cell cultures on microcarriers. *Journal Biotechnology* 92: 67-72.

11.Kallel H, Jouini A, Majoul S, Rourou S (2002). Evaluation of various serum and animal protein free media for the production of a veterinary rabies vaccine in BHK-21 cells. *Journal Biotechnology,* 95: 195-204.
12.Olenych S.G., Moussallem, M.M., Salloum D.S., Schlenoff, J.B., Keller T.C.S. Fibronectin and cell attachment to cell and protein resistant polyelectrolyte surfaces. *Biomacromolecules* 6, 3252-3258, 2005.
13. Winter C., Schwegman-Weβels C., Cavanagh D., Neuman U., Herrler G. Sialic acid is a receptor determinant for infection of cells by avian *Infectious bronchitis virus*. *Journal of General Virology* 87, 1209-1216, 2006.
14. Casais R., Dove B., Cavanagh D., Britton P. Recombinant avian infectious bronchitis vírus expressing a heterologous spike gene demonstrates that spike protein is a determinant of cell tropism. Jounal Virology, 77 9084-9089 2003.
15.Cardoso TC and Pilz D (2004). Wild rabies virus detection by plaque assay from naturally infected brains in different species. *Veterinary Microbiology,* 103: 161-167.
16.Bussereau F, Flamand A, Pese-Part D.(1982). Reproducible plaquing system for rabies in CER cells. *Journal Virological Methods,* 4: 277-282.
17.Reed LJ and Muench H. (1938). A simple method of estimating fifty per cent end points. *American Journal Hygiene,* 27: 493-497.

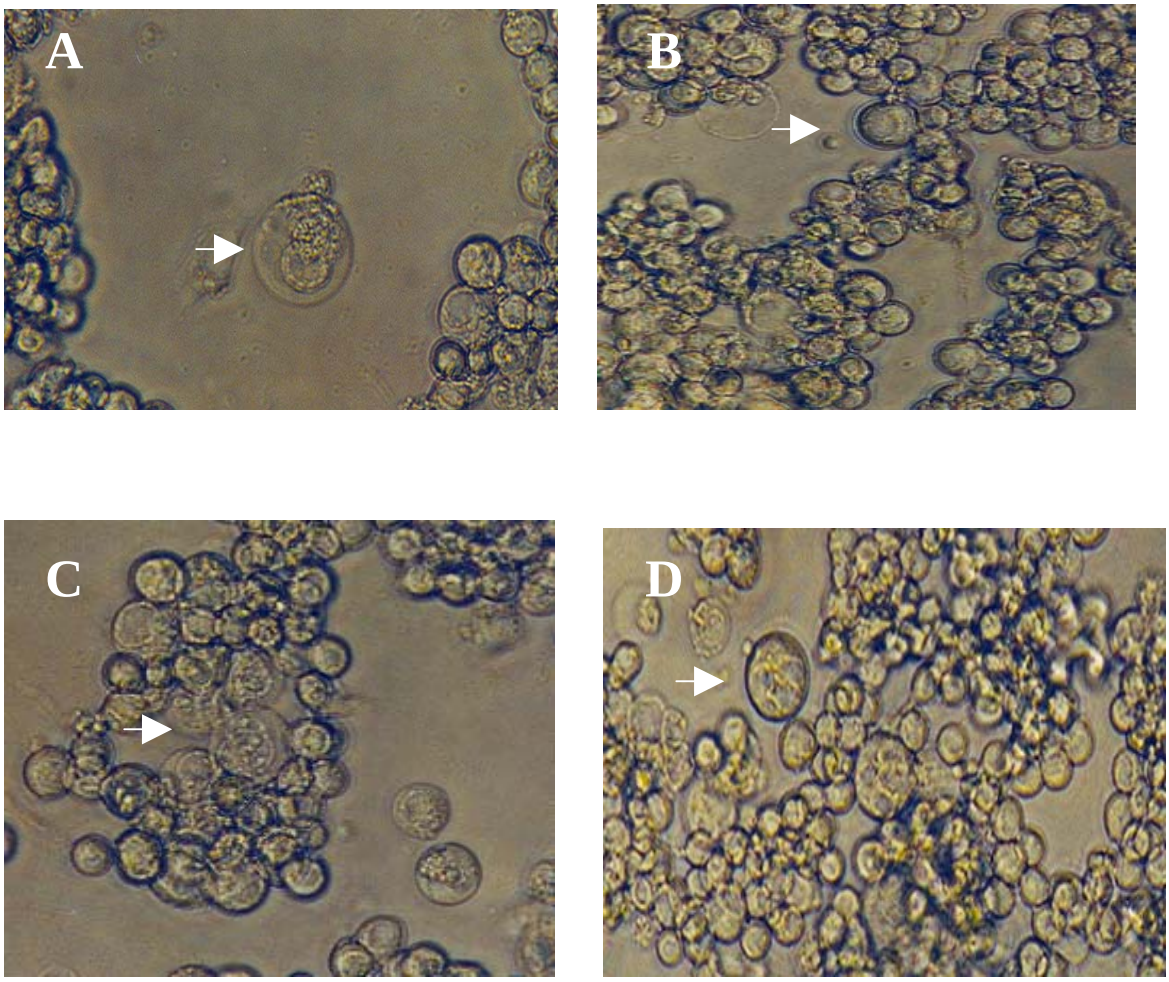

Figure 1- Cytophatic effect observed by M_{41} infection on CKC monolayers grew in serum free medium added by: 1,75µg/ml (A), 2,5µg/ml (B), 5µg/ml (C) and 10µg/ml (D).

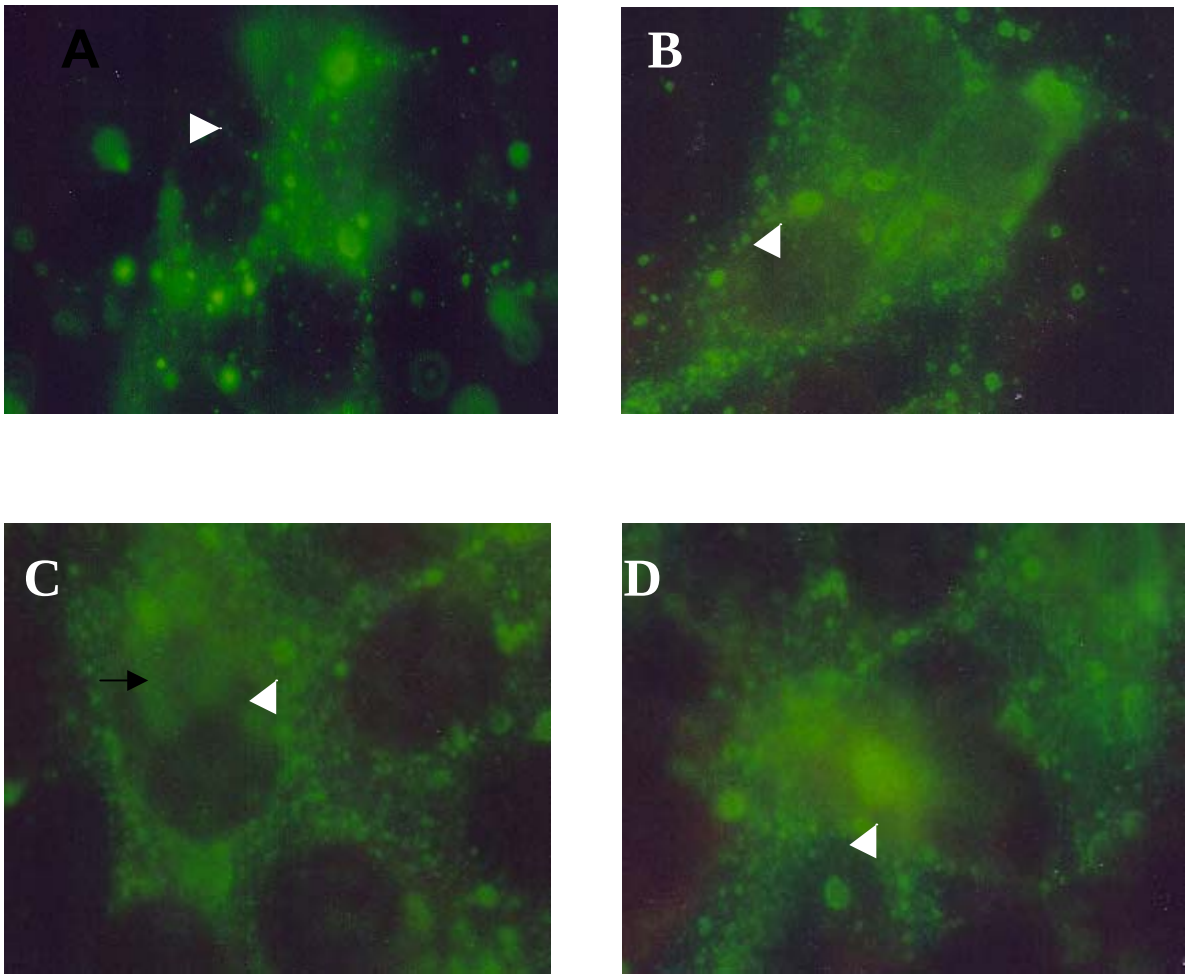

Figure 2- Fibronectin expression observed on CKC monolayers grew in serum free medium added by: 1,75µg/ml (A), 2,5µg/ml (B), 5µg/ml (C) and 10µg/ml (D).

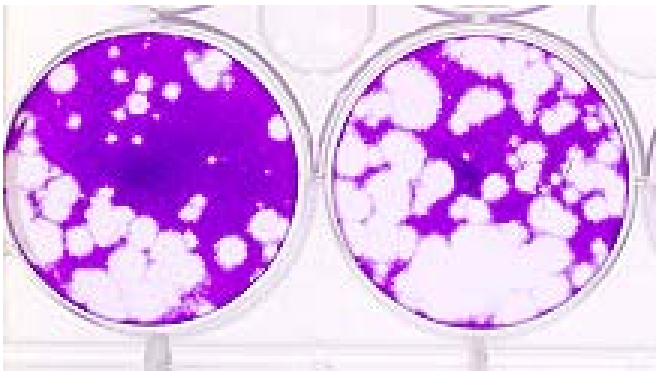

Figure 3- Plaquing of M_{41} in CKC cells grew in serum free medium.

VARIATION IN EFFECTS OF INFECTIOUS BRONCHITIS VIRUS ON THE REPRODUCTIVE PERFORMANCE OF INDIVIDUAL LAYING HENS: AN EXPERIMENTAL STUDY

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SUMMARY

This paper describes experimental infections to determine the effects of infectious bronchitis Massachusetts strain M41 on female chickens at 34 weeks of age. Following the intratracheal inoculation of 34-week old SPF hens in full lay, virus was detected in the oviduct between days 3-6 and immunofluorescence staining showed virus only in oviduct epithelial cells on day 6. Histological examination of affected oviducts revealed deciliation of the epithelium soon after infection, infiltration with lymphocytes and plasma cells and some replacement of tubular glands with fibrous tissue. Effects on egg production and quality were widely variable between individuals. Some birds laid continuously throughout, while others paused for varying lengths of time varying between less than 10 days to, in one instance, 74 days before recommencing to lay. Internal and shell quality were also variable after the return to lay, although shell abnormalities tended to be consistent for any one bird.

INTRODUCTION

Much has been written on the effects of infectious bronchitis virus (IBV) in the respiratory tract of the chicken and also its effects on the kidney. However, relatively little has been published on the effects of the virus on the female reproductive tract, despite the fact that IB is a major cause of loss of egg production and egg quality. Two occasions are critical in the life of the female: the first is soon after hatch, when infection in chicks without maternal antibodies to some infecting viruses can lead to oviduct abnormalities at maturity, and the second and best known, at sexual maturity, with loss of production and egg quality.

Sevoian and Levine (1957) in a comprehensive study described the fall in production with long-term consequences and the histological changes in the oviduct following IBV infection. However they demonstrated that water and feed deprivation could also cause a similar loss of egg production, but unlike after IBV infection, eggs were again

laid in expected numbers when they were restored. Other publications have shown the effects on the whole flock.
This paper describes an experimental infection in 34 week old SPF hens which was designed to determine the fate of individual hens after infection.

MATERIALS AND METHODS

Chickens
SPF light hybrid hens of 34 weeks of age, were housed in single cages for egg monitoring.

Virus: Massachusetts M41 was grown in fertile eggs with a titre of $\log_{10}$ 6.3 EID_{50}/ml.

Experimental design
A total of 36 hens were inoculated with 0.5ml of allantoic fluid intratracheally. The course of the respiratory infection was followed. Egg production was monitored daily and eggs were collected for examination of shell abnormalities and weighed. They were broken out and the albumen height measured. The internal quality was measured using a Haugh Unit calculator.
At intervals after infection, two birds were killed each time. Respiratory and reproductive tract samples were taken for virus isolation and immunofluorescence and histopathological staining of oviduct regions (ovary, infundibulum, magnum, isthmus and shell gland.

Virus in the tissues
Isolation was done by passage of tissue material in up to three 9 day old fertile eggs and immunofluorescence staining was done on snap-frozen tissues cut on a cryostat and stained using a hyperimmune anti-IBV chicken serum and FITC-labelled anti chicken globulin.

Histopathology
After fixation, tissues samples were embedded in paraffin wax, and sections were stained with haematoxylin and eosin or by Weigert and van Giessen stain for fibrous tissue.

Egg collection
Eggs from individual hens were collected daily and identified with the bird number. The performance of each hen was recorded. Shell abnormalities were noted and eggs were weighed individually. Each egg was broken out onto a glass plate and the height of the albumin surrounding the yolk was measured in mm with a micrometer. Using an egg quality calculator, the internal egg quality was determined from the weight and albumin height.

RESULTS

Virus in the tissues
Distribution of virus in the tissues is shown in Table 1. Titratable levels of virus were detected in trachea, lung and kidney up to day three postinfection (pi) and virus

persisted until day nine in trachea and kidney. Virus was present in the blood on all occasions up to day nine.
Virus was also detected by isolation form all levels of the reproductive tract on each occasion but titratable amounts were found in the uterus on days one and three and in all regions except ovary on day six. A very high titre (>5.0 logs) was found in the uterus at this time. Immunostaining showed virus in the surface epithelium at al levels of the oviduct on day six only (Figure 1).

Gross changes in the oviduct
Within a few days of ceasing production, at necropsy, the oviducts of infected hens regressed. Depending on the period after the onset of the laying pause, the ovary could still appear functional, with ova being shed into the body cavity (days after), or regressed also (weeks after). In some instances, the ovary was clearly becoming active again, before the oviduct.

Oviduct histopathology
Changes were very similar in different regions of the major secretory regions of the oviduct (magnum, isthmus and uterus) under particular study. From day 6 pi, there was epithelial damage and loss of ciliated cells (Figure 2B – compare control Figure 2A). Changes occurred in the glandular tissue, where the secretions appeared trapped in dilated glands. Between the tubular glands was an infiltration of lymphocytes and plasma cells. Epithelial damage persisted and by day 9 pi, lymphoid foci were seen deep in the tissue (Figure 2C). By day 21, the epithelium had still not recovered (Figure 2D). At this time, fibrous tissue was demonstrated by Weigert and van Giessen stain, apparently replacing some of the tubular glands (Figure 3).

Egg production for the flock
Figure 4 shows the effect on egg production of infection with IBV at 34 weeks of age. There was an immediate fall in production from around 73% to 11% in two weeks. Then a gradual climb was seen to a peak at 43 weeks but the production never reached the expected level as shown by the controls. The return to laying was accompanied by an increase in the numbers of abnormally shelled eggs.

Variation in production of individuals
Four birds laid continuously through the period of observation (100 days) while others experienced pauses in production which were widely variable. Figure 5 shows the wide variation in lengths of pause for 12 birds. Many of the others were killed for pathogenesis studies before they returned to lie. The length of pause varied from 13 days up to 75 days.

Egg quality
This was very variable between birds. For the shells, abnormalities ranged from misshape, thin shells, stress lines or chalk-like deposits. For any individual bird, the abnormalities were consistent for consecutive eggs laid, suggesting permanent damage to the shell gland. Internal quality was seen in the form of thin albumin ('watery whites') and this was also variable between birds.
Individual laying performances

The wide variation in effects of IBV infection on laying performance is given by some examples of individuals. Figure 6 shows the performance of one of the hens that laid consistently throughout. All the eggs laid during the period of observation were of good internal quality (Haugh Unit value of more than 60) and all shells were normal. The bird whose data are shown in Figure 7 went out of production after day 3 and paused for 18 days before returning to lay consistently apart form a second short later pause and producing eggs of normal quality.
Figure 8 shows the performance of an individual which stopped laying after three days then did not recommence until 77 days post infection, laying poor quality eggs. Figure 9 shows another which after a pause of about three weeks, resumed laying but produced eggs of poor internal quality with abnormal shells.

DISCUSSION

The results of this study show that following infection of SPF hens with virulent IBV via the respiratory tract, there is an initial period of viral replication in the respiratory tissues and kidneys, followed by a secondary burst of replication in the reproductive tract and in particular, all levels of the oviduct. Confirmation of the site of replication was provided by immunofluorescence staining which revealed that it is the ciliated epithelial cells on the surface of the oviduct only that are affected by virus. This replication results in high titres of infectious virus especially at about six days after infection. Since virus was detected consistently in the blood, albeit at low levels, it appears that the haematogenous route is the main means of spread of virus between respiratory tract and oviduct.
Although virus replicates in the oviduct epithelial surface only, it appears that the infection sets off a sequence of events which result in the closing down of the functioning of the oviduct and later the ovary, with ultimately, a reduction in size of both. These changes are not understood at all. At the microscopic level also, several other associated changed occur in the tissues which influence egg production and quality. One of the most important is the loss of surface epithelial cells which renders the surface susceptible to other infections. The normal epithelium contains glands which secrete ovomucin which is antibacterial. The loss of these glands thus allows bacteria, especially form the cloaca, to move antieriorly up the oviduct and this will be further encouraged since the mucociliary blanket in the oviduct is destroyed.
This could lead to colonisation by intestinal bacteria of the egg material not accepted by the oviduct but released into the abdominal cavity when it closes down, resulting in egg peritonitis.
There is a cellular immune response which has been shown previously (Sevoian and Levine, 1957), both diffuse and later, focal. We have shown that IBV infection of the oviduct is associated with locally produced IgG and IgA (Dhinakar Raj and Jones, 1966). These presumably assist in the clearance of virus form the oviduct, although in this experiment, virus detection was studied up to day nine only, when it was still present in untitratable amounts. In the longer term, the apparent replacement of tubular gland tissue by fibrous tissue as shown by Van Giesen's stain (Pierson et al., 1957), with associated malfunctioning of secretory processes of some regions of the oviduct, could explain the internal and external egg abnormalities.

