# Competitive ELISAs confirm that equine arteritis virus-infected horses develop antibodies to the M viral envelope protein

INAUGURAL-DISSERTATION
zur Erlangung des Doktorgrades beim
Fachbereich der Veterinärmedizin
der Justus-Liebig-Universität Gießen

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Eingereicht über das Institut für Virologie
der Justus-Liebig-Universität Gießen
im Fachbereich vertreten durch: Prof. Dr. Heinz-Jürgen Thiel

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In memory of

Susan Ellenor Haines

(23.03.1968-18.01.2004)

**“You better stand tall**
**when they are calling you out,**
**don’t bend, don’t break;**
**Baby don’t back down.”**
(John Bon Jovi)

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## 1. Abbreviations

| | |
|---|---|
| aa | amino acids |
| Ala | alanine |
| Arg | arginine |
| Asn | asparagine |
| ATTC | American Type Culture Collection |
| BALB/c | “Bagg Albino” inbred mice strain |
| ˚C | degree Celsius |
| cDNA | complementary deoxyribonucleic acid |
| c-ELISA | competitive enzyme-linked immunosorbent assay |
| Cys | cysteine |
| d-ELISA | direct enzyme-linked immunosorbent assay |
| $dH_2O$ | distilled water |
| EAV | equine arteritis virus |
| EDTA | ethylene-diamine tetra-acetic acid |
| ELISA | enzyme linked immunobsorbent assay |
| EVA | equine viral arteritis |
| g | relative centrifugal force |
| Gln | glutamine |
| Gly | glycine |
| Gp | glycoprotein |
| h | hour |
| His | histidine |
| IFA | immunofluorescense assay |
| Ig | immunoglobulin |
| Ile | isoleucine |
| kDa | kilodalton |
| l | liter |
| Leu | leucine |
| Lys | lysine |

| | |
|---|---|
| M | Mol |
| MAb | monoclonal antibody |
| MEM | minimal essential medium |
| Met | methionine |
| min | minute(s) |
| ml | milliliter |
| MLV | modified live virus |
| M.O.I. | multiplicity of infection |
| mRNA | messenger ribonucleic acid |
| nsp | non structural protein |
| OD | optical density |
| OPD | o-phenyldiamine |
| ORF | open reading frame |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PFU | plaque forming units |
| pH | pondus hydrogenii (measurement of hydrogen ion concentration) |
| Phe | phenylalanine |
| Pro | proline |
| qs | quantitate to |
| RNA | ribonucleic acid |
| rpm | rounds per minute |
| RT-PCR | reverse transcription-polymerase chain reaction |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| Ser | serine |
| sg | subgenomic |
| T | temperature |
| Thr | threonine |
| TRS | transcription regulating sequence |
| Tyr | tyrosine |
| µl | microliter |

## 2. Introduction

Descriptions of the disease equine viral arteritis can be found as far back as the late eighteenth and early nineteenth century, where it is referred to as “pinkeye”, “infectious or epizootic cellulites”, “influenza erysipelatosa”, “Pferdestaupe”, “Rotlaufseuche” and “equine influenza” (Bergmann, 1913; Burki and Gerber, 1966; Clark, 1892; Hofer and Steck, 1976; Mumford, 1985; Plateau and Jacquet, 1888). The disease, which affects all equines including horses, donkeys and mules, is caused by equine arteritis virus (Hofer and Steck, 1976; Newton et al., 1999; Paweska and Barnard, 1993; Paweska, 1994). The virus was first isolated in 1953, during an extensive outbreak of respiratory disease and abortion on a Standardbred breeding farm near Bucyrus, Ohio USA (Doll et al., 1957). After isolation of the virus from the lungs of an aborted fetus and the description of characteristic vascular lesions, EVA was distinguished from equine influenza (equine influenza virus) and equine rhinopneumonitis (equine herpes virus), which cause similar clinical syndromes (Doll et al., 1957a).