Perhaps the most striking finding from this investigation was the wide range of effects on the laying performance of individual hens, despite all having received the same dose of virus via the respiratory tract. Since the effects on egg production were being studied in the same experiment as the
pathogenesis, it was not possible to follow the production performance of all hens in the experiment. However, there was sufficient new detail to illustrate wide differences, ranging from a small number of hens which laid good quality eggs uninterruptedly throughout, while at the other extreme, a hen did not lay for 74 days but then resumed to produce eggs with abnormal shells and thin albumin but at a normal rate. In between was a range of pauses, with the return to lay often (but not always) characterised by poor quality eggs.
The reasons for this wide variation in effects are not known. More detailed study is needed to provide explanations. The reasons could relate to genetics, to different immune responses (innate or adaptive) in the oviduct or simply to the amount of virus reaching the reproductive tract from the primary respiratory site of replication. What ever, the reason(s), it is likely that this variation occurs in commercial flocks, so that the familiar fall in production for the whole flock due to IB actually conceals many different individual effects on laying performance.

REFERENCES

Dhinakar Raj, G. and Jones, R. C. (1996) Local antibody production in the oviduct and gut of hens infected with a variant strain of infectious bronchitis virus. Veterinary Immunology and Immunopathology 53, 147-161.

Pierson J. Van Alten, Richard A. Fennell (1957) Histogenesis and histochemistry of the digestive tract of chick embryos The Anatomical Record, 127, 677-695.

Sevoian, M. and Levine, P. P. (1957) Effects of infectious bronchitis on the reproductive tracts, egg production and egg quality of laying chickens. (1957) Avian Diseases, 1, 136-164.

Table 1. Distribution of IBV in the tissues of hens infected with IBV.

Days p.i.	1	3	6	9
Trachea	3.2[a]	3.2	+[b]	+
Lung	2.5	3.3	+	-[c]
Kidney	1.3	3.1	+	+
Blood	+	+	+	+
Ovary	+	+	+	+
Infundibulum	+	+	1.8 F[d]	+
Magnum	+	+	3.0 F	+
Isthmus	+	+	3.5 F	+
Uterus	3.1	3.6	>5.0 F	+

Results are mean titres or consensus +/- values
from two birds killed each time

[a]: Virus titre (log_{10} EID_{50}/ml; [b]: virus positive but untitratable
[c]: no virus isolated: [d]: Immunofluorescence staining of tissues

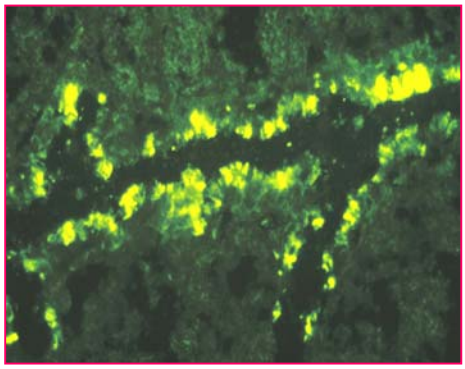

Figure 1. IBV in the epithelial cells of the magnum region of the oviduct, 6 days pi. Immunofluorescence stain.

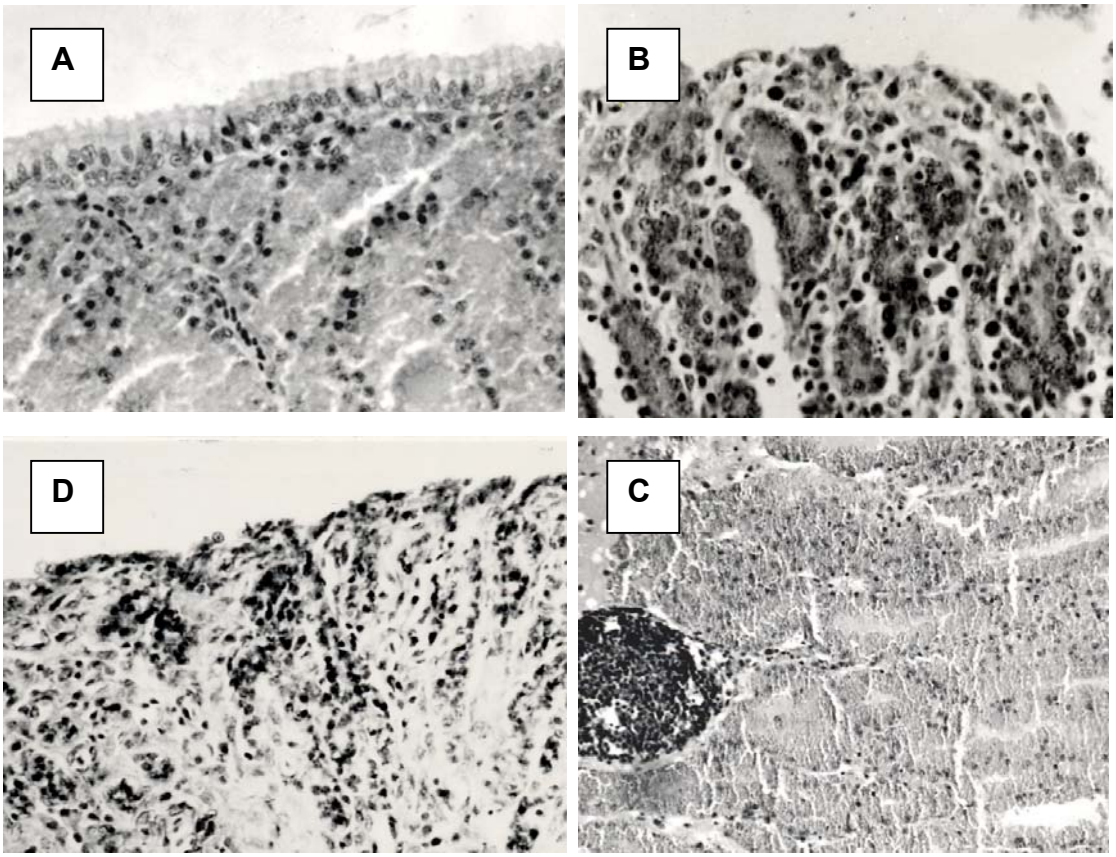

Figure 2. Histopathological changes in the oviduct after IBV infection.
A: Normal tissue. B: 6 days pi, loss of epithelial cells, dilation of tubular glands and diffuse cellular infiltration. C: 9 days pi: a lymphoid focus deep in the tissues. D: 21 days pi. Epithelium still damaged and fibrous tissue in the deeper layers.

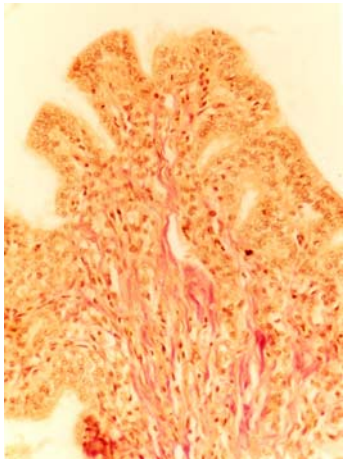

Figure 3. Replacement of tubular glands with fibrous tissue. 21 days pi.

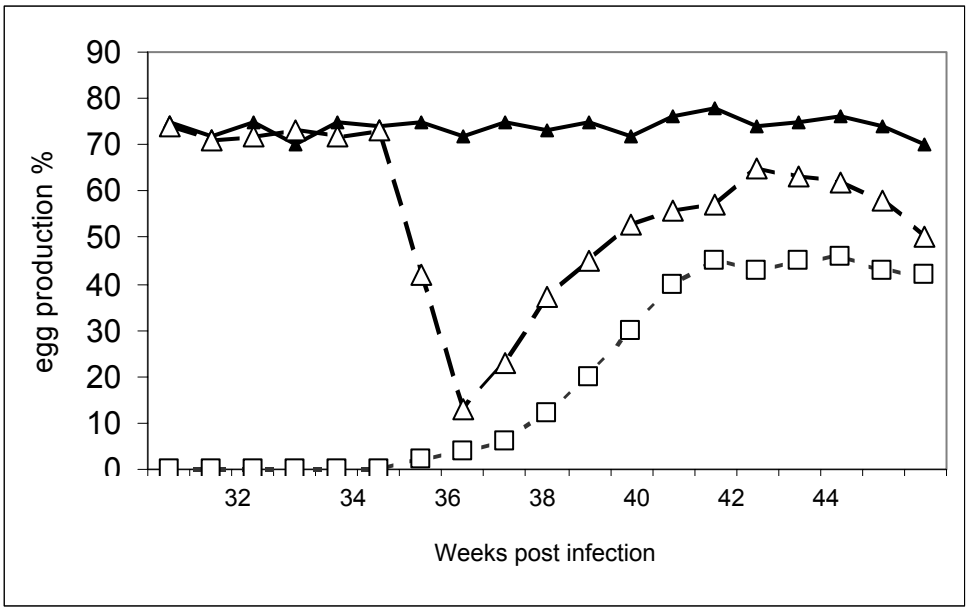

Figure 4. Graph of total egg production for the flock infected with IBV at 34 weeks.

▲ Controls; △ IBV infected birds; □ abnormal eggs laid

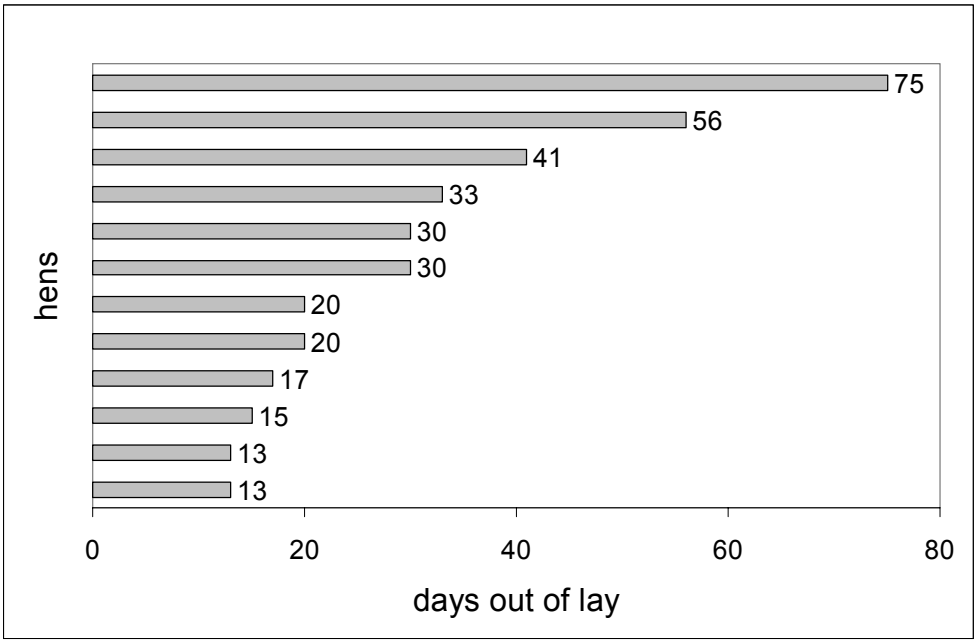

Figure 5. Variation in length of pause in laying of 12 hens after infection with IBV.

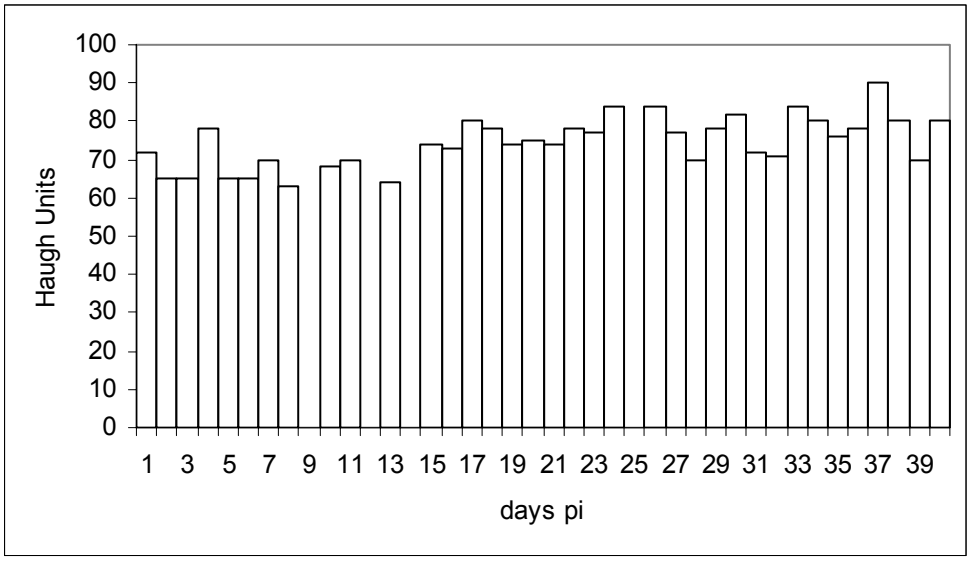

Figure 6. Laying performance of a hen which laid normal eggs continuously throughout, despite being infected with IBV.
Each column represents an egg and its height indicates its Haugh Unit value. 60 or above is normal.

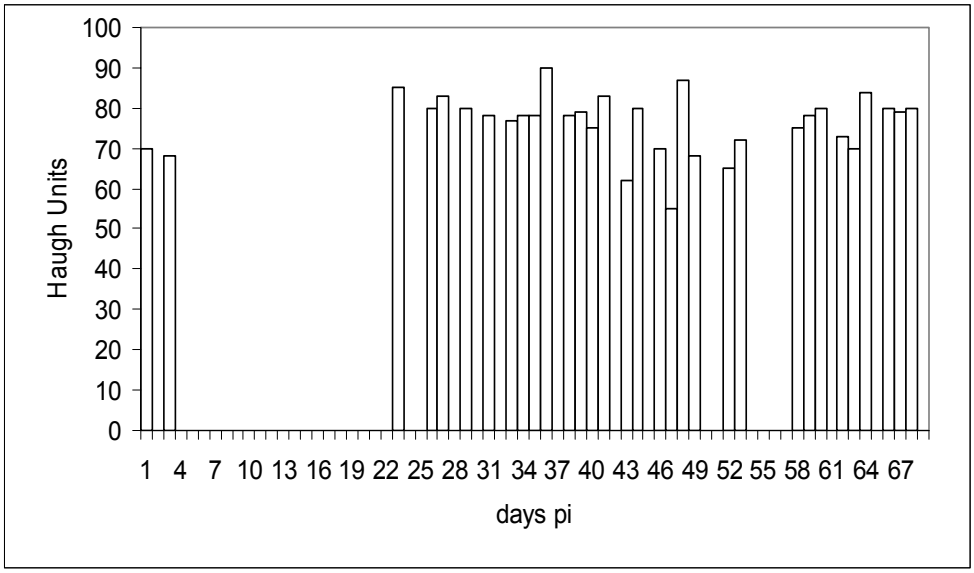

Figure 7. This bird went out of lay on day 3 and returned to lay normal eggs after a pause of 18 days.

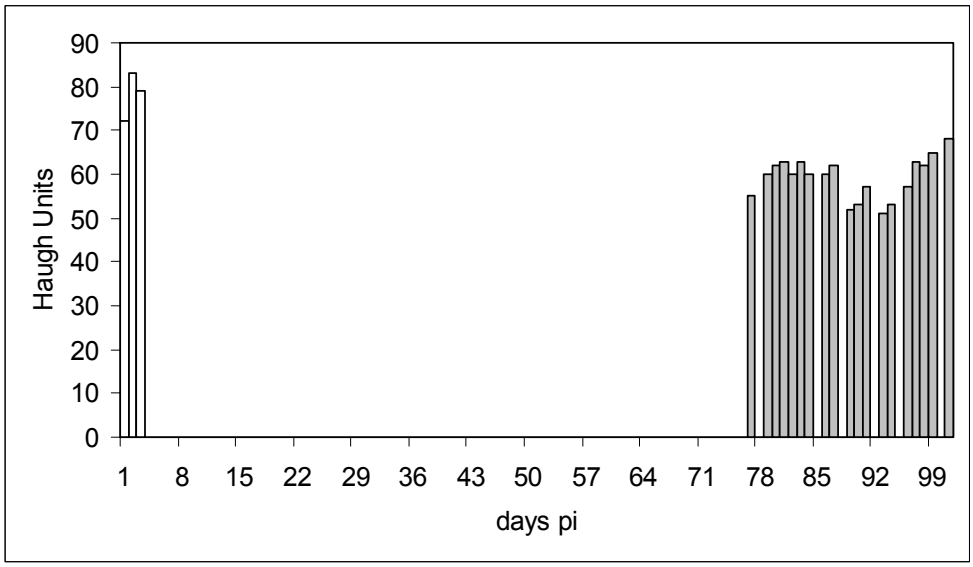

Figure 8. This hen stopped laying after 3 days and did not recommence until day 77 pi. Eggs laid after this were abnormal in shape (grey columns) and had generally poor internal quality.

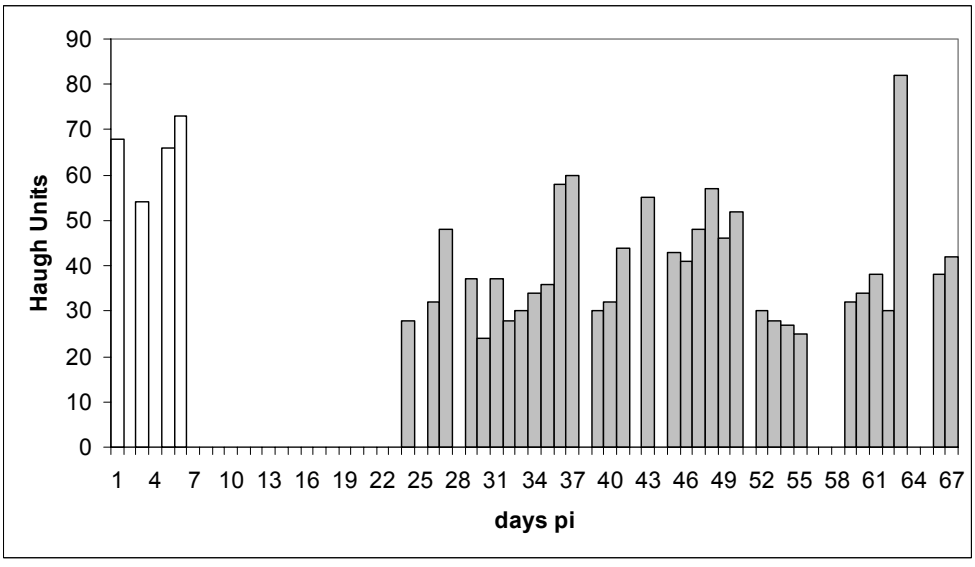

Figure 9. This hen paused for 3 weeks and then laid eggs with abnormal shells and very variably internal quality.

AVIAN INFECTIOUS BRONCHITIS VIRUS AND AVIAN METAPNEUMOVIRUS ASSOCIATED WITH ORCHITIS IN ROOSTERS WITH DECREASED FERTILITY

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SUMMARY

This research aimed to evaluate the presence of *IBV* and *APV* in testis of roosters from a Brazilian poultry breeders flock with epididymal stones and low fertility. Samples of testis, trachea and lungs of breeders aged 57 weeks were positive to *IBV* by a PCR targeted to the 3'UTR and testis samples were also positive to *APV* by a PCR targeted to the G gene of this virus. The inoculation of testis samples in chicken embryonated eggs via the allantoic cavity route resulted in curled, hemorrhagic and stunted embryos. The allantoic fluid was submitted to a PCR aimed to amplify 706bp of the region coding for the S1 subunit of IBV S gene and sequence analysis of the amplified fragment reveled a close relationship with D274 strain. Histopathological examination of the testis showed infiltration of plasma cells and lymphocytes and epididymal stones. These results indicate that *IBV* and *APV* may have played a role on the pathogenesis of the testicular disease described herein and gives some light to measures aimed to reduce the impact of male reproductive diseases in poultry industry.