Today EAV is geographically wide spread (Chirnside, 1992) and most European and North American countries have EAV-seropositive animals, due to international movement of horses for breeding or competition (Hullinger et al., 2001; McCollum and Bryans, 1973; McKenzie, 1988; Timoney, 1992). Over the last years, there has been an apparent increase in the number of confirmed outbreaks of EVA worldwide, suggesting that EVA is an emerging disease (Timoney, 1986). Tests of randomly selected horse sera in Germany showed an increase of EAV antibodies from 1.8% in 1987/1988 to 24.8% in 1994 (Kaaden et al., 1996). EU Community measures were confined to import restrictions for carrier stallions from non-EU countries. In March 1995 the German State Veterinary Service changed the status of EVA and it was declared as a notifiable disease (Eichhorn et al., 1995; Kaaden et al., 1990). This reflects an international trend, triggered by the significant economic impact EVA outbreaks can have, especially in the horse breeding and racing industries.

Reliable testing is the key to continued safe movement of horses for breeding and athletic competition. The current officially recognized serological test is the serum neutralisation test, which is highly specific but suffers from the disadvantages that it is

slow and time consuming. ELISA-based serological assays offer considerable theoretical advantages for the serological testing of horses for previous exposure to EAV. Thus the development of a more convenient and rapid test for serologic diagnosis of EAV infection of horses has been a consistent research goal in recent years (Balasuriya et al., 2002; Chirnside et al., 1995a; Hedges et al., 1998; Inguiez et al., 1998; Kondo et al., 1998).

The objective of this study was to further evaluate the serological response of horses to EAV infection by comparing data obtained with known EAV-positive and negative horse sera and several competitive ELISAs to different EAV structural proteins (E, $G_L$, M and N) and non structural proteins (nsp1). This required that MAbs first be produced to the M and nsp1 proteins of EAV.

# 3. Literature review

## 3.1 Equine arteritis virus

### 3.1.1 Classification

Equine arteritis virus was first designated as a ribolipovirus and grouped into the "hemoviruses" along with hog cholera and bovine viral diarrhea viruses (Burki, 1965; Burki and Gerber, 1966). This group of viruses is not arthropod-transmitted, although Breese and McCollum et al. (1970) initially considered EAV to be an arbovirus based on its morphological characteristics.

During further characterization some morphological and physicochemical properties similar to alphaviruses (Horzinek et al., 1971; Hyllseth et al., 1970; Maess et al., 1970; Magnusson et al., 1970) and flaviviruses (Burki, 1970; Burki et al., 1972; Hyllseth, 1970a) were identified. Two comparative studies found that EAV was distinct from viruses in those two groups (Horzinek, 1973; Horzinek, 1973a; Horzinek, 1981). On the basis of morphologic criteria such as size, an isometric core and the presence of an envelope, it was proposed that EAV be classified as a non-arthropod-borne togavirus along with the pestiviruses and rubiviruses, lactate dehydrogenase-elevating virus (LDV), simian hemorrhagic fever virus (SHFV) and cell fusion virus (Porterfield et al., 1978). EAV, LDV and SHFV were subsequently assigned in 1984 to a new genus arterivirus within the family *Togaviridae* (Westaway et al., 1985). However, the structural proteins of LDV and EAV were known to differ from those of the togaviruses (Plagemann, 1996; van der Zeijst et al., 1987). Sequence analysis as well as characterization of the replication of these viruses in the last decade showed that EAV is evolutionarily related to viruses from the coronavirus-like superfamily (den Boon et al., 1991; Seybert et al., 2000; Snijder et al., 1993). In 1996, during the 10th International Congress of Virology (Jerusalem), EAV, LDV, SHFV and porcine reproductive and respiratory syndrome virus (PRRSV; Meulenberg et al., 1994), were included in the family *Arteriviridae* (Cavanagh, 1997).

The basis for the classification of arteriviruses as an independent family was their distinct biological properties, including their unique structural proteins, the virion morphology, and their ability to establish persistent infections. Furthermore sequence

studies revealed an unexpected relationship with members of the coronavirus family at the level of genome organization and expression (den Boon et al., 1991; de Vries et al., 1990; Godney et al., 1993; Kuo et al., 1992; Meulenberg et al., 1993; Murtaugh et al., 1995; Thiel et al., 1993). The common link is reflected in the genome expression of both families, which includes the generation of a nested set of sg mRNAs. This fact led to the establishment of the order Nidovirales, which includes the arterivirus and coronavirus families (Cavanagh, 1997).