INTRODUCTION

Infectious bronchitis virus (IBV) is an epitheliotropic virus that causes acute and severe diseases of the respiratory, renal, intestinal and female reproductive tracts of chickens and broilers (Uenaka & Kishimoto, 1998; Cook et al., 2001; Cavanagh & Naqi, 2003;).

IBV-caused diseases occur with high morbidity and, in some cases – not few- mortality rates that causes large economical losses to the poultry industry. Infectious bronchitis virus replicates mainly in the epithelium of the oviduct of the female chicken resulting in decline in the quality and production of eggs (Crinion et al., 1971; Dhinakar & Jones, 1997).

The genus *Metapneumovirus*, in which one can find the species *Avian metapneumovirus* (*aMPV*) belongs to the subfamily *Pneumovirinae* within the

Paramixoviridae (Pringle, 1998). Based on neutralization patterns and molecular analysis, metapneumoviruses are subdivided in four different groups (A, B, C and D) (Bäyon-Auboyer et al., 2000; Hess et al., 2004).
aMPV infections are also associated with swollen head syndrome in chickens (Jones et al, 1991). Furthermore, reduced laying performance is reported in broiler breeders in association with swollen head syndrome (Perelman et al., 1988).
In previous studies, it was determined that several factors could alter the production of fertile sperm, including the presence of stones in the epididymal region, more specifically in the efferent ductules (Janssen, 2000). Others have shown that roosters with stones could have lower testis weight and decreased daily sperm production (Boltz et al., 2004).
The present article reports the association of IBV and APV as the possible cause of epididymal stones and decreased fertility in roosters in a Brazilian poultry breeder flock.

MATERIALS AND METHODS

Case history and samples

Samples of testis, trachea, kidneys and lungs of 6 roosters from a poultry breeding flock with respiratory disease, facial edema, nephritis and decreased fertility aged 57 weeks originated from Sao Paulo State, Southeastern Brazil, were collected in May 2005 and sent to the Laboratory of Avian Pathology of the Sao Paulo University in order to determine the cause of the respiratory and reproductive problems related with low fertility, low hatchability and epididymal stones found at the necroscopic procedure. Each pool of 12 testis, 12 kidneys, 12 lungs and 6 tracheas was treated as one individual sample.
Samples were homogenized (20% w/v) in PBS 0.01 M, pH 7.4 (PBS) and clarified at 1,500 x g for 15 minutes, the supernatant collected to analysis.
IBV screening by a universal polymerase chain reaction
Primers UTR 41+, UTR 31- and UTR 11- and reaction conditions described by Cavanagh et al. (2002), were used with some modifications to amplify a 179-bp fragment of the 3' untranslated region (3' UTR) of avian coronaviruses.

Avian metapneumovirus detection

All samples were screened for aMPV with primers G6- and G1+ and reaction conditions described by Cavanagh et al. (1999), with some modifications, to amplify 268-bp or 361-bp fragments, corresponding to the subtypes A and B, respectively, of the G gene of the virus.

Virus isolation and propagation

The pool of testis was used for virus isolation in embryonated chicken egg. A suspension of sample was filtered with 0.45 μm and 0.22 μm membranes, added 10 mg/μL of Gentamicin and inoculated by the allantoic cavity route of 9- to-11-day-old SPF embryonated chicken eggs obtained from Biovet™ Laboratory (Vargem Grande Paulista – Brazil). The inoculated eggs were incubated at 37ºC and candled daily to check for embryo viability. Allantoic fluids were harvested 48-72 hours post inoculation and four blind serial passages were performed in a similar way and screened for IBV and aMPV by the PCR assays as described above.

PCR to the IBV S1 coding-region
The set of primers S1OLIGO 5'and CK2 (Kwon and Jackwood, 1995; Keeler et al, 1998; Kwon et al, 1993) and reaction conditions described by Ziegler et al., 2002., with some modifications, were used to amplify a product of approximately 706 bp of the region coding for the S1 subunit of the S gene of *IBV* from the testis pool passed in embryonated eggs, now on named strain IBV/Brazil/2005/USP-01.

DNA Sequencing and phylogenetic analysis
The PCR product corresponding to the S1 region from strain IBV/Brazil/2005/USP-01 was purified with the QIAquick Spin Miniprep kit (Qiagen™) and submitted to bi-direcional DNA sequencing with.DYEnamic ET Dye Terminator (Amersham Biosciences™) according to the manufacturer's instructions, the sequences resolved in a MegaBACE™ 1000 (Amersham Biosciences ™).
A Neighbor-joining distance tree with Kimura- 2- parameter model was built with Mega 2.1 (Kumar et al., 2001) with 1000 Bootstrap replicates with the sequence from strain IBV/Brazil/2005/USP-01 aligned by the Clustal/W method with Bioedit (Hall, 1999) with 62 IBV homologous sequences retrieved from GenBank (Accesion numbers are shown in figure 1).
The sequence obtained to strain IBV/Brazil/2005/USP-01 was deposited in GenBank under the accession number DQ355995.

Histopathological examination
Testis specimens (n=12) were individually fixed in 10% formalin, processed routinely, sectioned and stained with hematoxylin and eosin (H&E).

RESULTS

IBV screening
All the samples (separate pools of testis, kidneys, trachea and lungs) were positive to IBV after the PCR aimed to the amplification of a 179 bp of the 3'UTR region of the virus. No contamination was found, as negative controls produced no bands.

Avian metaneumovirus detection
The PCR aimed to amplify the G gene resulted positive only to the pool of testis, with a fragment of 268-bp, which corresponds to the serotype A of aMPV. No contamination was found, as negative controls produced no bands.

Virus isolation and propagation
Inoculated embryos presented lesions after four serial passages, the lesions consisting of curled, hemorrhagic and stunted embryos, usually in the third day post inoculation. IBV, but not aMPV isolation was confirmed by PCR as described.

PCR to the IBV S1 coding-region
The PCR targeted to the S1 coding-region showed a fragment of approximately 706 bp for strain IBV/Brazil/2005/USP-01. Negative controls produced no bands after electrophoresis.

Phylogenetic analysis
The partial S1 sequence of the IBV/Brazil/2005/USP-01 segregated in the same cluster containing IBV strain D274, Cal99 and Ark 99 and field strains detected in China, Korea and Spain, apart from the clusters formed by the vaccine strains H120, Ma5, M41 and Connecticut and distant from strains DE072 and 793B. (Figure 1)

Histopathological examination
The histopathological lesions founded in the testis samples consisted on infiltration of plasma cells, lymphocytes and cell debris surrounding the affected ductules (Figures A-C). The columnar epithelium of the efferent ductules was shortened. There were epididymal stones in the efferent ductules, whose epithelium was composed by a thin envelope of squamous cells, continuous to the efferent ductile epithelium.
Furthermore, the seminiferous ductules were atrophic and showed a low concentration of viable spermatozoids.

DISCUSSION

Evidences of Infectious Bronchitis Virus and Avian metapneumovirus have been found in testis of roosters with low fertility and respiratory problems in a breeder flock with poor reproductive performance, associated with epididymal microscopic lesions. The economic impact of epididymal lesions to the poultry industry is potentially quite severe, because its affects directly the fertility (Mahecha, 2002).
The *IBV* strain detected by PCR was isolated and grown in chicken embryonated eggs, inducing evident signs of avian infectious bronchitis in the embryos, what means that not only was the viral genome detected, but also that such strain was replicating in the testis cells, what resulted in the microscopic lesions found in these, such as destruction of the efferent ductules,, destruction of the epididymus, seminiferous tubules and the presence of stones in the epididymus, similar to a previous report by Boltz et al. (2004).
Data from previous studies support that the epididymal stones found in the testis samples analyzed herein are the ultimate cause of a fall in fertility in the birds studied. It's well established that one of the abnormalities that can lead to low fertility in roosters is the formation of calcium stones in efferent ducts (Janssen et al., 2000). Once stones are formed in the efferent ductules of the epididymal region, the production and viability of the sperm is seriously compromised, as the efferent ductules are responsible for reabsorption of fluid from the testis and any damage of this function leads to fluid accumulation, fluid back-pressure, testicular atrophy and infertility (Janssen, 2000; Mahecha, 2002).
Taking this into account, one can hypothesize that *IBV* replication in testis cells of the roosters sampled in the present study caused the severe microscopic lesions that, at chronicle stage, resulted in the formation of stones in the epididymus and caused the clinically observed fertility decrease.
Phylogenetic analysis revealed that IBV/Brazil/2005/USP-01 clustered closely to some *IBV* strains from Europe and Asia, but far apart from strain H-120, the only one that, in an attenuated form, is used in Brazil for anti-infectious bronquitis vaccination. Considering this clustering pattern, an H120-vaccine-derived pathogenesis of the lesions here described can be promptly discarded, but a larger number of sequences with similar history must be obtained in order to track back the origin of

IBV/Brazil/2005/USP-01 and its relationship with other *IBV* strains that prevail in Brazil.

In the case of the aMPV strain detected in the same testis samples, one needs to emphasize that viral detection was carried out with by a PCR that showed the presence of viral RNA rather than viral replication in the tissues analyzed, what means that the significance of the positive PCR result without viral isolation in terms of detecting an active infection can not be made with the results obtained herein, as proposed by Cook (2000). Nonetheless, due to the tropism of aMPV by cells of the reproductive tract of hens, it can be suggested that the effects of aMPV in the reproductive organs of the male must still be considered as possible.

Experimental studies aimed at the reproduction of the disease in roosters, using the IBV and aMPV are needed in order to establish a definitely association of these viruses with decreased fertility in roosters.

REFERENCES

Bäyon-Auboyer, M.H.; Arnauld, C.; Toquin, D. and Eteradossi, N. Nucleotide sequences of the F, L and G protein genes of two non-A / non-B avian pneumoviruses (APV) reveal a novel APV subgroup. Journal of General Virology, 81: 2723-2733. 2000.

Boltz, A.D.; Nakai, M.; Bahr, J.M. Avian infectious bronchitis virus: A possible cause of reduced fertility in the rooster. Avian Diseases, 48: 909-915. 2004.

Cavanagh, D.; Mawditt, K.; Britton, P.; Naylor, C. J. Longitudinal field studies of infectious bronchitis virus and avian pneumovirus in broilers using type-specific polymerase chain reactions. Avian Pathology. v. 28, p. 593-605. 1999.

Cavanagh, D., K. Mawditt, D. de B. Welchman, P. Britton and R.E. Gough. Coronaviruses from pheasants (*Phasianus colchicus*) are genetically closely related to coronaviruses of domestic fowl (infectious bronchitis virus) and turkeys. Avian pathol. 31:81-93. 2002.

Cavanagh, D. and Naqi.. Infectious Bronchitis, In: Y.M., Saif; Barnes, H.J.; Glisson, J.R.; Fadly, A.M.; McDougald, L.R. and Swayne, D.E. (ed). Diseases of Poultry, 11th ed. Iowa State University Press, Ames. p. 101-119, 2003

Cook, J.A.K. Avian pneumovirus infections in turkeys and chickens. Review. The Veterinary journal, 160: 118-125. 2000.

Cook, J.K.; Chesher, J.; Baxendale, W.; Greenwood, N.; Hugging, M.B. and Orbell, S.J. protection of chickens against renal damage caused by a nephropathogenic infectious bronchitis virus. Avian Pathology. 30: 423-426, 2001.

Crinion, R.A.P.; Ball, R.A. and Hofstad, M.S. Pathogenesis of oviduct lesions in immature chickens following exposure to infectious bronchitis virus at one day old. Avian Diseases, 15(1):32-41. 1971.

Dhinakar, G and Jones, R.C. Growth of infectious bronchitis virus vaccines in oviducts derived from oestrogen-treated chicks and embryos. Vaccine, 15(2), 163-168. 1997.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41: 95-98.

Hess, M.; Huggins, M.B.; Mudzamiri, R and Heinez, V. Avian pneumovirus excretion in vaccinated and non-vaccinated specific pathogen free laying chickens. Avian Pathology, 33(1): 35-40. 2004.

Janssen, S.J.; Kirby, J.D.; Hess, R.A.; Rhoads, M.; Bunick, D.; Bailey, K.L.; parsons, C.M.; Wang, H.; Bahr, J.M. Reduced fertility in roosters with epididymal calcium stones. Poultry Science, 79: 568-574. 2000.

Jones, R.C.; Naylor, C.J.; Bradbury, J.M.; Savage, C.E.; Worthington, K and Willians, R.A. Isolation of turkey rhinotracheitis-like virus from broiler breeders in England. Veterinary Record, 129: 509-510. 1991.

Keeler, C.L. Jr.; Reed, K.L.; Nix, W.A. and Gelb, J. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S-1) gene. Avian Diseases, 42: 275-284. 1988.

Kwon, H.M.; Jackwood, M.W. and Gelb, J. Jr. Differentiation of infectious bronchitis virus serotypes using the polymerase chain reaction and restriction fragments length polymorphism analysis. Avian Diseases, 37: 194-202. 1993.

Kwon, H.M. and Jackwood, M.W. Molecular cloning and sequence comparison of the S1 glycoprotein of the Gray and JMK strains of avian infectious bronchitis virus. Virus Genes, 9: 219-229. 1995.

Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software.Bioinformatics, 17(12):1244-5.2001

Mahecha, G.A.B.; Oliveira, C.A.; Balzuweit, K. and Hess, R.A. Epididymal lithiasis in roosters and efferent ductule and testicular damage. Reproduction, 124: 821-834. 2002.

Pringle, C.R. Virus taxonomy. Archives of virology, 143: 1449-1459. 1998

Uenaka, T. and I. Kishimoto. Intracloacal infection with avian infectious bronchitis virus. Avian Pathology, 27: 309-312. 1998.

Wilding, G.P.; Baxter-Jones, C. and Grant, M. Ciliostatic agent found in Rhinotracheitis. Virus record, 118: 735. 1986.

Ziegler, A.F.; Ladman, B.S.; Dunn, P.A.; Schneider, A.; Davison, S.; Miller, P.G.; Lu, H.; Weinstock, D.; Salem, M.; Eckroade, R.J.; Gelb, J.Jr. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Diseases, 46: 847-858. 2002.

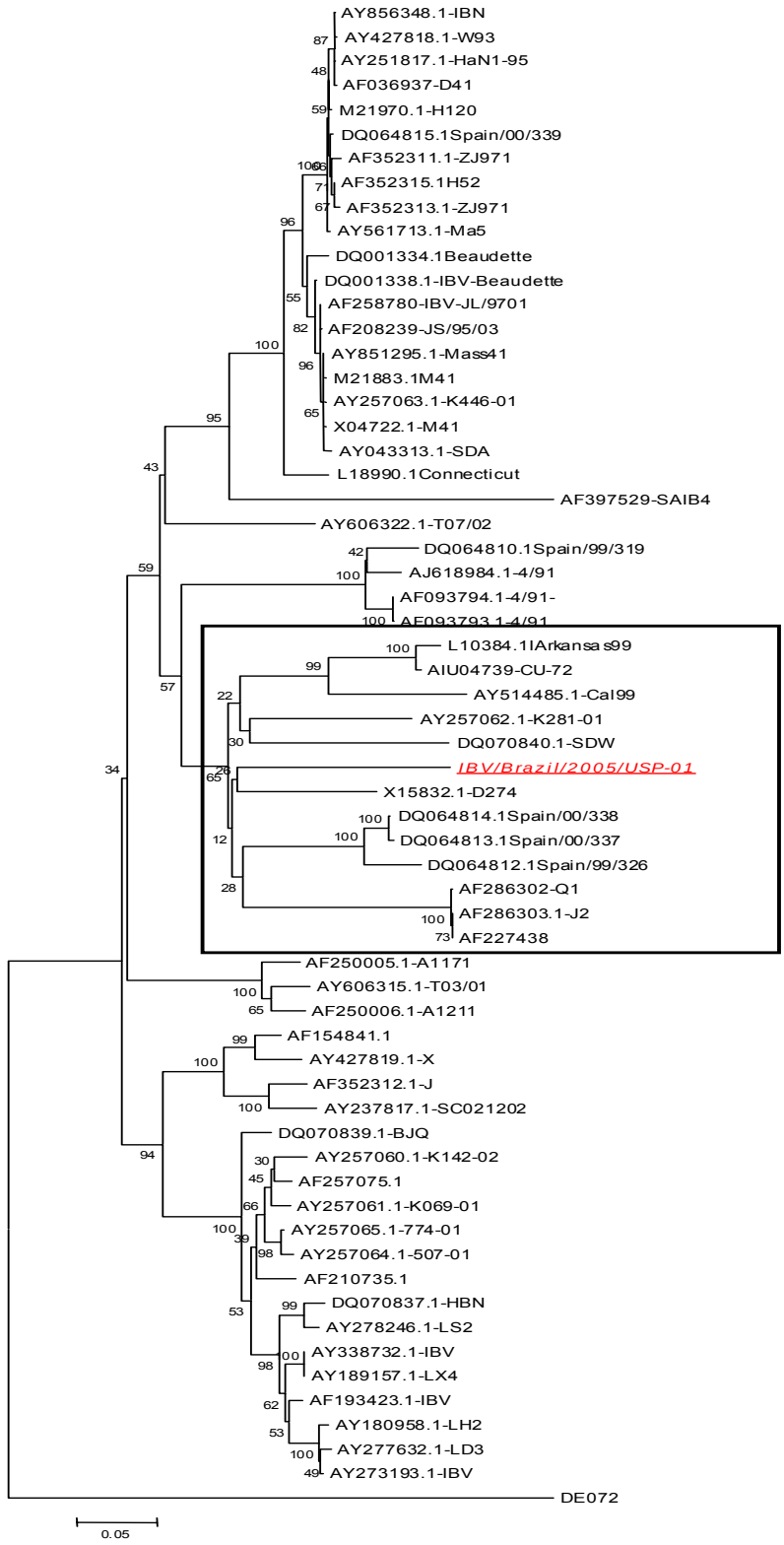

Figure 1 - Phylogenetic tree based on S1 coding region of *IBV* strains from different countries and *IBV* reference strains showing strain IBV/Brazil/2005/USP-01 (bold, underlined). Numbers at each node are bootstrap values; the bar represents the number of nucleotide substitutions per site.

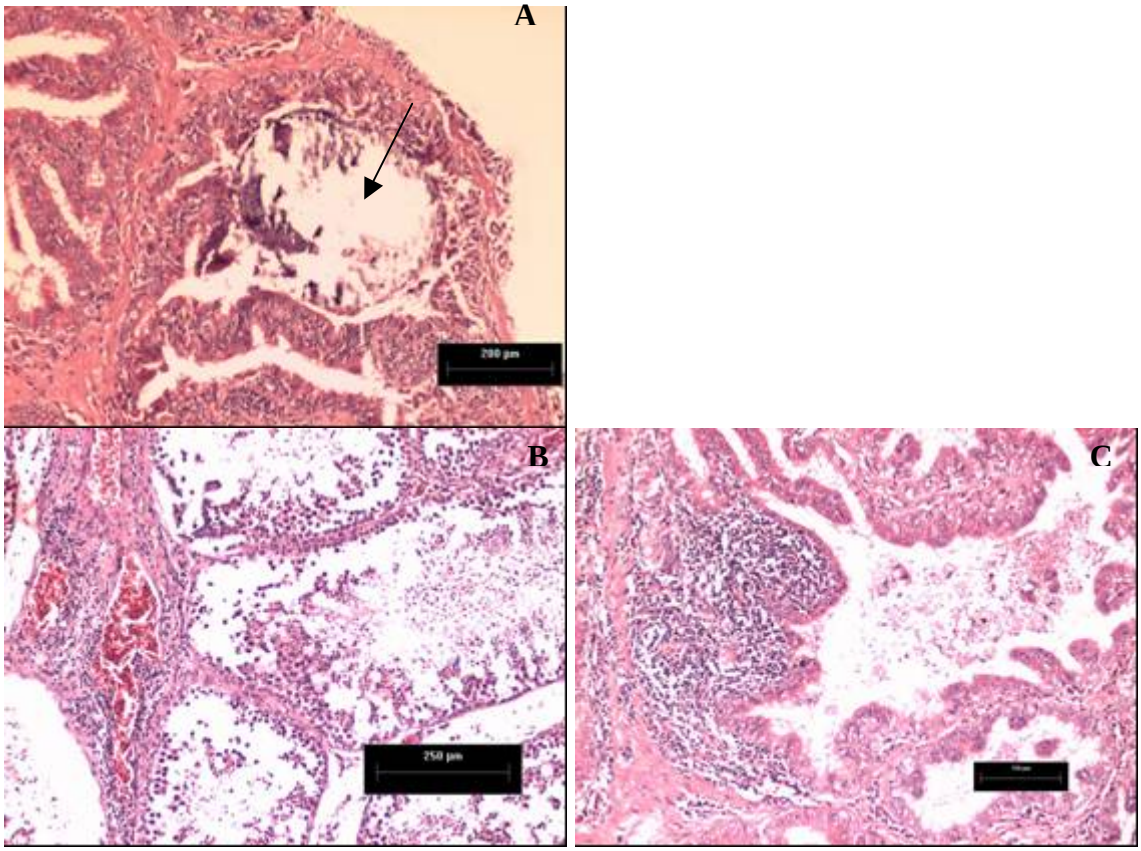

Figure 2 - Histopathological findings in testis of rooster with decreased fertility from which IBV and aMPV was isolated. A; B and C. A. Presence of stone (arrow) and inflammatory infiltrate; B. Seminiferous ductules showing low concentration of viable spermatozoids; C. Seminiferous ductules showing cells debris and inflammatory infiltrate (arrow). H&E staining, 100x magnification.

IMMUNODEFICIENCY IN CHICKENS INFLUENCES THE PATHOLOGIC OUTCOME OF INFECTIOUS BRONCHITIS

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Respiratory disease cases submitted to the Alabama State Veterinary Diagnostic Laboratory have yielded a high frequency of infectious bronchitis virus (IBV) isolations as compared with other respiratory pathogens. The restriction fragment length polymorphism (RFLP) profiles of numerous of these IBV isolates have shown identity with the RFLP profiles of vaccine strains extensively used in this region. One possible explanation is the hypothesis that viral immunodeficiency in chickens plays a role in the epidemiology and outcome of IBV infection. The immunosuppressive agents infectious bursal disease virus (IBDV), affecting principally B cell responses, and chicken anemia virus (CAV), affecting immature T cells in the thymus and mature T cells in secondary organs, are ubiquitous in the poultry industry. We evaluated the effects of viral immunodeficiency on the outcome of infectious bronchitis virus (IBV) infection in chickens as a hypothetical cause for failure of adequate protection in vaccinated chickens. Initially we investigated infectious bronchitis virus (IBV) isolations from outbreaks of respiratory disease in association with the presence of thymic and/or bursal atrophy in more than 320 submissions in the period 1997-2002. The number of IBV isolations was consistently higher in broilers aged 27 through 43 days, coinciding with lymphocytic depletion of bursa and/or thymus, providing circumstantial evidence that lymphocytic depletion and respiratory disease caused by IBV may be linked (Fig. 1). We then experimentally evaluated the effects of viral immunodeficiency (caused by CAV and IBDV) on the outcome of IBV infection. SPF chickens were either inoculated with CAV and IBDV at day 7 of age and subsequently inoculated with an Ark-type IBV isolate at day 15 of age or inoculated at day 15 of age with IBV only. A similar percentage of immunodeficient and immunocompetent chickens inoculated with IBV suffered from respiratory disease at 5 days post-inoculation (DPI). During the following days immunocompetent birds recovered while a significantly higher percentage of birds of the immunodeficient group maintained respiratory signs of IBV. By day 16 after IBV inoculation, none of the immunocompetent chickens exhibited respiratory signs, while 1 of 17 chickens of the CAV+IBDV infected group (6.3%) still showed respiratory signs. Histopathological

evaluations of trachea and larynx showed IBV characteristic hyperplasia and mononuclear infiltration accompanied by epithelial deciliation and necrosis. Based on lesion scores, lesions in the trachea of immunodeficient chickens were significantly more severe (P<0.05) than those of immunocompetent chickens at 14 DPI. Immunocompetent chickens showed significantly increased mononuclear cell infiltration at 9 DPI followed by a linear decline which achieved a plateau on day 19 after IBV infection. Immunodeficient chickens showed a less pronounced mononuclear cell infiltration at 9 DPI which was maintained throughout the experimental period. Detection and quantitation of IBV RNA in trachea and lachrymal fluid showed higher levels and more persistent IBV in immunodeficient chickens. A lower rate of decline of IBV RNA was clearly demonstrated in lachrymal fluid of immunodeficient chickens compared to immunocompetent chickens. Furthermore, IBV RNA was readily detected in lachrymal fluids of immunodeficient chickens through day 20 after IBV inoculation, while in immunocompetent birds IBV RNA was no longer readily detectable at 14 DPI. Collectively, these results suggest that viral immunodeficiency alters the outcome of IBV infection and may be playing a relevant role in IBV epidemiology.

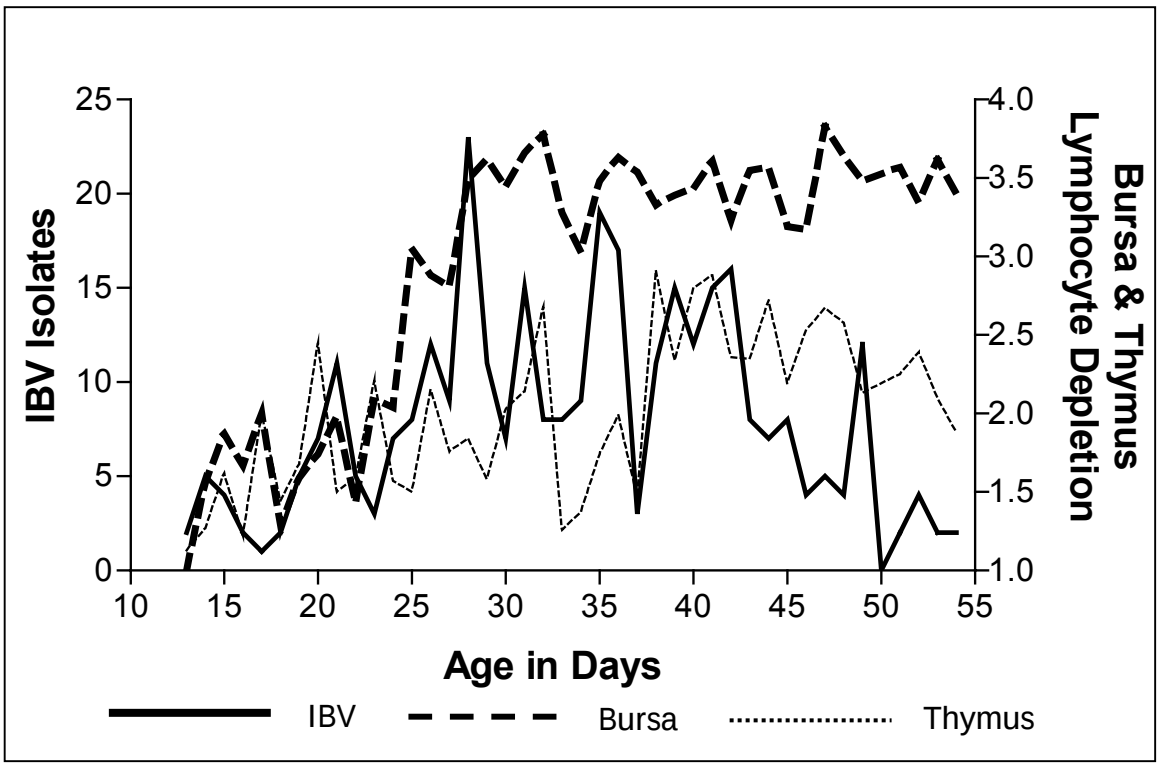

Fig. 1. Infectious bronchitis virus (IBV) isolations from broiler cases of respiratory disease in association with the presence of thymic and/or bursal atrophy in 320 submissions in the period 1997-2002.

Full length article will be submitted for publication elsewhere.

EFFECTS OF CAV AND/OR IBDV ON IBV INFECTION AND IMMUNE RESPONSES

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SUMMARY

We evaluated the effects of the immunosuppressive viruses chicken anemia virus (CAV) and infectious bursal disease virus (IBDV) on infectious bronchitis virus (IBV) infection and immune response. CAV and IBDV infection each delayed the IBV-specific local antibody response, prolonged respiratory signs, and increased viral persistence. However, the extent and kinetics of the effects of each immunosuppressive virus on IBV infection were distinct.

INTRODUCTION

Chicken anemia virus (CAV) infection results in depletion of cytotoxic and helper T cells (Adair, 2000) and prevents development of virus-specific cytotoxic T-lymphocyte responses (Markowski-Grimsrud & Schat, 2003) against other viruses. Although chickens older than 2 weeks of age are not susceptible to anemia caused by CAV, they still exhibit the immunosuppressive effects of CAV infection. Furthermore, elimination of the ability to generate an antibody response to CAV by infection with infectious bursal disease virus (IBDV) (Rosenberger & Cloud, 1989) or bursectomy (Hu et al., 1993) abrogates "age resistance." IBDV infects B cells and results in impaired antibody responses (Lukert & Saif, 2003). Chickens infected with one or both of these immunosuppressive viruses are more susceptible to diseases caused by other pathogens (Adair, 2000; Lukert & Saif, 2003).

Cytotoxic T-lymphocytes play an important role in IBV clearance following primary infection (Collisson et al., 2000), and memory T cells are important for protective immunity (Pei et al., 2003). Mucosal antibodies (IgA in tears) have been shown to be related to protective immunity (Toro & Fernandez, 1994), but this relationship is still controversial (Gelb et al., 1998). Our previous epidemiological studies indicated that respiratory disease caused by infectious bronchitis virus (IBV) in commercial chickens is frequently associated with lymphocytic depletion of the thymus and/or bursa (Toro et al., 2006). Our previous experimental studies showed that infection with CAV and IBDV eight days prior to IBV infection results in reduced lacrimal

antibody production, prolonged respiratory signs, and increased viral persistence (Toro et al., 2006). However, infection with CAV fourteen days prior to inoculation with an IBV vaccine strain had no effect on respiratory signs or length of IBV persistence and little effect on the kinetics of local IBV-specific antibody development (van Santen et al., 2004b). The present study experimentally examined the individual effects of CAV or IBDV infection on the outcome of IBV infection with a virulent IBV field isolate.

MATERIALS and METHODS

Five experimental groups of specific pathogen free leghorn chickens were used: uninfected control, IBV, IBDV + IBV, CAV + IBV, and IBDV + CAV + IBV. CAV (2 X 10^6 $TCID_{50}$ low passage Alabama isolate 03-4876; van Santen et al., 2004a) was inoculated intramuscularly and IBDV (10^4 CID_{50} APHIS strain) was inoculated orally into chickens of the appropriate groups at 7 days of age. Eight days later, at 15 days of age, chickens were inoculated via nasal and ocular routes with a low passage Alabama Ark-type IBV isolate (10^7 EID_{50} AL/4614/98). Respiratory signs were monitored every three days beginning 6 days post-IBV inoculation (DPI). Tear samples were collected from ten chickens of every group every three days beginning 5 DPI. Serum samples were collected from five chickens from each group 19 and 23 DPI. IBV-specific IgA in tears and IBV-specific antibody in sera were determined by ELISA. IBV genomes in tears were detected and quantified by RT-PCR. Five to six chickens from each group were necropsied 6, 9, 14, 19, and 23 DPI. Samples of trachea were obtained for IBV genome detection and quantification by RT-PCR. The number of IBV-specific IgA and IgM-secreting cells in the Harderian glands were determined by ELISPOT 6 and 9 DPI.

RESULTS

Respiratory signs (nasal and tracheal rales) persisted longer in a higher percentage of chickens in CAV + IBV- and IBDV + CAV + IBV-infected groups compared to chickens infected with IBV alone. The proportion of IBDV + IBV-infected chickens with nasal and tracheal rales was similar to chickens infected with IBV alone throughout the experiment. However, at 9 DPI, respiratory signs appeared more severe in IBDV + IBV-infected chickens than in chickens infected with IBV alone.
The systemic antibody response to IBV was poor in all groups. None of the groups were 100% seropositive (S/P ratio > 0.2) 23 DPI. IBV-specific IgA in tears was detectable at 5 and 8 DPI and reached maximum levels 11 DPI in chickens infected with IBV alone. The appearance of IBV-specific IgA in tears was delayed in all 3 groups infected with immunosuppressive viruses. In IBDV + IBV-infected chickens and IBDV + CAV + IBV-infected chickens, maximum levels of IBV-specific IgA were lower than in chickens infected with IBV alone. However, peak IBV-specific IgA levels in tears of chickens infected with CAV ultimately exceeded those of all other groups, including chickens infected with IBV alone. The number of IBV-specific IgA secreting cells in Harderian glands of IBV-infected chickens was over 200-fold greater 9 DPI than in uninfected controls and increased approximately 2–fold between 9 and 14 DPI. The number of IBV-specific Ig-A secreting cells was lower in IBDV + IBV infected chickens than in chickens infected with IBV alone at 9 DPI, but was

equivalent at 14 DPI. In contrast to the higher levels of IBV-specific IgA found in tears of CAV + IBV infected chickens 14 DPI, the number of IBV-specific IgA-secreting cells in Harderian glands of this group was lower at 14 DPI than in chickens infected with IBV alone. Harderian glands of IBDV + IBV infected chickens contained more IBV- specific IgM-secreting cells 14 DPI than those of other groups.
We found tears to be more sensitive for IBV genome detection and quantification than tracheal homogenates. In chickens infected with IBV alone, IBV genomes were detected in 100% of tear samples through 11 DPI, whereas IBV genomes were detected in 100% of tracheal homogenates only through 6 DPI. The number of IBV genomes in tears decreased more slowly in chickens infected with immunosuppressive viruses than in chickens infected with IBV alone. Eight DPI, geometric mean levels of IBV genomes of tears of all three immunosuppressive virus-infected groups were similar to each other and approximately 10-fold greater than in chickens infected with IBV alone. Eleven DPI, IBV genome levels in tears of CAV + IBV infected chickens were higher than in chickens infected with IBV alone, but lower than the two groups infected with IBDV.

DISCUSSION

Our results confirm and extend the results of our previous study (Toro et al., 2006), which showed that inoculation with IBDV + CAV eight days prior to IBV inoculation resulted in reduced antibody response, prolonged respiratory signs, and increased viral persistence. We showed that either CAV or IBDV infection delays antibody response to IBV, delays clearance of IBV, and prolongs respiratory signs. The kinetics of the effects of CAV infection are somewhat different from those of IBDV infection. Both delay local IBV-specific antibody development, but IBDV infection results in a lower level of IBV-specific IgA, while CAV infection results in higher peak levels of IBV-specific IgA. The delay of development of local antibody in CAV-infected chickens could be due to effects of CAV on helper T cells and/or cytokine expression. Higher antibody levels in CAV-infected chickens might be due to higher levels of helper T cell activity as the chickens recover from CAV infection (Adair et al., 1991) as well as increased stimulation of B cells due to higher viral load. Viral load 8 DPI is similar in chickens infected with either CAV or IBDV, and higher than in chickens infected with IBV alone. However, at 11 DPI the viral load in CAV + IBV-infected chickens is lower than in IBDV + IBV-infected chickens. This sudden clearance of IBV in CAV + IBV-infected chickens might be due to recovery of T cell activity (Adair et al., 1991) and/or high local antibody levels detected at 11 DPI.

Our results suggest that immunosuppression due to infection with either CAV or IBDV or both viruses could influence respiratory disease caused by IBV in the poultry industry.

REFERENCES

Adair, B. M. (2000). Immunopathogenesis of chicken anemia virus infection. *Developmental and Comparative Immunology* 24**,** 247-255.

Adair, B. M., McNeilly, F., McConnell, C. D., Todd, D., Nelson, R. T., & McNulty, M. S. (1991). Effects of chicken anemia agent on lymphokine production and lymphocyte transformation in experimentally infected chickens. *Avian Diseases* 35**,** 783-792.

Collisson, E. W., Pei, J., Dzielawa, J., & Seo, S. H. (2000). Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. *Developmental and Comparative Immunology* 24**,** 187-200.

Gelb, J., Jr., Nix, W. A., & Gellman, S. D. (1998). Infectious bronchitis virus antibodies in tears and their relationship to immunity. *Avian Diseases* 42**,** 364-374.

Hu, L. B., Lucio, B., & Schat, K. A. (1993). Abrogation of age-related resistance to chicken infectious anemia by embryonal bursectomy. *Avian Diseases* 37**,** 157-169.

Lukert, P. D., & Saif, Y. M. (2003). Infectious bursal disease. 11th ed. *In* "Diseases of Poultry" (Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald, and D. E. Swayne, Eds.), pp. 161-179. Iowa State Press, Ames, Iowa.

Markowski-Grimsrud, C. J., & Schat, K. A. (2003). Infection with chicken anaemia virus impairs the generation of pathogen-specific cytotoxic T lymphocytes. *Immunology.* 109**,** 283-294.

Pei, J., Briles, W. E., & Collisson, E. W. (2003). Memory T cells protect chicks from acute infectious bronchitis virus infection. *Virology* 306**,** 376-384.

Rosenberger, J. K., & Cloud, S. S. (1989). The effects of age, route of exposure, and coinfection with infectious bursal disease virus on the pathogenicity and transmissibility of chicken anemia agent (CAA). *Avian Diseases* 33**,** 753-759.

Toro, H., & Fernandez, I. (1994). Avian infectious bronchitis: specific lachrymal IgA level and resistance against challenge. *Journal of Veterinary Medicine, Series B* 41**,** 467-472.