### 3.1.2 Morphology and physicochemical properties

EAV is an enveloped single-stranded positive sense RNA virus (Horzinek, 1981; Maess et al., 1970; Magnusson et al., 1970). The virion is a spherical, 50-65 nm particle with a 30-35 nm icosahedral core with a lipid envelope (Horzinek et al., 1971). The envelope includes 12-15 nm diameter ring-like structures (Hyllseth, 1973) and its membrane is derived from a pre-Golgi compartment (Plagemann, 1996). The morphology of extracellular virus particles was established by negative and positive staining of purified virus preparations (de Vries, 1994; Horzinek et al., 1971; Hyllseth, 1973; Maess et al., 1970). The reported size of the virus particle varies from 50 to 65 nm, depending on the purification and staining method used (Burki, 1966; Maess et al., 1970 Maess et al., 1970a; Magnusson et al., 1970). De Vries (1994) demonstrated tiny “spikes” on the viral surface and showed a small fraction of virus particles to contain two nucleocapsids by using cryo-electron microscopy.

The buoyant density of EAV has been determined using sucrose, cesium chloride and potassium tartrate gradients (Hyllseth, 1970; Hyllseth, 1973; Maess et al., 1970). Depending on the substrate used, the infectivity peak varied from 1.155 – 1.19 g/cm$^3$ (Iwashita ad Harawasana, 1987; Maess et al., 1970; van der Zeijst and Horzinek, 1975; Zeegers et al., 1976). The sedimentation coefficient of EAV particles is approximately 224 S +/- 8, whereas the nucleocapsid sediments at 158 S (van der Zeijst and Horzinek, 1975).

The EAV virions are highly unstable at a pH other than 6 to 7.5, or in solutions that contain low concentration of non-ionic detergents (Burki, 1966; Konishi et al., 1975). The virus remains viable during prolonged storage at -20 to -70°C (Crawford and

Henson, 1973). EAV quickly loses its infectivity when stored at temperatures of 4°C and higher. As shown for the Bucyrus and Bibuna strains (Konishi et al., 1975), incubation at 37°C or 50°C for 30 minutes had no dramatic effects on the virus infectivity whereas incubation at 60°C for 30 minutes reduced the virus titre by 4.0 to 5.75 $\log_{10}ID_{50}$.

Reports on the effect of trypsin on EAV are controversial. In a study by Burki and Gerber (1966) the virus was reported to be resistant, whereas Konishi et al. (1975) found a reduction of virus titre after trypsin treatment. EAV is inactivated by ether and chloroform (Burki, 1965; Doll et al., 1957a), sodium deoxylcholate 1 mg/ml (Konishi et al., 1975) and by incubation with phospholipase C for 30 minutes at 37°C (Hyllseth, 1973). The envelope can readily be removed by treatment with 1% Triton X-100 or other non-ionic detergents, which leaves pure nucleocapsid preparations (Horzinek et al., 1971).

### 3.1.3 Growth in cell culture

EAV, in contrast to the extreme cell type specificity of the other members of the *Arteriviridae* family, is not fastidious in its growth in vitro. The virus replicates well in primary cultures of endothelial, kidney, ovary and dermal cells (Burki, 1965; Klavano, 1987; McCollum et al., 1961; McCollum et al., 1962; McCollum et al., 1962a; Shinagawa et al., 1976; Wilson et al., 1962), as well as a variety of cell lines such as baby hamster kidney (BHK-21; Hyllseth, 1969; Maess et al., 1970), rabbit kidney (RK-13; McCollum et al., 1970), rhesus monkey kidney (LLC-MK2; Breese and McCollum, 1970), African green monkey kidney (VERO) and hamster lung cells (HmLu; Konishi et al., 1975). The appearance of the cytopathic effect (CPE) and titer of virus produced by different cell lines varies significantly. Rounding, vacuolation, increased optical density, refraction and detachment from the supporting surface characterize the CPE exhibited by EAV-infected cells. The CPE caused by EAV is used for quantity titration of virus in infected tissue cell culture and tissue culture fluids by endpoint titration dilution assay and plaque assays. The results obtained can depend on the passage history of the cells (Bagust, 1972; Fukunaga et al., 1981; Hyllseth, 1969; Konishi et al., 1975; Maess et al., 1970; McCollum, 1970; Radwan and Burger, 1973; van Berlo et al., 1980; Wilson et al.,

1962). Moore et al. (2002) showed that equine pulmonary endothelial cells can provide a relevant model for the in vitro characterization of the virulence determinants and pathogenesis of EAV.