Toro, H., van Santen, V. L., Hoerr, F. J., Li, L., van Santen, E., & Lockaby, S. (2006). *V. International symposium on avian corona- and pneumoviruses and complicating pathogens, Rauischholzhausen, Germany*.

van Santen, V. L., Hoerr, F. J., Joiner, K. S., Murray, C., Petrenko, N., & Toro, H. (2004a). Pathogenesis of chicken anemia virus: comparison of the oral and the intramuscular routes of infection. *Avian Diseases* 48**,** 494-504.

van Santen, V. L., Toro, H., Joiner, K. S., & Hoerr, F. J. (2004b). *IV. International Symposium on Avian Corona- and Pneumovirus Infections, Rauischholzhausen, Germany*.

THE H STRAIN OF AVIAN INFECTIOUS BRONCHITIS AND ITS USE AS A VACCINE

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SUMMARY

An avian infectious bronchitis virus (IBV) was isolated for the first time in The Netherlands in 1955. It was soon attenuated for use as the vaccine strain, H. Despite the development of other safe and effective IB vaccines, both live attenuated and inactivated ones, the H vaccine is still used effectively worldwide some 50 years after its first introduction. This paper will review its continued use, particularly in the light of its efficacy in providing cross protection against a number of the many IB serotypes or variants now causing disease problems throughout the world.

INTRODUCTION

Although infectious bronchitis (IB) was recognised as a major pathogen of chickens in many parts of the world following the first reports of the disease in US in the early 1930s (Schalk & Hawn, 1931), it was not diagnosed in the Netherlands until the mid 1950s. The first report in that country was in a flock of 6-week-old broilers showing respiratory problems and some mortality (Bijlenga, 1956). Bijlenga (Figure 1) was able to isolate the causal agent in embryonated eggs and then use this virus isolate to develop a vaccine strain by serial passage in embryonated chicken eggs (Bijlenga, 1960). The virus at the 52nd passage level subsequently became IB vaccine strain H52, and at the 120th passage level, the H120 vaccine strain; the H standing for the initial letter of the name (HUYBEN) of the owner of the broiler farm from which the virus was isolated. This farm is situated in the province of Brabant, in the southern part of the Netherlands and the letter H has continued to be used mistakenly as an abbreviation of “Holland” (Rosenberger et al., 1976; Winterfield et al., 1976; Owen et al., 1991).

The H vaccine strain, a virus of the Massachusetts serotype, quickly became an important part of vaccination programmes for the prevention and control of IB infections in chickens world wide. Although the less attenuated, H52 vaccine, has

now been largely replaced as a booster vaccine by oil-adjuvanted inactivated vaccines, the H120 strain has stood the test of time and is still widely used as a primary vaccine in broilers and as part of the vaccination programme for future layers and breeders. Over the years it has also become recognised that the H120 strain is efficacious in providing cross protection against some of the many different IB serotypes or variants now causing disease problems throughout the world.
After briefly describing the origin of the H120 vaccine this paper will consider the heterologous protection the vaccine is able to provide.

INITIAL DEVELOPMENT OF THE IB VACCINE STRAIN H

The initial isolate, having been confirmed as IBV, was passaged serially via the allantoic cavity of groups of 5-10 10-day-old embryonated eggs. By the third passage, embryo dwarfing, typical of IB infection, was observed. With continuing passage, the virus became embryo adapted. The dwarfing effect was observed until the 30th passage, but at higher passages embryo mortality occurred. By the 51st passage all the inoculated eggs had died within 48h of inoculation. It was therefore decided to test the virus at passage levels 25 and 52 as a potential vaccine. In experimentally inoculated broilers, both these passage levels induced strong antibody responses and, whilst passage level 25 virus caused respiratory disease, the 52 passage level did not. However, in field trials it was found that the passage 52 virus caused clinical signs and some mortality in birds up to approximately 3 weeks of age, although no reactions were observed when it was given to older birds or to hens in lay. This residual virulence suggested that the virus at the 52nd passage level was too virulent and further embryo passages were performed, to obtain virus at the 120th passage level. Extensive testing showed this virus to be sufficiently attenuated and it was developed as the H120 vaccine strain.

DURABILITY OF THE IB H VACCINE STRAIN

The H120 vaccine virus has been used successfully as a primary vaccine in broilers and for the initial vaccination of breeders and future layers for almost 50 years in most parts of the world. The exception to this is Australia, where the existence of unique lineages of IB serotypes (Sapats et al., 1996) has necessitated the development in that country of specific IB vaccines. One of the reasons for the continuing success of the H vaccine strain is the fact that field strains of the Massachusetts serotype have continued to cause disease in large areas of the world (Cavanagh & Naqi, 2003). However, a second, important factor is the evidence that the H vaccine strain can provide cross protection against other types of IBV.

HETEROLOGOUS CROSS PROTECTION PROVIDED BY IB VACCINE H STRAIN

The existence of many different IB serotypes or variants is one of the main reasons why IB infections are so difficult to control under field conditions, where flocks are likely to be exposed to different heterologous field strains. However, since the 1970s it has been known that the H vaccine strain has the ability to cross-protect against heterologous serotypes. Winterfield & Fadley (1975) and Rosenberger et al. (1976) reported that H53 or H52 vaccines respectively protected well against

challenge with several different IB serotypes commonly causing disease in USA at that time. Likewise, H strain vaccines, developed from intermediate passage levels, for example, passage 92, also have cross-protective capabilities (Winterfield et al., 1976). Higher egg passaged H strains have also been shown to possess cross protective ability, although Winterfield & Fadly (1975) found that as egg passage increased, cross protection waned, although not completely. However, the H120 vaccine has been found to induce some protection of the respiratory tract against heterologous challenge with several different European IB serotypes (Cook et al., 1999) (Table 1).

More recently, Gelb et al., (2005) used molecular techniques to compare the S1 sequence of field isolates of IB obtained in US and Israel and then performed protection studies in H120-vaccinated chickens. They found relatively poor protection with those isolates that showed the least S1 homology to the H120 vaccine strain. However, only partial protection was found against challenge with some isolates with high S1 homology to the H120 vaccine strain, for example IS/385/97 (Table 2).

In most studies, protection of the respiratory tract following challenge has been assessed either by attempting virus recovery from the trachea or by quantifying the damage caused to the epithelium of the trachea. However, in an attempt to mimic the situation commonly seen in the field, Cook et al. (1986) took a different approach. Chicks were vaccinated at 4-day-old with the H120 vaccine and challenged with a mixed infection comprising a pool of pathogenic *Escherichia coli (E coli)* isolates together with different heterologous IBV challenge strains, not of the Massachusetts serotype. Good cross protection was found following challenge with the *E coli* pool and some, but not all, the IBV variants examined (Table 3).

Further enhancement of cross-protection against several different IB variants was observed when H vaccine strains were given in combination with other IB vaccines. For example, Gelb et al. (1991) demonstrated cross protection when an “intermediate” H strain vaccine was used in combination with the Arkansas DPI strain and Cook et al. (1999) demonstrated the benefit of using the H120 vaccine in combination with a 4/91 vaccine.

PREVENTION OF IBV TRANSMISSION AFTER H120 VACCINATION

In the field, protection of the flock as a whole against transmission of an IB challenge virus is clearly more important than protection of the individual bird. However, in protection studies, efficacy is usually expressed as the percent of vaccinated birds protected against challenge. De Wit et al. (1998) used the H120 vaccine in a different approach to investigate immunity of the flock, rather than the individual bird. Using the concept of the reproduction ratio, defined as “the average number of new infections caused by one typical infectious bird during its entire infectious period”, they found that a single eye-drop vaccination with H120 reduced transmission among unvaccinated chickens significantly compared with transmission amount non-vaccinated birds. Interestingly, H120 is still the only IB vaccine to have been tested in this way, providing another example of how an “old” IB vaccine can meet a modern requirement, namely preventing IB transmission within a flock.

CONCLUSION

In the years since the IB H strain was developed for use as a vaccine in the 1950s there have been many changes in the IB disease situation worldwide. Many novel IB serotypes (and genotypes using molecular methods) have been identified; some of them with the ability to cause disease of severe welfare and economic importance. New IB vaccines, both of the Massachusetts serotype as well as ones based on some of the important IB variants that have emerged, have been developed. However, the H vaccine strain, particularly in the form of the H120 vaccine, has stood the test of time and is still widely used, some 50 years after its initial development.
Since that time, considerable advances in molecular biology and in the study of possible novel approaches for the development of avian and other animal vaccines have been made. It is interesting, therefore, to note that the apparently simple method used for attenuation of the H strain, namely serial passage through embryonated eggs, is still a method of choice for the development of effective live-attenuated IB vaccines (Gelb & Cloud, 1983; Jackwood et al., 2003).
The durability and efficacy of the H vaccine strain is therefore confirmed for protecting chickens, not only against challenge with the homologous serotype, but interestingly against some of the many heterologous serotypes that have been identified over the years. A final important point of note is that the vaccine has been found to conform to the rigorous safety standards now required of avian vaccines.

REFERENCES

Bijlenga, G. (1956). Het infectieuze bronchitis virus bij kuikens in Nederland aangetoond met behulp van ei-enting, dierexperimenteel onderzoek en serumneutralisatieproeven. (The infectious bronchitis virus of chicks detected in the Netherlands by means of egg-inoculation, animal experiments and serum neutralisation tests). Tijdschrift voor Diergeneeskunde, 81, 43-55.

Bijlenga, G. (1960). Untersuchungen über dei Wirksamkeit eines lebenden Impfstoffes gegen die infektiöse Bronchitis der Hühner mit einem am bebrüteten Hühnerei adaptierten, dem Trinkwasser zugefügten, autochthonen Virus-Stamm (Investigations on the activity of a living vaccine against infectious bronchitis of chickens with an embryonated egg-adapted autogenous virus strain applied in the drinking water). Doctor's thesis, Faculty of Veterinary Medicine, Berne, Switzerland, printed in the Netherlands by G. van Dijk, Breukelen, 1960.

Cavanagh, D. & Naqi, S. A. (2003). Infectious bronchitis. In Saif, Y. M., Barnes, H. J., Fadly, A. M., Glisson, J. R., McDougald, L. R. & Swayne, D. E. (eds) Diseases of Poultry, 11th edition (pp 101-119). Ames, Iowa State University Press.

Cook, J.K.A., Williams Smith, H. & Huggins, M.B. (1986). Infectious bronchitis immunity: its study in chickens experimentally infected with mixtures of infectious bronchitis virus and *Escherichia coli*. Journal of General Virology, 67, 1427-1434.

Cook, J. K. A., Orbell, S. J., Woods, M. A. & Huggins, M. B. (1999). Breadth of protection of the respiratory tract provided by different live-attenuated

infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotypes. Avian Pathology, 28, 477-485.

De Wit, J.J., de Jong, M.C.M., Pijpers, A. & Verheijden, J.H.M. (1998). Transmission of infectious bronchitis virus within vaccinated and unvaccinated groups of chickens. Avian Pathology, 27, 464-471.

Gelb, J. Jr. & Cloud, S. S. (1983). Effect of serial passage of an Arkansas-type avian infectious bronchitis virus isolate on clinical response, virus recovery and immunity. Avian Diseases, 27, 679-687.

Gelb, J., Wolff, J. B. & Moran, C. A. (1991). Variant serotypes of infectious bronchitis virus isolated from commercial layer and broiler chickens. Avian Diseases, 35, 82-87.

Gelb, J Jr., Weisman, Y., Ladman, B. S. & Meir, R. (2005). S1 gene characteristics and efficacy of vaccination against infectious bronchitis virus field isolates from the United States and Israel (1996-2000). Avian Pathology, 34, 194-203.

Jackwood, M. J., Hilt, D. A. & Brown, T. P. (2003). Attenuation, safety and efficacy of an infectious bronchitis virus GA98 serotype vaccine. Avian Diseases, 47, 627-632.

Owen, R. L., Cowen, B. S., Hattel, A. L., Naqi, S. A. & Wilson, R. A. (1991). Detection of viral antigen following exposure of one-day-old chickens to the Holland 52 strain of infectious bronchitis virus. Avian Pathology, 20, 663-673.

Rosenberger, J.K., Alphin, R.L. & Krauss, W.S. (1976). Cross-protection studies with a Holland strain of IBV. Avian Diseases, 20, 199-201.

Sapats, S. I., Ashton, F., Wright, P. J. & Ignjatovic, J. (1996). Sequence analysis of the S1 glycoprotein of infectious bronchitis viruses: identification of a novel genotypic group in Australia. Journal of General Virology, 77, 413-418.

Schalk, A. F. & Hawn, M. C. (1931). An apparently new respiratory disease of baby chicks. Journal of the American Veterinary Association, 78, 413-422.

Winterfield, R. W. & Fadly, A. M. (1975). Potential for polyvalent infectious bronchitis vaccines. American Journal of Veterinary Research, 36, 524-526.

Winterfield, R. W., Fadly, A. M. & Hoerr, F. J. (1976). Vaccination and revaccination with a Holland (H) strain of infectious bronchitis virus. Avian Diseases, 20, 369-374.

Figure 1. Dr Gosse Bijlenga, the Dutch Veterinarian who developed the IB H strain for use as a vaccine.

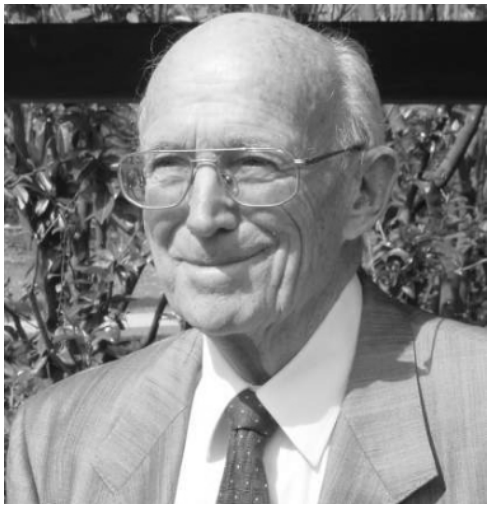

Table 1. Effect of vaccinating 1-day-old chickens with H120 and challenging them 5 weeks later with the homologous or heterologous IB serotypes.

IB challenge strain	% protection of respiratory tract	
	Not vaccinated	Vaccinated
Massachusetts (M41)	1	100
Italian 710	3	50
Italian 2149	5	67
Belgian B1648	5	70

Table 2. Protection provided by H120 vaccine against challenge with genotypic variants of IB isolated in Israel.

IB challenge strain	% S1 sequence homology to H120	% protection of respiratory tract	
		Not vaccinated	Vaccinated
IS/665/98 (Mass S1 genotype)	-	0	83
IS/385/97 (New variant)	96	16	58
IS/64714/96 (Variant 1)	66	16	58
IS/585/98 (Variant 2)	67	0	25
IS/720/99 (New variant)	65	0	36

IS = Israeli field isolated of IB

Table 3. Effect of vaccinating 4-day-old chickens with H120 and challenging them 3 weeks later with a pool of E coli strains and the homologous or heterologous IB viruses.

IB challenge strain	% mortality following challenge with IB & E coli	
	Not vaccinated	Vaccinated
Massachusetts (M41)	77	0
D207	23	0
Holte	14	0
UK 9	23	23*
UK 10	14	40*

* indicates poor protection

PREDICTIVE VALUE OF THE RESULTS OF AN α-IBV IgM ELISA FOR THE EFFICACY OF IBV VACCINATIONS IN THE FIELD

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SUMMARY

Infectious bronchitis virus (IBV) is, in spite of vaccination(s), a major cause of respiratory problems in broilers and of poor egg production in breeders and layers in the Netherlands. A possible cause of failure of the protection induced by vaccination(s) is an inadequate application of the vaccine. The availability of a simple and cheap method to check or estimate the efficacy of an IBV vaccination would be an asset to the poultry industry.
In this field study, the efficacy of IBV vaccinations was compared with the results of the α-IBV IgM ELISA on sera that were collected at several intervals post vaccination of the same broilers. The predictive value of the IgM ELISA was determined for the efficacy of the vaccination. The results that groups which showed at least 50% positive sera at 10 days post vaccination in the IgM ELISA had a protection of at least 89% against challenge. Groups of broilers with a low level of IgM positives showed an average protection of 43% with a range from 0% to 85%. These results show that the IgM ELISA can be used as an indicator of the efficacy of the IBV vaccination.

INTRODUCTION

Infectious bronchitis virus (IBV) is, in spite of vaccination(s), a major cause of respiratory problems in broilers and of poor egg production in breeders and layers in the Netherlands. Possible reasons of failure of the protection induced by vaccination(s) are e.g. a.) heterologous challenges, b.) immunesuppression, c.) very short or long interval between vaccination and challenge and d.) inadequate vaccine application. The mass application of IBV vaccines in the field is known for its many variations in a.) application technique (eyedrop, spray, water, atomist), b.) water (quantity, quality, temperature), c.) dosage, and d.) combination of different vaccines (e.g. IBV with Newcastle Disease vaccines). Many of these factors can have a negative effect on the efficacy of the vaccination. Therefore, a simple and cheap method to check the vaccination would be an asset to the poultry industry.

To determine the protection induced by the vaccination in the field by challenge experiment is not practical. If a vaccination induces a humeral response, serology can be used for checking the vaccination by detection of a seroconversion using paired serum samples. This strategy is hampered for vaccinations in presence of maternally derived antibodies (MDA).
An alternative strategy for serodiagnosing an IBV vaccination would be to look for the presence of an IBV-specific IgM response. In contrast to the two other immunoglobulin classes, IgG and IgA, that are produced in response to IBV vaccination and infection, IBV-specific IgM response is short-lived (Gillette, 1974; Mockett & Cook, 1986; Martins *et al*., 1990, 1991; Toro *et al*, 1994; De Wit *et al*, 1998) Detection of IgM is therefore indicative of a recent infection or vaccination.
Results of a IgM-capture ELISA, specific for IgM directed against IBV, were reported in Avian Pathology (De Wit *et al*, 1998). The specificity of the α-IBV-IgM ELISA was 99.0%, the sensitivity based on an experimental vaccination (H120) of 9-week-old SPF chickens was 83 to 100%, depending on the days post vaccination. The IgM responses were rapid (first IgM after 3 to 5 days) and transient (about 2 weeks) and therefore indicative for an acute IBV infection or vaccination.
A field study (De Wit, 1998) showed that about half of 80 broiler flocks did not show a detectable IgM response after IBV vaccination, which might indicate that part of the vaccinations might not have been successful.
In this study, the correlation between the efficacy of IBV vaccinations and the IgM response after vaccination was determined in 12 groups of broilers originating from 6 broiler flocks. The IgM response after vaccination of 129 flocks was also determined.

MATERIALS and METHODS

IgM ELISA

The ELISA was performed as described by De Wit *et al*. (1998). Briefly, a monoclonal antibody specific to chicken IgM was used as catching antibody and coated onto microtitre plates. Subsequent steps included the addition of test serum, IBV-antigen or control-antigen, enzyme labelled anti-IBV monoclonal antibody, and enzyme substrate.

Vaccinations

From 6 broiler flocks that were going to be (for the first time) vaccinated with a Mass (containing) vaccine at about 14 days of age, 10 birds per flock were transported to GD and housed in negative pressure isolators. These birds were vaccinated with one dose of the same vaccine as the other birds of that flock at 14 days of age by eye-drop. From each of the flocks, 10 birds that had been vaccinated at the farm (spray or water application), were transported to GD and housed in isolators. Blood samples were taken at day of vaccination, 7, 10 and 14 d.p.v. and stored in -20°C until testing.