The kinetics of EAV adsorption to different cell lines at different temperatures has been described (Higgins, 1993; Hyllseth, 1969; Radwan et al., 1973). Experiments have shown that approximately 50% of the inoculated plaque forming units of virus adsorb to BHK-21 cells after 1h of incubation at 37°C and almost 100% after 2h. One step growth experiments have shown that the first release of progeny virus is after 6 to 8h and peaks between 12 and 20h post infection (Inoue et al., 1975; Tsukamoto et al., 1982; van Berlo et al., 1982; van Berlo et al., 1986; van Berlo et al., 1986a), often with titers that exceed $10^8$ $TCID_{50}$ / ml. In RK-13 cells progeny virus appears at 8h after infection when incubated at 37°C, and a second round of replication begins at 18 to 24 h and peaks at 36 to 48h with a titer of $>10^7$ $TCID_{50}$/50 µl (Balasuriya et al., 1993; de Vries et al., 1994).

### 3.1.4 Molecular biology

#### Viral genetics and replication

The first indication that EAV is an RNA virus was evidenced by the ability of 5-iodo-2-deoyuridine to inhibit viral replication (Burki, 1965; Burki, 1966). Additional evidence was provided by the labelling of the viral genome with [$^3$H]uridine (Breese et al., 1971; Rawanda et al., 1973). Van der Zeijst and Horzinek (1975) demonstrated that the EAV genome is an infectious, single-stranded RNA molecule with an estimated molecular weight of $4x10^6$, as determined by polyacrylamide-agarose-gel electrophoresis (PAGE) and a $S_{20,W}$ value of 48 in isokinetic sucrose gradient in 0.1 M saline. RNA extracted from purified virus was infectious, but following RNase treatment the infectivity of the extracted RNA was completely abolished. Actinomycin D did not inhibit EAV replication (Van der Zeijst and Horzinek, 1975). These studies indicated, therefore, that EAV genomic RNA is positive sense.

The EAV genome includes 12,687 bases and contains at least nine open reading frames (ORFs; den Boon et al., 1991). ORFs 1a and 1b are located within the 9.7 kb at the 5'end of the genome and encode the viral replicase (van der Meer et al., 1998; Snijder et al., 1994; Snijder et al., 1993a). The ORFs 2a, 2b, 5, 6, and 7 encode the five structural

proteins (see 3.1.5), E, Gs, $G_L$, M and N, whereas ORFs 3 and 4 encode two poorly characterised glycoproteins, Gp3 and Gp4 (Chirnside et al., 1993; deVries et al., 1992).

**Attachment and entry**

The attachment and entry pathway of *Arteriviridae* is not yet fully characterized. The M and $G_L$ proteins were assumed to serve the viral targeting function. Studies using an infectious cDNA clone of PRRSV with a chimeric M protein of other arteriviruses showed that the surface structures composed by arterivirus M and $G_L$ ectodomains do not determine viral tropism (Verheije et al., 2002). Arteriviruses can replicate in several cell lines that are not susceptible to infection with virus particles, after transfection with genomic RNA (Inada et al., 1991; Meulenberg et al., 1996). This finding, together with the fact that certain cell lines that are not susceptible to LDV infection become susceptible after infection with murine leukemia virus (Inada and Yamazaki, 1991), suggests that cell tropism is determined by the presence of an as yet unidentified receptor on the cell surface. Studies on PRRSV have shown that low pH appears to be required for virus entry, which most likely occurs through the standard endocytic route (Kreutz et al., 1996). The entry was blocked by cytochalasin D (Glaser et al., 1997), a compound that inhibits receptor-mediated endocytosis.