Challenge

All birds were challenged between 14 and 20 days after vaccination by eye-drop (10^4 EID_{50} M41 in 0,1 mL per bird).

Determination of protection
The level of protection was determined using the ciliostasis test on 5 trachea rings per bird. The tracheas are placed in HMEM medium immediately after electrocuting of the chickens. Subsequently, 5 rings (equally divided over the total length of the trachea) are cut and placed in medium and placed at 37ºC. The level of ciliostasis is determined independently by two technicians between 1 and 4 hours after electrocution. The level of beating of the cilia per ring was expressed as 0 (0% beating of cilia), 1 (> 0 - 25% still beats), 2 (> 25 - 50% still beats), 3 (> 50 - 75% beats) or 4 (> 75 - 100% of the cilia beats). One bird can score between 0 and 20 (5 rings time score 4).

Field sera
From 129 flocks (120 broiler, 6 pullets, 3 rearing broiler breeder flocks) at least 10 sera were collected between 7 and 14 days post IBV vaccination. The broiler flocks were all vaccinated between 14 and 19 days of age. One rearing flock was vaccinated at 11 days of age, the other flocks were vaccinated between 11 and 14 weeks of age. The variables included age (11 days to 14 weeks), vaccine (7 different IBV vaccines were used), dosage (0.5 to 2.1 dose per bird), use of stabiliser, way of application (eye-drop, spray, atomist or water), and combinations of vaccines (non, 2 IBV vaccines, one IBV and a ND vaccine, or 2 IBV vaccines and a ND vaccine).

RESULTS

The 6 groups of broilers that had been vaccinated in the isolator by eye-drop showed at least 50% positive sera at 10 d.p.v in the IgM ELISA and all groups showed a protection between 89% and 100% against challenge (Figure 1, ■). The 6 groups of broilers that had been vaccinated at the farm (Figure 1, ♦) showed on average 7% positives in the IgM ELISa and an average protection of 43% with a range from 0% to 85%.
The results of the IgM ELISA in sera of the 129 flocks sampled between 7 and 14 days post vaccination are listed in figure 2. It showed that 44% of these flocks had less than 10% positives after vaccination. 26% of the flocks had at a IgM response between 50 and 100%.

DISCUSSION

The comparison of the IgM response post IBV vaccination and the efficacy against challenge in the 12 groups of broilers showed that groups which showed at least 50% positive sera at 10 days post vaccination in the IgM ELISA had a protection of at least 89% against challenge. Groups of broilers with a low level of IgM positives showed an average protection of 43% with a range from 0% to 85%. Although the absence of detectable amounts of IgM post vaccination does not prove the vaccination didn't induce (local) protection, it is not considered to be a good sign. It could mean that the vaccination was not or less effective.
The results of the 129 vaccinated flocks showed that 44% of these flocks had less than 10% positives after vaccination. The results of these 2 field studies suggest that the application of IBV vaccines in the field is less simple than many people think. More important, the average efficacy of the IBV vaccinations could be improved

considerably. The main question is however, how to achieve this. With the use of the IgM ELISA, it is relatively easy and cheap to compare and optimize different IBV mass application methods under field conditions. The IgM response of large groups of farms with different application methods (application method, application machines, dosage, amount of water, additives, etc) can be compared to find the critical factors that influence the efficacy of IBV vaccinations.
This might result in a significantly higher average efficacy of IBV vaccinations under field conditions, what normally would result in less respiratory disease outbreaks, less secondary infections and condemnations, less need of antibiotics, more first quality eggs and progeny.

REFERENCES

Davelaar, F.G., Noordzij, A., & Donk, J.A. van der (1982). A study on the synthesis and secretion of immunoglobulins by the Hardian gland of the fowl after eyedrop vaccination against infectious bronchitis at 1 day-old. *Avian Pathology*, 11: 63-79.

De Wit, J.J. (1998). Detection of IgM by ELISA after IBV vaccination*. Proceedings of the International symposium on infectious bronchitis and pneumovirus infections in Poultry. Rauischholzhausen, Germany*, pp 361-365.

De Wit, J.J., Mekkes, D.R., Koch, G. & Westenbrink, F. (1998). Detection of specific IgM antibodies to infectious bronchitis virus by an antibody-capture ELISA. *Avian Pathology*, 27, 155-160.

Gillette, K.G. (1974). Avian infectious bronchitis: demonstration of serum IgG and IgM neutralising antibody by sucrose density-gradient centrifugation and mercapto-ethanol reduction. *Avian Diseases*, 18: 515-525.

Martins, N.R. da Silva, Mockett, A.P.A., & Cook, J.K.A. (1990). A method for the rapid purification of serum IgM for the diagnosis of recent viral infections of chickens. *Journal of Virological Methods*, 29: 117-126.

Martins, N.R. da Silva, Mockett, A.P.A., Barrett, A.D.T., & Cook, J.K.A. (1991). IgM responses in chicken serum to live and inactivated infectious bronchitis virus vaccines. *Avian Diseases*, 35: 470-475.

Mockett, A.P.A., & Cook, J.K.A. (1986). The detection of specific IgM to infectious bronchitis virus in chicken serum using an ELISA. *Avian Pathology*, 15: 437-446.

Toro, H., Hidalgo, H., & Collingwood-Selby, A.N. (1994). Lachrymal antibody response of specific pathogen free chickens and chickens with maternal immunity after infectious bronchitis virus vaccination. *Preventive Veterinary Medicine*, 18: 267-274.

Figure 1.

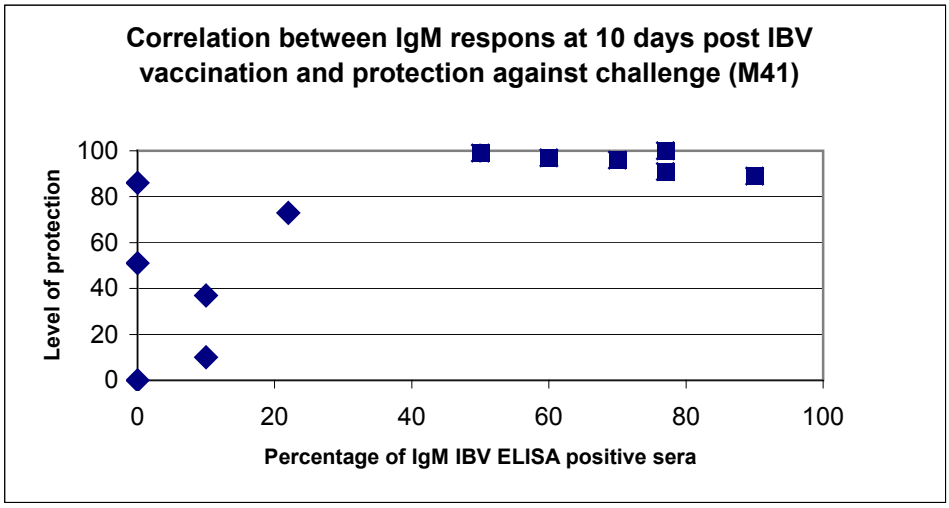
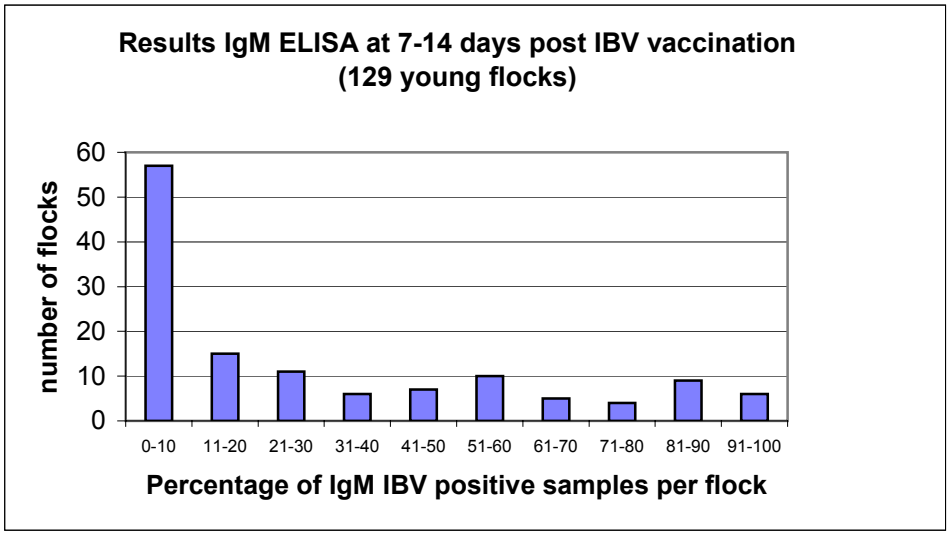


Figure 2.



THE COMPATIBILITY OF INFECTIOUS BRONCHITIS, NEWCASTLE DISEASE AND AVIAN METAPNEUMOVIRUS VACCINES

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SUMMARY

An experiment was performed to determine if an Avian metapneumovirus (AmPV) vaccine could be given together with Infectious Bronchitis (IB) and Newcastle Disease (ND) vaccines. It was demonstrated that the AmPV vaccine could still provide complete protection against virulent AmPV challenge when co-delivered with the IB vaccine and ND vaccine.

INTRODUCTION

AmPV, IBV and NDV cause significant disease issues in most poultry industries worldwide. Live AmPV, NDV and IBV vaccines are given to different types of chickens depending on their location and disease pressure. Significant advantages would be gained if these vaccines could be given at the same time in the hatchery. However, the vaccines must be shown to be compatible according to regulatory guidelines and if not, need to be given at least two weeks apart. Previous workers have shown that there is a significant interaction between AmPV, IBV and NDV vaccines even when only two out of the three vaccines are co-delivered (Thornton & Muskett, 1975; Cook *et al*., 2001; Ganapathy *et al*., 2005). The purpose of this work was to determine if the three vaccines could be co-administered to day old chicks without affecting the efficacy against a virulent AmPV challenge.

MATERIALS AND METHODS

Chickens

Specified Pathogen Free (SPF) White Leghorn chickens (Lohmann, Germany) were used. All chickens were contained in negative pressure isolators during the experiments. Chick starter crumbs and water were provided ad libitum during the course of the experiments.

Virus strains

Vaccines.
AmPV (strain 11/94, subtype B AmPV, Nobilis Rhino CV®), IBV (strain H120, Massachusetts serotype, Nobilis H120®), NDV (strain C2, Nobilis ND C2®) vaccines were used at the manufacturers recommended doses. The vaccines were delivered either alone or mixed in various combinations via the oculonasal (o.n) route in 0.1ml of diluent.

Virulent viruses.
For AmPV challenge a virulent subtype B AmPV strain was used via the oculonasal route in 0.1ml.

Assessment of AmPV challenge
Protection against AmPV challenge was assessed for each chick according to the criteria set out by below for up to 10 days post challenge. Mean scores per bird are presented.

1. slight nasal discharge *(nares pressed)*
2. moderate nasal discharge
3. copious nasal discharge
4. turbid nasal discharge
5. ocular discharge
6. swollen sinus

Statistical analyses
A two-tailed T test was used to compare serological responses. P-values of less than 0.05 were considered to be significant.

Experimental design.
Twenty SPF chickens were vaccinated via the ocularnasal route at day old with various combinations of IBV H120, NDV C2 and AmPV RhinoCV vaccines. The chickens were challenged with virulent AmPV virus via the ocularnasal route at 6 weeks of age. The chickens were observed for clinical signs of AmPV over a 10 day period.

RESULTS.

To determine if the co-delivery of IBV and NDV vaccines affected the efficacy of the AmPV vaccine, the AmPV RhinoCV vaccine was given with the IBV H120 and NDV C2 vaccines. None of the birds showed signs of respiratory disease post vaccination indicating that the trivalent vaccine combination was safe for delivery to one day old birds. Complete protection against clinical signs of AmPV was achieved when the IBV H120 and NDV C2 vaccines were co-delivered with the AmPV RhinoCV vaccine. In contrast birds vaccinated with IBV H120 and NDV C2 alone and the non-vaccinates had clinical signs of AmPV.

DISCUSSION

The purpose of these experiments was to investigate the compatibility of the AmPV, IBV and NDV vaccines when co-delivered to SPF chickens. These viruses are known to grow in the respiratory tract of chickens so it is expected that there would be competition for the sites of infection and replication. Indeed, previous reports have demonstrated that interference can occur when combination of only two out of three of these vaccines are given together (Thornton & Muskett, 1975; Cook *et al*., 2001; Ganapathy *et al*., 2005). Surprisingly it was found that the combination of the 3 vaccines could be given without affecting the efficacy of the AmPV vaccine. Further investigations would be required to determine how the vaccines interacted with each other and whether the compatibility of these vaccines is associated with their specific characteristics, such as dose and level of attenuation.

REFERENCES

Cook, J.K.A., Huggins, M.B., Orbell, S.J., Mawditt, K & Cavanagh, D (2001) Infectious bronchitis virus vaccine interferes with the replication of avian pnemovirus vaccine in domestic fowl. Avian Pathol. 30, 233-243.

Ganapathy K, Cargill P, Montiel E, Jones RC. (2005) Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. Avian Pathol. 34:297-302.

Thornton DH, Muskett JC (1975) Effect of infectious bronchitis vaccination on the performance of live Newcastle disease vaccine. Vet Rec. 96:467-8

PROTECTION BY MATERNALLY DERIVED ANTIBODIES AND VACCINATION AT DAY OF HATCH AGAINST EARLY CHALLENGE WITH IBV SEROTYPE D388

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SUMMARY

Strains of infectious bronchitis virus (IBV) serotype D388 were first detected by the Animal health Service in December 2003 and have shown to be a major cause of respiratory problems and nephritis in broilers and other young chickens and of poor egg production in breeders and layers in the Netherlands and surrounding countries.
In this study, the efficacy was determined of 5 different IBV vaccination programmes in layer breeders in inducing maternally derived immunity (MDI) against an early challenge with IBV D388 of the progeny. In 2 of these groups, the efficacy of an additional vaccination at day of hatch was determined.

INTRODUCTION

Since December 2003, a new serotype (Table 1) of IBV (D388) has been detected in The Netherlands and surrounding countries. It has been involved in respiratory disease, nephritis and drops in egg production and egg quality. Part of flocks that were infected at very young age showed false layers at later age.
Experiments by the GD (Animal Health Service, Deventer, The Netherlands) showed that a). D388 infections in young birds caused serious damage in respiratory tract, kidney and oviduct, b). Mass vaccines at day 1 provided only moderate protection against D388 (genotype QX), and c). Mass vaccines (incl. combi) at day 1 and 793B-like vaccine at 14 days provided a reasonable to high protection at 4 weeks of age.
The experiments and field situation showed that protection in the first few weeks of life depends very much on the presence of sufficient D388-neutralising maternally derived antibodies (MDA). An experiment was performed to see which vaccination schedule in (layer) breeders provided the highest level of protection against an early challenge with D388 of the progeny. The breeders were challenged too.

MATERIALS AND METHODS

Vaccinations and serology
Five groups of SPF layer pullets were housed in negative pressure isolators and vaccinated according to Table 2. Blood samples were collected at several ages for determining the level of D388 neutralising antibodies using the VNT. Starting at 4 weeks after application of the inactivated vaccines, eggs were collected and hatched. Part of the progeny was challenged at 6 days of age (experiment A). Progeny of 2 groups of parents were vaccinated at day 1 and challenged at 10 days of age (experiment B). At the end of the experiment, the breeders themselves were challenged with D388.

Challenge
All birds were challenged by eye-drop (10^4 EID_{50} D388 in 0,1 mL per bird).

Determination of protection
The level of protection in the trachea was determined using the ciliostasis test on 5 trachea rings per bird. The tracheas are placed in HMEM medium immediately after electrocuting of the chickens. Subsequently, 5 rings (equally divided over the total length of the trachea) are cut and placed in medium and placed at 37ºC. The level of ciliostasis is determined independently by two technicians between 1 and 4 hours after electrocution. The level of beating of the cilia per ring was expressed as 0 (0% beating of cilia), 1 (> 0 - 25% still beats), 2 (> 25 - 50% still beats), 3 (> 50 - 75% beats) or 4 (> 75 - 100% of the cilia beats). One bird can score between 0 and 20 (5 rings x score 4).
The level of protection in the kidney was determined using the immuneperoxidase test .

RESULTS

Serology
The level of D388 neutralising antibodies is listed in table 3. The breeders that had not been vaccinated with an inactivated vaccine did not or hardly develop D388 neutralising antibodies. Group E that had been primed with D388 developed the highest level of D388 antibodies. The 3 other groups developed intermediate titres with the general rule that more strains (3 versus 2) or more vaccinations (2x inactivated) resulted in somewhat higher titres.

Experiment A
The level of protection of the unvaccinated progeny for the different groups of breeders that were challenged at 6 days of age is listed in Table 4.

Experiment B
The level of protection of the vaccinated progeny for the progeny of breeder groups B and C that were challenged at 10 days of age is listed in Table 5.

Challenge breeders
The level of protection in the breeders at 23 weeks post the first application of the inactivated vaccines is listed in Table 6.

DISCUSSION

In general, an higher level of virus neutralising antibodies (VNA) against D388 in breeders correlated with higher level of protection in progeny against early challenge. The protection of the breeders themselves showed the same kind of result. The MDA provided a certain level of local protection in the trachea as shown in experiment A. The local protection at 10 days of age in experiment B (progeny of groups B and C (with MDA) itself was vaccinated at day of hatch) was not much higher than in experiment A (unvaccinated progeny). This indicates that a (heterologous) vaccination of the progeny at day 1 did not result in a clear rise in local protection against early challenge. This confirms the results of earlier experiments which showed that a heterologous vaccination at day 1 does not provide a sufficient protection against challenge with D388. This means that the (level of) MDA are very important most of the protection of young birds against early challenge.
Use of (booster by) inactivated vaccines in the breeders resulted in a higher level of VNA against D388. Boostering with an inactivated vaccine composed of 3 IBV serotypes resulted in higher VNA than by boostering with an inactivated vaccine with 2 strains. However, the highest titers and protection of the progeny were achieved by a primary infection with D388 and boostering by an inactivated vaccine with 3 IBV strains. This shows that use of an homologous D388 vaccine might be preferred.