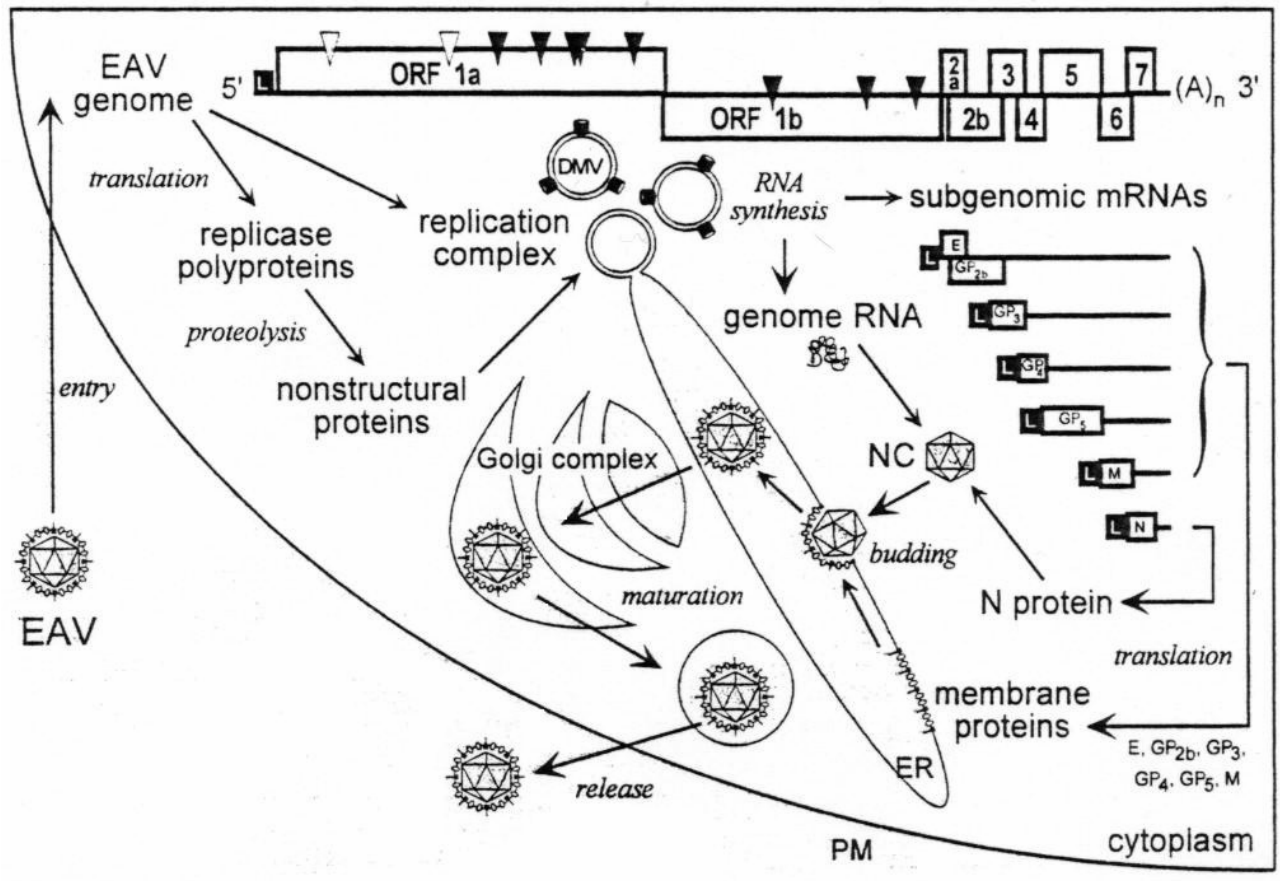
**Genome replication**

The replication cycle of EAV occurs entirely in the cytoplasm of infected cells (see Figure 1). A genome-length negative strand is generated that serves as the template for genome replication. Furthermore, nested sets of complementary sg negative strands and sg mRNAs are generated (Snijder and Meulenberg, 1998). The transcripts in each set are both 5'- and 3'- coterminal with each other and with the corresponding genome-length RNA. There is little known about the signals involved in the genome replication. Recently, natural and synthetic defective interfering RNAs have been generated for EAV (Molenkamp et al., 2000). Those RNAs invariably require at least 300 nucleotides from the 3' and 5' genome termini for efficient replication. The interaction of the signals with the host proteins or viral proteins is largely uncharacterized. It appears that four proteins, which have yet to be identified, interact with transcripts representing the corresponding region of the EAV negative strands, suggesting the involvement of a common set of host

factors in the initiation of arterivirus positive-stranded RNA synthesis (Hwang and Brinton, 1998).