Table 1. One-way neutralisation of IBV D388

Antiserum	Virus	Neutralisation titer
Homologues D388	D388	2048
Negative (SPF)	D388	6
M41	D388	11
D274	D388	32
D1466	D388	22
D8880	D388	32
D3128	D388	64
D3896	D388	11
793B	D388	45
Arkansas	D388	45
Conn.	D388	22
Iowa	D388	16

Table 2. Vaccination schedule of 5 groups of layer breeders

Group	Age of vaccination			
	13	15	19	23
A	Mass + D274*	793B*	-	-
B	Mass + D274	793B	Inact (M41 + D274)**	-
C	Mass + D274	793B	Inact (M41 + D274 + D1466)***	-
D	Mass + D274	793B	Inact (M41 + D274 + D1466)	Inact (M41 + D274 + D1466)
E	D388	-	Inact (M41 + D274 + D1466)	-

* 1 dose of live vaccine by eye-drop
** 1 dose of an commercially available Inactivated IB vaccine with M41 and D274 antigen
*** 1 dose of an commercially available Inactivated IB vaccine with M41, D274 and D1466 antigen

Table 3. Results of VNT D388 in vaccinated breeders

Group		Mean log2 VNT titer (SD)		
		Day 0 (age 13 weeks)	4 weeks post first Inact	10 weeks post first Inact
A	Live	<7	7.0 (1.6)	<7
B	Live, IB2	<7	8.9 (1.2)	8.8 (1.3)
C	Live IB3	<7	9.0 (1.7)	9.3 (2.2)
D	Live, 2x IB3	<7	9.8 (1.8)	10.1 (1.2)
E	D388, IB3	<7	11.5 (1.2)	11.3 (1.0)

Table 4. Results of D388 challenge at 6 days of age of unvaccinated progeny of all breeder groups

Breeder group		Challenge	Protection (% of 10 birds)			
			Trachea, ciliostasis	Kidney, Immunoperoxidase test		
			5 d.p.c.	5 d.p.c.	8 d.p.c.	11 d.p.c.
NC	-	-	100	100	100	100
PC	-	D388	0	90	50	40
A	Live	D388	50	100	70	60
B	Live, IB2	D388	50	80	30	100
C	Live IB3	D388	60	100	70	100
D	Live, 2x IB3	D388	60	100	70	100
E	D388, IB3	D388	100	100	90	100

Table 5. Results of D388 challenge at 10 days of age of vaccinated (day of hatch) progeny of breeder groups B and C

Group / Vaccin (day 1)	Challenge	Trachea, % cilia movement	Protection (% of birds)				
			Trachea, % cilia movement	Kidney, Immunoperoxidase test			
		0 d.p.c.	5 d.p.c.	5 d.p.c.	8 d.p.c.	11 d.p.c.	18 d.p.c.
- (NC)	-		100	100	100	100	100
- (PC)	D388		9	80	30	60	70
Mass/D274	-	84					
Mass/D274	D388		64	92	100		
Mass	-	98					
Mass	D388		78	100	92		
793B	-	92					
793B	D388		54	100	100		

Table 6. Results of D388 challenge of the different groups of layer breeders

Group	Vaccinations	Positive IPT IBV, 5 d.p.c.		Positive IPT IBV, 8 d.p.c.	
		trachea	kidney	trachea	Kidney
A	Live	1/5	4/5	0/4	0/4
B	Live, IB2	2/5	4/5	1/4	0/4
C	Live IB3	0/4	1/4	0/4	0/4
D	Live, 2x IB3	1/4	1/4	0/4	1/4
E	D388, IB3	0/5	0/5	0/4	0/4

EMBRYO VACCINATION AGAINST INFECTIOUS BRONCHITIS VIRUS.

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SUMMARY

Vaccination of embryos against infectious bronchitis virus (IBV) could bring significant advantages to the poultry industry. We tested recombinant IBVs based on the Beaudette strain expressing the Beaudette spike protein (Beau-R), the spike protein from the virulent M41 strain (Beau-R-M41(S)) or the spike protein from the virulent 4/91 strain (Beau-R-4/91(S)) for their potential as vaccines for 18-day-old embryos. A high dose of virus was inoculated *in ovo* and the pathogenicity was assessed by observing the effect on hatch plus clinical signs post hatch. The recombinant IBV strains did not reduce hatchability or cause clinical signs post hatch. Beau-R-M41(S) gave a high level of protection of the embryos against virulent M41 challenge. These results are promising for the development of embryo safe efficacious IBV vaccines for *in ovo* application.

INTRODUCTION

Infectious bronchitis virus (IBV) causes respiratory disease in domestic fowl and can also cause kidney and reproductive disease. Conventional vaccines are live viruses that have been attenuated by passage in embryonated eggs and are usually given at one day of age or later. Depending on their attenuation status the vaccines can give high levels of protection against virulent though can also cause post vaccinal reactions. The molecular reasons for the attenuation and ability to protect remain unknown.

Embryo vaccination against Marek's disease is common in the USA and South America. Embryo vaccination saves on labour charges so can be cost effective for large hatcheries. As most strains of IBV are lethal to embryos little work on embryo vaccination with IBV has been done. One group has shown that *in ovo* vaccination with IBV can be successfully accomplished, although the strain reduced hatch at relatively low titres (Wakenell & Sharma, 1986).

Using a reverse genetics approach molecularly defined IBV viruses can be produced (Casais et al 2001; Casais et al., 2003; Britton *et al*., 2005). An IBV of known sequence based on the Beaudette strain has been prepared (Casais *et al*., 2001) and the spike gene of the M41 IBV virus swapped for Beaudette spike gene (Casais *et al.*, 2003). This altered the cell tropism of the strain (Casais *et al*., 2003), though the pathogenicity was not altered (Hodgson *et al.*, 2004). This strain was able to afford significant protection to vaccinated birds against challenge with a virulent M41 strain. The aim of this work was to determine whether Beaudette strain-based IBVs produced by reverse genetics could be used as *in ovo* vaccines.

MATERIALS AND METHODS

Embryonated eggs

Specified Pathogen Free (SPF) White Leghorn chicken embryonated eggs (Lohmann, Germany) were used. All chicks were hatched in a contained environment and transferred to negative pressure isolators for the remainder of the experiments. Chick starter crumbs and water were provided *ad libitum* during the course of the experiments.

Virus strains.

Candidate vaccine strains

The Beau-R and Beau-R-M41(S) strains were prepared using reverse genetics as described previously (Casais et al 2001; Casais et al., 2003). The Beau-R-4/91(S) strain was prepared using a modified technique (Britton et al., 2005). The viruses were grown on primary chick kidney cells and the titres of the viruses were established in 9-to 12-day-old embryonated eggs.

IBV challenge strain

For the IBV challenge the Massachusetts serotype M41 strain was given by the oculonasal route (0.1ml/bird) at a dose of $10^{3.0}$ EID_{50} per bird.

Vaccinations

For embryo vaccination, eggs on the 18^{th} day of embryo development were inoculated into the amniotic fluid using a 20 gauge, 2.5cm long needle. 0.1ml of the virus dilution was inoculated. Placebos received 0.1ml of the diluent. Eggs were hatched in separate incubators. Hatch was assessed after 21.5 days of incubation.

Assessment of clinical signs post hatch

Examination of general health was done daily during the course of the experiments. Nasal discharge was assessed by gently squeezing the nares of the chicks and determining if any fluid was visible.

Assessment of ciliostasis

Assessment of ciliostasis was done as previously described (Cook et al., 1999).

Statistical analyses

A two-tailed T test was used to compare ciliary activity and serological responses. P-values of less than 0.05 were considered to be significant.

Embryo vaccination experiments
SPF eggs were inoculated with high doses of Beau-R or Beau-R-M41(S) (experiment 1). A placebo vaccinated group was included. Live chicks were counted at 21.5 days of incubation and groups of at least ten chicks formed. Examination of nasal discharge was made at 6 days post hatch. The chicks were challenged with virulent M41 IBV at 4 weeks. At 5 and 7 days post challenge half of the birds in each group were euthanized, their tracheas removed and assessed for ciliary activity. In experiment 2 SPF eggs were inoculated with a high dose of Beau-R-4/91(S) or a placebo and live chicks counted at 21.5 days of incubation.

RESULTS

Embryo vaccination with IBV – the effect on hatch
High doses of Beau-R or Beau-R-M41(S) did not effect the hatch in comparison to a placebo vaccination. The Beau-R and Beau-R-M41(S) gave slightly lower hatch than the placebo vaccinates (Table 1). A high dose of Beau-R-4/91(S) also did not greatly affect hatch (Table 2) and it should be noted that differences in hatch could be due to the fact that separate hatching units were used for each group.

Clinical observations post hatch
Beau-R or Beau-R-M41(S) did not cause any morbidity during the post hatch observation period. Nasal discharge was not detected in birds vaccinated *in ovo* with either Beau-R or Beau-R-M41(S) suggesting that these viruses were not pathogenic.

Efficacy of the candidate vaccines
The remaining birds were challenged 4 weeks post hatch with virulent M41 virus and the ciliary activity tested on days 5 and 7 post challenge (table 1). Based on ciliary activity of 50% or less being protected, 80% and 100% of the Beau-R-M41(S) *in ovo* vaccinated birds were protected at 5 and 7 days post challenge respectively. In contrast 0% and 30% of the Beau-R *in ovo* vaccinated birds were protected at 5 and 7 days post challenge respectively. None of the mock-vaccinated birds were protected.

DISCUSSION

Embryo vaccination against Marek's disease is commonplace for broiler birds in the USA. We tested three rIBVs, one a molecular clone based on the Beaudette strain (Beau-R) the others again based on the Beaudette strain but expressing the M41 spike protein (Beau-R-M41(S)) derived from the virulent M41 virus or the 4/91 spike protein (Beau-R-4/91(S)) derived from the virulent 4/91 strain.
The results demonstrate that on the basis of the hatch rate and clinical signs post hatch that the rIBVs were safe for delivery *in ovo*. This is an unusual finding since many IBV strains greatly reduce the hatch rate when given *in ovo* (I Tarpey, unpublished observations).
In addition to being safe, the efficacy tests using a respiratory challenge with the virulent IBV M41 strain, showed that the Beau-R-M41(S) gave high levels of protection suggesting a positive effect of the M41 spike protein expressed by this

virus. This agrees with the results of challenge experiments when chicks had been vaccinated with the recombinant IBVs (Hodgson et al., 2004).
In summary, this work demonstrates the utility of the reverse genetics approach for preparing recombinant IBVs and shows that vaccines based on rIBVs of the Beaudette strain, are safe for the *in ovo* vaccination of SPF embryos. Further work will be required to confirm the potential use of these candidate vaccines in the field.

REFERENCES.

Britton, P, Evans, S, Dove, B, Davies, M, Casais, R, Cavanagh, D. (2005). Generation of a recombinant avian coronavirus infectious bronchitis virus using transient dominant selection. J Virol. Methods **123**, 203-11.

Casais R, Thiel V, Siddell SG, Cavanagh D, Britton P. (2001) Reverse genetics system for the avian coronavirus infectious bronchitis virus. J Virol. 75: 12359-69.

Casais R, Dove B, Cavanagh D, Britton P. (2003) Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol. 77: 9084-9.

Cook, J.K.A, Orbell, S.J., Woods, M.A. & Huggins, M.B. (1999) Breadth of protection of the respiratory tract provided by different live attenuated infectious bronchitis vaccines against challenge with infectious bronchitis viruses of heterologous serotype. Av. Path. 28, 477-485.

Hodgson, T., Casais, R., Dove, B., Britton, P., and Cavanagh, D. (2004) Recombinant infectious bronchitis coronavirus Beaudette with the spike protein gene of the pathogenic M41 strain remains attenuated but induces protective immunity. J. Virol 78, 13804-13811.

Wakenell, P.S. and Sharma, J.M. (1986) Chicken embryonal vaccination with avian infectious bronchitis virus. Am J Vet Res. 47:933-8

Table 1: Embryo vaccination with recombinant IBVs, analysis of hatch, clinical signs, serological responses and protection from virulent challenge.

Vaccine	Hatch rate (%)	Protection (based on ciliary activity post challenge)	
		Day 5	Day 7
Beau-R	73	0	30
Beau-R-M41(S)	82	80	100
Placebo	85	0	0

Fertile SFP eggs were inoculated with Beau-R, Beau-R-M41(S) or a placebo. Hatch was assessed at 21.5 days of incubation. All birds were challenged with virulent M41 at 4 weeks post hatch and the ciliary activity determined at 5 and 7 days post challenge.

Table 2: Embryo vaccination with recombinant IBVs: Analysis of hatchability.

Vaccine	Hatch rate (%)
Beau-R-4/91 (S)	67
Placebo	80

Fertile SFP eggs were inoculated with Beau-R-4/91(S) or a placebo. Hatch was assessed at 21.5 days of incubation.

SUMMARIES OF THE SESSIONS

Session: AVIAN PNEUMOVIRUS - EPIDEMIOLOGY, VIROLOGY and DIAGNOSIS

Francois-Xavier Le-Gros, Merial, Lyon, France

Epidemiology

Various confirmations were obtained from the presentations in this section: A and B types are still relevant for Europe and Latin America in chickens in turkeys, and C type in turkeys for some states of USA and in wild waterfowls. This C type was also described in France in commercial ducks a few years ago.
Cecchinato *et al*.(Bologna-Italy) also presented the genetic evolution of type B Italian isolates clearly related to time although 98% identity was conserved over 20 years. The genetic identity between chicken and turkey isolates was also confirmed.

General virology

After the description of a reverse genetic system in aMPV type A two years ago, two teams from the Universities of Georgia and Maryland (Qingzhong Yu *et al*., Dhanasekaran *et al*.) presented a comparable system for the type C. It is combining the expressions of the RNP complex (N,P,M2,L proteins) via relevant plasmids under the control of the T7 vaccinia polymerase, and of a full RNA sequence aMPV mini-genome that can be modified. Applications of the system will help in defining the essential elements for the viral replication, its attenuation and its immunogenicity, leading to safer and more effective vaccines. Vector vaccines could also be derived from the aMPV using this system as demonstrated by the successful expression of an inserted GFP gene.
Another tool to study the molecular biology of the virus was presented by T. Cardoso (Araçatuba-Brazil) relating a work from Ferreira *et al* (Campinas-Brazil): This is the use of “silencing” RNA that inhibits or compete with the translation of aMPV mRNA. This application was demonstrated successful against the expressions of N and N+F proteins, which were also associated with a diminution of infectious titres in cell cultures of a Brazilian type A aMPV isolate.

Diagnosis

Guionie *et al.* (AFSSA Ploufragan – France) described a real time PCR system that is specific for the four known types of aMPV, and relatively quantitative. This will allow easier and more extended studies on the prevalence of the various types in the field, and namely answer the question whether C and D types are still present in Europe.
However ELISA tests are still the most widely used in the field to diagnose infection or control vaccine take. Brown *et al*. (Liverpool- UK) described an ELISA test using a recombinant aMPV protein expressed in E. Coli. The concept was tested with a highly antigenic F region fused with a his-tag component that allows for purification on nickel columns. This technology offers a higher stage of purification than classical methods and could help in improving the specificity and sensitivity of ELISA tests.

Session: AVIAN PNEUMOVIRUS - PATHOGENESIS AND IMMUNITY

Elena Catelli, University of Bologna, Ozzana Emilia, Italy

Three papers were presented in this session.
The first presentation reported research that is in progress concerning mucosal immunity in avian metapneumovirus infections. Professor Sharma gave a concise update of the immuno-pathogenic mechanisms during APV infections then focused on their recent studies concerning the quantification and the identification of the lymphoid cells of the B cell phenotype infiltrating the respiratory mucosa lining the turbinates. He evaluated the response to the infection with three different strains of APV subtype C. B cells secreting IgM, IgG or IgA were identified and quantified with immunohistochemistry. Moreover, IgA was detected in the tracheal washes. After exposure to the virus there was a significant increase in the number of the B cells secreting IgG and IgA, but not significant changes in the IgM. Moreover, the number of IgA cells, as well as secreting IgA, appeared earlier following the second exposure to the virus compared to the first exposure. The protective role of the surface IgA needs to be investigated.
Dr Rautenschlein reported results of experimental investigations into the pathogenesis of avian metapneumovirus in broiler type chickens using A and B subtypes. She also described results of field observations. She found that under experimental condition both APV subtypes induced only mild respiratory signs which peaked at 6 days post inoculation. APV B type seems to replicate to a greater extent compared with the A type and was found in a larger range of tissues. It also induced a higher antibody response. During the discussion, possible relation of this results with the B strain used and with the sensitivity of the serological or molecular tests were discussed. The field observations gave an indication of the difficulties that are encountered facing APV infection in chickens, often related to the inconsistent virus detection and isolation from field outbreaks and the interpretation of the serological findings.
Dr. Guionie summarized the major points of some experimental trials intended to study the pathogenesis of the four APV subtypes A, B, D and C, isolated from turkeys or ducks, in turkeys and Muscovy ducks. Serological data, clinical signs and viral excretion after experimental infections were presented. SPF Muscovy ducklings were proven to be resistant to the infection with APV subgroups A, B and D. Clear clinical signs, viral re-isolation and seroconversion were lacking. When infected with APVs belonging to C subgroup, they showed different behaviour depending on whether APV C originating from ducks (French isolate) or from turkeys (Colorado strain) were used. In the first case they did develop disease, in the second case just seroconversion and virus excretion was observed. A possible role of ducks in APV C viral spreading was hypothesised. SPF turkeys conversely were fully susceptible to APVs A, B, D and C Colorado strain, but quite resistant to APV Subtype C isolated from ducks. In this case the turkeys showed minor clinical signs and none or slight specific seroconversion. It was not possible to re-isolate the virus from the trachea. The existence of an extra-tracheal site of replication was suggested by the authors and this was a matter for discussion.

Session: AVIAN PNEUMOVIRUS - VACCINATION

J.J. (Sjaak) de Wit, Animal Health Service (GD Deventer), Deventer, The Netherlands

Four papers were presented in this session. Dr Banet-Noach (Israel) presented a study with Vero cells that had survived an inoculation with AMPV subtype A, B or C. These cells were the basis of 3 stable cell lines that appeared to be chronically infected with AMPV. Staining of these cells with an anti-AMPV immunofluorescence test showed that these cells produced AMPV antigen. Addition of new Vero cells resulted in a CPE in the added cells. A first animal experiment showed that vaccination with these apparently chronically infected cells induced a certain level of protection against challenge with AMPV. Further work is needed and planned for studying the mechanism of the chronic infected and more understanding of the interaction between the virus and Vero Cell and the potency as vaccine.

Dr Catelli (Italy) presented a paper about AMPV vaccinations under field conditions. Under experimental conditions, AMPV vaccines cause no or only mild clinical signs. The vaccine virus is only detectable for a short time. In the presented field study, the vaccine was detectable for 2 till 6 weeks, suggesting a suboptimal application of the vaccine.
One of the re-isolates of the vaccines (confirmed by sequencing) was tested for pathogenicity and showed to be as pathogenic as a virulent field strain, showing that reversion to virulence can happen relatively fast with AMPV vaccines. During the discussion this was confirmed by Dr Naylor (UK), who has seen reversion to virulence of an AMPV vaccine in only 4 bird passages.

The last two papers were presented by Dr Naylor (United Kingdom). The first paper shoed the results of a study that started with the clone purification of a AMPV vaccine to improve the stability of the vaccine. The plaque purification of an AMPV vaccine candidate resulted in several clones from which several were not inducing protection anymore. Comparison of the complete genome of these non-protecting and protecting clones showed that the relevant mutations were most likely located in the region of the G gene (SH-G interregion).
In the second paper Dr Naylor showed his magic's with reverse genetic techniques. With a new variant of reverse genetics, replacements of the F gene of a non-protective strain by the F gene of a protective strain, resulted in a protecting strain. Three strains that were not inducing ciliostasis in poults still induced full protection against challenge.

The conclusion I draw from this session is that high quality work is performed on the fundamental understanding of AMPVirus and the translation of this knowledge into the development of AMPV vaccines. However, a (vaccination) chain is as strong as its weakest link. The application of these products under field conditions might be a link that needs more attention.

Session: AVIAN CORONAVIRUS – EPIDEMIOLOGY I

Jane K A Cook, Huntingdon, United Kingdom

Six papers were presented in this session, reviewing the IB situation in different parts of the world.

The first 2 papers, from the group at Mérida University, Yucatán, México, studied disease in back-yard chickens in rural communities. These flocks are very important economically and socially. 80-90% of families maintain such poultry, but rarely use veterinary services and have little knowledge of the disease status of their birds. *Dra S Ramírez-González* reported the results of a serological study to determine the true incidence rate of Infectious bronchitis (IB), Newcastle Disease (ND) and *Mycoplasmas* in backyard flocks in rural communities in Yucatán, using local Creole birds as sentinels – their slow growth rate making them very useful for such studies. There was no evidence of ND infection, but antibodies to *Mycoplasma gallisepticum and synoviae* were found. Using HI tests with both M41 and a local isolate (SIN6YUC/MEX/96) as antigens, a high incidence of IB antibodies was found. The second paper, given by *Dr E Gutiérez Ruiz* reported experimental studies on the virulence of the above mentioned local isolate for 1-day-old commercial broilers and 3-week-old commercial layer pullets, both alone and together with *E coli.* The IB virus was defined as of low virulence for the broilers and medium virulence for the layer pullets. These two papers highlight the importance of backyard flocks in the epidemiology of IB; the risks they pose to commercial birds, and *vice versa.*

The third paper was given by *Dr H Montassier,* on behalf of workers at UNESP, Jaboticabal, EMBRAPA, Concórdia and University of San Paolo, Brazil. Brazil is one of the world's major poultry producing countries and IB has been a serious problem there in recent years. This paper reported an analysis of part of the S1 glycoprotein of IBVs obtained, mainly from broilers, but also from layers, in the south and south east of the country; some vaccinated, some not. In addition to Massachusetts, four distinct genotypes were identified. Major amino acid changes were detected and the conclusion drawn that different genetic variants are co-circulating in Brazil.

In a paper given by *Mariette Ducatez*, Institute of Immunology, Luxembourg, we heard of molecular epidemiological studies performed by PCR in collaboration with colleagues in China and Nigeria. IBV was detected in most samples from live bird markets in China. Four genetic groups were found; one closely related to the H120 vaccine strain. Seven IBVs obtained from commercial flocks in Nigeria were also studied. Interestingly, a close genetic relationship was found between them and the IBVs from China. We were reminded of the extensive trade in poultry between these two countries.

Dr K Domanska-Blicharz, NVRI, Pulawy, Poland, reported results showing that a nested RT-PCR, using primers to amplify a fragment of the N gene, was more sensitive that a conventional one based on the 3' UTR fragment. Analysis of IBV isolates from 1997-2006 showed that both the 793/B and QX genotypes are present

in Poland, as well as two isolates which showed little genetic relationship to the others and which formed separate branches of a phylogenetic tree.

Finally a paper was presented by *Dr L Renstrom*, Uppsala, Sweden. IB was previously a notifiable disease in Sweden, with an eradication policy for infected flocks and IB vaccination was not permitted. However, from the mid 1990s, a number of distinct IB outbreaks were reported in all types of commercial chickens. Transport of infected birds and the movement of empty cages from infected farms were both implicated in the spread of infection. S1 sequence analysis of IB isolates showed both at least two distinct outbreaks time wise, as well as the presence of two unrelated IBVs, present at the same time. Serological studies also indicated a high incidence of infection in clinically healthy birds and necessitated a change of policy to allow IB vaccination.

The papers presented in this session demonstrate to large amount of molecular analysis of IBVs being performed in many laboratories worldwide and confirm the number and diversity of IBV genotypes that exist, whilst the experiences in Sweden emphasise the difficulty of country or area maintaining its IB-free status when surrounded by IB-infected areas. Whilst advances in molecular biology are providing tools for performing excellent molecular analyses of IB variants, it is important to relate the findings of such studies to the disease situation in the field and to methods of control.

Session: AVIAN CORONAVIRUS – EPIDEMIOLOGY II

Silke Rautenschlein, School of Veterinary Medicine Hannover, Hannover, Germany

Four papers were presented in this session, which focused on the epidemiology of IBV in Germany, Italy and Spain. Problems regarding diagnosis of IBV were discussed. Dr. Philipp (Germany) presented the general diagnostic scheme for the detection of IBV, which included consideration of clinical signs, pathology, and virus detection including species and serotype identification. Problems related to IBV diagnostics were pointed out such as field application of various vaccines with different serotypes, lots of cross reactions between strains, and sensitivity of the RT-PCR, which may vary depending on the serotype. Recent field observations beginning at 2004 in Germany indicate that the most prominent IBV strains detected were 7/93 B, Mass-type, D1466, It-02, and QX-type.

Dr. Beato (Italy) presented a paper on health problems caused by circulating Chinese variants of IBV in northern Italy between 2004 and 2006. The following methodology was applied for the surveillance programme: placement of specific pathogen-free sentinel birds; virus-isolation in embryonated chicken eggs; electronmicroscopy, and finally molecular characterisation of the isolates. In 2004 they detected for the first time QX-type isolates. From 44 IBV positive farms out of 68 investigated ones, 11 farms were positive of QXIBV, which included 3 backyard flocks. The isolated QX-type IBV showed 99 % homology in the S1-gene sequence analysis with the Chinese QX-strain.

Dr. Zanella presented the isolation of a new serotype of IBV in Italy. The following observations were obtained in the field: mild respiratory and ‘enteric signs’, kidney lesions. Virus isolates were passed 8 times in embryonated chicken eggs, and characterized further in the neutralization test with monospecific antisera and molecular methods. A new virus isolate was identified (AZ-40/05). This IBV showed high sequence homology in the S1-gene with QXIBV.

Dr. Dolz (Spain) presented the evolution of the 4/91 genotype in Spain. 4/91 was shown to be the most common serotype in Spain. 13 field isolates from Spain were further characterized, and the S1-gene was sequences. Based on these sequences the isolates were integrated in an IBV phylogenetic tree with British, French and Iranian isolates. All 13 isolates were grouped with 4/91. Three different IBV-subgroups were identified for these 13 Spanish isolates. It is speculated that the 4/91 strains in Spain may have different ancestors.

The final discussion led to the conclusion, that the epidemiological data are important in order to understand more about the prevalence and evolution of IBV not only in Europe but also world-wide. This knowledge is prerequisite to adjust continuously IBV vaccination schemes. At the moment it is not possible to assign a specific IBV serotype to a clinical picture. More research is needed to understand the meaning of the sequence variation between IBV isolates. Furthermore, it will be necessary in the future to standardize the nomenclature of the various IBV strains from the different countries.

Session: AVIAN CORONAVIRUS – EPIDEMIOLOGY III

Haroldo Toro, Auburn University, Auburn, Alabama, USA

Four papers were presented in this session. Two of these papers reported on surveillance of coronavirus infections in domestic poultry and two presented evidence for new coronaviruses affecting wild bird species.

Dr. Worthington presented a survey on infectious bronchitis virus by RT-PCR in selected European countries started in 2002. Their survey shows that Mass and 793B strains are highly prevalent in the European poultry industry. The high prevalence of these serotypes is associated with the extensive use of strains of these types for vaccination. The Italy-02 strain wild type strain has become less prevalent in many European countries with the exception of Spain. On the other hand, several Chinese genotypes, one of which is called QX serotype has increased its prevalence in many countries of Europe included in the study except Spain and the UK. There seems to be a need for a good vaccine to protect chickens against QX-like strains.

Dr. Cavanagh presented data on prevalence of coronaviruses and astroviruses in turkey populations in Great Britain. Prevalence of turkey coronavirus was at least 50% and the presence of this pathogen was associated with enteric disease. On the other hand, the presence of astroviruses, although highly prevalent (30-40%) in the flocks, was not always associated with disease. It seems that more experimental information is required to assess the role of astroviruses in disease.

The threat of a flu pandemic has provided opportunities to search for other avian pathogens in the sampled materials. In this context, Dr. Jonassen provided evidence for a novel coronavirus infecting ducks, geese, and pigeons in Norway. The agent was identified and characterized by molecular procedures. The isolation of this novel coronavirus is necessary to better understand its role in disease of wild birds.

Drs. Gough and Cavanagh jointly presented data on the isolation and characterization of a new coronavirus from psittacine birds. The virus was isolated from a green-cheeked Amazon parrot showing a dilated proventriculus. This was the first demonstration of a coronavirus affecting psittacines.

Session: AVIAN CORONAVIRUS - VIRUS PROPERTIES

Dave Cavanagh, Institute for Animal Health, Compton Laboratory, Compton, UK

A common factor linking papers in this session was gene sequences, although the objectives of the authors varied.

Ching-Ho Wang and Antonio Zanella addressed a topic that has not received as much attention as it should: the changes that occur to the genome of infectious bronchitis virus (IBV) during attenuation of pathogenicity by passage in embryonated eggs. In other words, what is the molecular explanation of attenuation? Antonio and colleagues had investigated part of the S1 gene, which encodes the most variable of the IBV proteins. Of the three attenuated strains examined, only one had an amino acid change in this region. Even then, it was only a single change. He concluded that probably changes in other genes were responsible for attenuation. Ching-Ho also reported sequence for three attenuated strains, this time for the ~7.5 kb of the genome that included gene 2 (encoding the S protein) and all the genes downstream to and including the 3' untranslated sequence (3' UTR). This revealed: up to 7 changes in S1; 2 to 3 in S2; 1 to 3 in the M protein; zero or 1 in the E protein; and none in the N protein. There was no consistent pattern to the location of the changes. This data is going to be very useful as we manipulate the genome of IBV to understand the attenuation process.

A major difference between now and the first symposium in this series is that we are now much more aware of coronaviruses in avian species other than the chicken and turkey. Some papers related to this aspect were presented on the previous day, with more in the present session. I reported the work of Francesca Culver and Regina D'Arce, being sequence for the ~7.5 kb of the pheasant coronavirus (PhCoV) genome that included gene 2 (encoding the S protein) and all the genes downstream to and including the 3' untranslated sequence (3' UTR). The new data, that for the S, M and N genes, confirmed that PhCoV has no remarkable genomic features that would distinguish it from IBV. Even the S1 protein is no more different from that of IBV than is exhibited amongst serotypes of IBV. Taken together with recent studies elsewhere, a picture is emerging of a 'cloud' of avian coronaviruses with a demonstrably similar genome organisation and much conservation of protein sequences, and which have overlapping host ranges.

Recombination is often claimed to be behind some of the sequence similarities and discontinuities observed amongst IBV strains. I described the work of Sousan Izadkhasti who had used our reverse genetic system to test the hypothesis that recombination within the S gene would generate viable recombinants. Sousan showed that approximately 50% of the recombination events that occurred between the S genes of IBV strains Beaudette and B1648 resulted in viable virus. Of the four recombinants studied in depth, three replicated as well as the B1648 strain which had donated most of the S sequence of those recombinants. Thus whilst I still believe that it is difficult to claim with certainty that a given field strain arose by recombination between stated parents, our work shows that recombination within the S gene does generate viable viruses at high frequency.

Laura Villarreal reported evidence for a very exciting development, namely a new coronavirus associated with diarrhoea in chickens in many regions of Brasil. The exciting element is that this virus is not IBV, and would appear to be very different from IBV. Although it has a 3'UTR like that of IBV and other Group 3 coronaviruses, none of the IBV oligonucleotide primers tried by Laura in RT-PCRs for any gene gave a product, indicating extensive differences compared with IBV. Even PCR primers based on a very conserved region of gene 1 of known avian coronaviruses did not give a product. Corresponding gene 1 primers based on sequence of Group 2 coronaviruses e.g. bovine coronavirus, did give a product, the sequence of which confirmed it as being a coronavirus. Primers for other Group 2 virus genes did not give PCR products. So, the nature of the genome downstream of gene 1, apart from the extreme 3' end, is a mystery. Clearly much more needs to be done with this virus. Who knows, may be this virus is as prevalent – and as economically important - as IBV?

Session: AVIAN CORONAVIRUS - VIRUS PROPERTIES AND DIAGNOSIS

Richard C. Jones, University of Liverpool, Leahurst, Neston, UK

A paper on the use of a chick embryo-related cell line maintained in serum-free medium and used for the assay of infectious bronchitis virus as presented by Tereza Cardoso and colleagues from UNESP, Sao Paolo, Brazil. The M41 strain produced a clear cytopathic effect and serum neutralisation tests could be done conveniently using this sytem. Plaquing of the virus was also done easily. This cell free medium shown promise for cell cultures without the need for bovine serum.

Another paper from Brazil, given by Dr M. Montassier and colleagues from Jabotical, described a lectin-capture (LC) RT-PCR, which has the speed and convenience of ELISA and the sensitivity of PCR. Allantoic fluids and tissue materials were examined and the LC RT-PCR proved more sensitive than a capture ELISA for detecting IBV. The sensitivity of the LC RT-PCR remains to be compared for sensitivity with the conventional RT-PCR.

Kannan Ganapathy from the University of Liverpool, presented a paper reviewing some aspects of IBV or APV detection in clinical material. IBV was detected in selected tissues of chickens killed at 10 days after infection with Mass IBV and examined at intervals up to 24 hours after storage at fridge temperature. Virus persisted in all tissues and RT-PCR was the most sensitive, with tracheal organ cultures and eggs less sensitive. Intestine was the most successful source of virus at 24 hours. Other studies shoed that after infection of chicks with virulent or attenuated avian pneumovirus, it was detectable in cloacae swabs. The role of enteric APV in pathogenesis is unknown. Finally, comparison of single or pooled treatment of tracheal swabs from infected birds showed discrepancies which probably depend on the virus concentration in the positive swabs.

Christine Winter and colleagues of the Hannover Veterinary School showed that alpha 2,3 linked sialic acid serves as a receptor determinant on cultured cells for the Beaudette and Massachusetts IBV strains. Prior treatment of organ cultures with neuraminidase before infection with IBV resulted in longer lasting ciliary activity compared to untreated cultures. The authors suggest that sialic acid plays an important role not only for cultures cells but also in determining the pathogenesis of infection of chickens with IBV.

Session: AVIAN CORONAVIRUS - PATHOGENESIS AND IMMUNOLOGY

Jadgev Sharma, University of Minnesota, St. Paul, Minnesota, USA

Dr. Jones inoculated M41 Massachusetts IBV in 36 SPF hens and noted that there was great variation between hens in the response to the virus. This result cautioned that when you see a fall in the egg production in a flock. there may be a wide variation between individual hens within the flock.

Dr. Villarreal evaluated the presence of IBV and APV in the testes and other tissues of 57-week-old roosters from a flock with a history of epidydmal stones and low fertility. IBV was located in the testes, trachea and lungs. APV was localized only in the testes. Testes homogenate was inoculated in chicken embryos. The allantoic fluid had IBV. These results provided circumstantial evidence that both IBV and APV may play a role in the testicular diseases in roosters.

Dr. Toro and colleagues examined the effect of immunosuppression on the pathogenesis of IBV. Immunosuppression was induced by infection with CAV and IBDV. Tracheal and nasal rales lasted longer, virus persisted longer, lesions were more severe and recovery occurred later in the immunosuppressed birds than in intact birds.

Dr. van Santen and colleagues used a similar immunosuppression model to examine pathogenesis of IBV. Immunosuppression prolonged IBV-induced respiratory distress and delayed the appearance of IgA in tears. An interesting observation was that IgA levels in tears and anti-IBV antibodies in the circulation were higher in CAV + IBV birds than in IBV alone birds.

Session: AVIAN CORONAVIRUS - VACCINATION AND CONTROL

Erhard F. Kaleta, Justus Liebig University of Giessen, Giessen, Germany

This session comprizes five papers on vaccination and monitoring of the immune responses following application of vaccines.

Dr. J.K.A. Cook presented a brief historical review on the origin and subsequent worldwide use of the H strain of infectious bronchitis. This strain was separated as early as 1955 into two substrains of differing passage levels and is probably the most often strain for the vaccination of youg chicks and asdult birds. The two differing levels of attenuation and the broad range of cross protection of other IB variants resulted successful prevention of IB associated losses. Dr. Cook made it also clear, that for the time being vaccination with the H strains will play a dominant role in the chicken area.

Unfortunately, Dr. Ji-Yong Zhou from China was unable to attend and present his contribution on a newly developed “edible vaccine” against infectious bronchitis.

Dr. J.J. de Wit presented a contribution on the serologic response of IB vaccinated chickens by using an α-IBV IgM ELISA. He analysed the data in an attempt to reach predictive values that would allow to estimate the degree of protection and by doing so, to contribute to the efficacy of IBV vaccination in the field.

Compatibility of vaccines following oral or parenteral applications is together with efficacy a factor of dominant importance. Dr. I. Tarpey presented his experience of simultaneous and successive vaccinations using vaccines against infectious bronchitis, Newcastle disease and avian pneumovirus. All three viruses replicate in the respiratory epithelium of the upper respiratory tract and negative interference is likely to occure. However, the vaccines under his study were unnocious following simultaneous application and provided full protections as shown by challenge experiments.

Dr. J.J. de Wit focused in his second talk on the effects of maternal antibodies that were induced by five different vaccination schedules. A newly developed strain “D388” provided protection against homologous challenge.

Dr. I. Tarpey presented data on embryo vaccination. . Earlier studies have clearly demonstrated that all IBV vaccine strains are lethal for embryos and can not be applied to embryos at any age. His major point is the development of a new recombinant of theBeaudette strrain and the spike protein of the virulent M41 and importance of exact timing of vaccination with respect to the embryonic development at 18 days of incubation. Initial date indicate that the recombinant is promising in terms of innocuity and efficasy.

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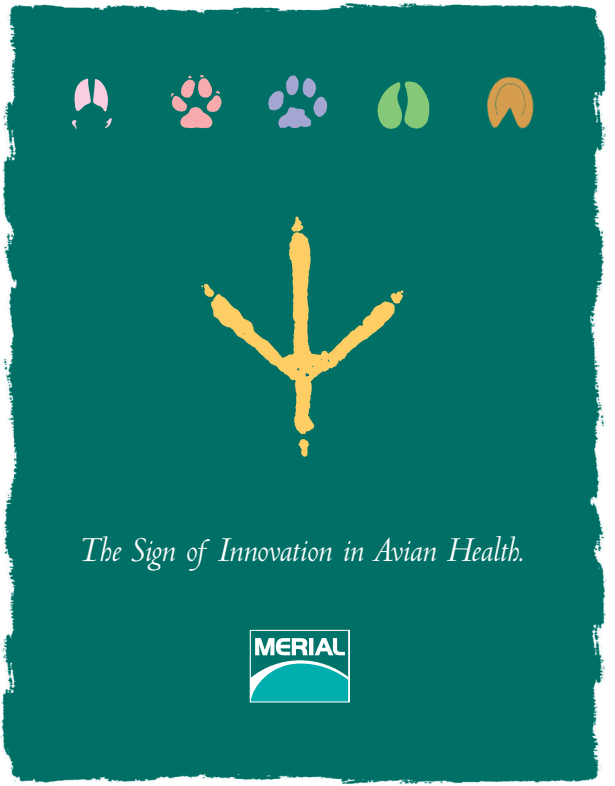

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