Figure 1: Overview of the life cycle of EAV

The genome organization and the replicase cleavage sites (arrowheads) are shown at the top of the figure. At the right the nested set of subgenomic (sg) mRNAs and their common leader sequence (L) are illustrated. DMV, double membrane vesicle; ER, endoplasmic reticulum; NC, nucleocapsid; PM, plasma membrane. (Snijder and Meulenberg, 2001)



**Subgenomic mRNA transcription**

EAV and other members of the *Arteriviridae* characteristically generate a nested set of sg mRNAs during their replication cycle from which the genes in the 3' end of the genome are expressed. The mRNAs have a 5' terminal leader sequence that is derived from the 5' end of the genome, and they are 3' coterminal. Based on studies of EAV replication, it was postulated that arterivirus sg mRNAs are synthesized by a discontinuous transcription mechanism (den Boon et al., 1995). The presumed common ancestry of the arterivirus and the coronavirus replicase genes supports this concept. A substantial number of models for nidovirus sg mRNA synthesis have been proposed. Recently, a direct proof of base pairing between the positive leader transcription-regulating sequence (TRS) and the negative body TRS was obtained from reverse genetic studies using an EAV infectious cDNA clone (van Maarle et al., 1999). The stability of this duplex was shown to be a crucial factor in the synthesis of subgenomic mRNA of arteriviruses (Pasternak et al., 2003). Introducing site-specific mutations in the positive leader TRS can be compensated by introduction of the same mutations in the positive body TRS. The model of discontinuous negative-stranded synthesis was supported by TRS sequences found in mRNAs generated by TRS mutants with partial transcription activity (van Maarle et al., 1999).

**Translation and posttranslational processing**

The first step in EAV replication is the translation of the virus genome that yields two large replicase polyproteins, ORF1a and ORF1b protein. A ribosomal frame shift is required for the translation of ORF 1b, immediately before ORF 1a translation is terminated (den Boon et al., 1991). Two signals that promote this event are contained in a region overlapping ORFs 1a and 1b. This region is a so-called slippery knot sequence, which is the actual frame shift site, and a downstream RNA pseudoknot structure. A frame-shifting efficiency of 15 to 20% was observed, using a reporter gene construct for EAV (den Boon et al., 1991). In the post-translational phase the ORF 1b polyprotein is cleaved at least ten times by three different ORF 1a-encoded proteases. In combination with the ribosomal frame shift, this leads to the generation of 12 processing end products and a large number of processing intermediates. The end products are named non-structural proteins 1 to 12, nsp1-8 are generated by ORF 1a and 10-12 by ORF 1b. Nsp9

consists of a small, ORF 1a-encoded N-terminal domain, and a large C-terminal part that is encoded by the 5'-proximal region of ORF 1b. The EAV protease domains are located in nsp1 (29kDa), nsp2 (61kDa), and nsp4 (21kDa). These proteases and their cleavage sites are well conserved (Snijder et al., 1995). Both, nsp1 and 2 contain a cysteine autoprotease domain that mediates, probably exclusively in cis, their rapid release from the polyprotein. The N-terminal replicase subunit, nsp1, was shown to be dispensible for genome replication but absolutely required for sg RNA transcription (Tijams et al., 2001). The nsp1 protease has been characterized as a papain-like cysteine protease (PCP; Snijder et al., 1992; denBoon et al., 1995a), with residues Cys-164 and His-230 forming a putative catalytic dyad in EAV. Nsp1 consists of two papain-like protease domains and a predicted N-terminal zinc finger (Tijams et al., 2001). The nsp2 cysteine protease is an unusual cysteine protease showing similarities in the amino acid sequence to papain-like and chymotrypsin-like proteases (Snijder et al., 1995). Nsp4, a serine protease, is the prototype of the 3C-like serine proteases, a novel group of chymotrypsin-like enzymes (Snijder et al., 1996). The EAV replicase subunits localize to the perinuclear region of the infected host cells, where they are associated with the intracellular membranes, exclusively or predominantly with those of the endoplasmatic reticulum (Balasuriya et al., 2000; Pederson et al., 1999; van Dinten et al., 1996; van Dinten et al., 1997). This complex has been shown to be the site of de novo viral RNA synthesis by electron microscope studies (Balasuriya et al., 2000; Faaberg and Plagemann, 1995). Parts of nsp1 are the only exception regarding the perinuclear localization of replicase subunits. Fractions of nsp1 have been found in the host cell nucleus, especially during the earlier stages of infection (Tijams et al., 2002). The fact that an autoimmune response against Golgi-antigens has been reported in mice after infection with EAV, may offer an approach to determine the mechanism by which such responses develop and become of biological importance (Weiland and Weiland, 2002). After infection with EAV, host cell membranes are modified into vesicular double membrane structures that carry the *Arteriviridae* replication complex (Balasuriya et al., 2000; Porterfield et al., 1978). Predictions relating to the functions of the ORF 1b encoded replicase subunits are largely derived from comparative sequence analysis. An in vitro assay demonstrated that nsp10 has a duplex unwinding activity (Senne et al., 1985). ORF 1b encodes two additional

conserved domains that appear to be unique to nidoviruses: a conserved domain in nsp11 and a Cys-His-rich domain in the N-terminal region of nsp10, upstream of the helicase. A replacement of Ser-2429 by Pro in nsp10, rendered the EAV full-length cDNA clone non-infectious owing to an almost complete block of sg mRNA transcription (Crawford et al., 1973; van Dinten et al., 1997) The mutant replicated its genomic RNA efficiently, showing that replication and sg mRNA transcription are distinct processes.

### 3.1.5 Structural proteins

After a number of conflicting, often contradictory reports, research has shown that arteriviruses possess six or seven structural proteins, which is unusually large compared to other positive stranded RNA viruses (Chirnside, 1992; de Vries et al., 1992; Dobbe et al., 2001; hyllseth, 1973; Iwashita et al., 1987; van Berlo et al., 1986; Zeegers et al., 1976). The E protein of EAV (7kDa) is encoded by ORF 2a, a gene conserved in all arteriviruses. The E protein is a small, hydrophobic nonglycosylated protein that is thought to be an integral membrane protein with an uncleaved signal anchor sequence in the central part of the molecule (de Vries et al., 1992; Snijder et al., 1999).

The ORF 2b encoded minor envelope protein was designated Gs (Gp2; Lepage et al., 1996). It is a typical class I membrane protein of 25kDa that contains complex type N-glycans when incorporated into virions. It appears to be indispensable for virion replication, since its deletion rendered the EAV full-length cDNA clone non-infectious (van Dinten et al., 1997). The Gs protein occurs in infected cells in four monomeric conformation as well as disulfide linked homodimers. Only the homodimers are incorporated into virus particels. The fact that the protein is retained in the ER upon individual expression in vivo (de Vries et al., 1995a; Meulenberg and Petersen-den Besten, 1996) indicates that it probably requires other structural proteins and/or virus assembly for transport from the ER to the Golgi complex.

The major glycoprotein ($G_L$; 30-42kDa) of EAV is N-glycosylated and includes 255 amino acids; it is also referred to as GP5. The putative ectodomain is 95 residues long and possesses a single N-linked polylactosamine side chain (de Vries et al., 1992). Some strains of EAV however, have an additional N-glycan chain (Glaser et al., 1995).

The $G_L$ ectodomain is an important antigen that is recognized by neutralizing and non-neutralizing antibodies in the horse (Chirnside et al., 1995; Chirnside et al., 1995a), and it may function in both receptor binding and membrane fusion (de Vries, 1994). Neutralization resistant variants (escape mutants) of EAV contained minor mutations or even substantial deletions in this region of ORF 5 (Balasuriya et al., 1995a; Balasuriya et al., 1999a; Larsen et al., 2001). Sequence analyses of the $G_L$ protein have shown that there is a high diversity amongst field and laboratory strains of EAV (Balasuriya et al., 1995a; Larsen et al., 2001). The number of glycosylation sites differs between strains (Glaser et al., 1995).

The major envelope protein (M; 16kDa; 162 aa) is the most conserved structural protein of the arteriviruses. It is encoded by ORF 6 and so also is referred to as GP6. It is a hydrophobic polypeptide and the N-terminal half contains three potential membrane-spanning regions that may imply a $N_{exo}C_{endo}$ orientation (Snijder and Meulenberg, 1998). The poor immunogenicity of the M protein is probably explained by the fact that only a short stretch of only 10 to 18 aa residues is thought to be exposed at the virion surface. Recently Jeronimo and Archambault (2002) discovered that the C-terminal portion of the M protein that includes aa 88 to 162, is recognized by EAV specific antisera. One of the membrane-spanning fragments is thought to function as a signal sequence. Little is known about the function of the M protein, although it probably plays an important role in virus assembly and budding, as shown for coronaviruses. The M protein accumulates in the endoplasmatic reticulum, where it forms disulfide-linked heterodimers with the $G_L$ protein (Crawford et al., 1996; Faaberg et al., 1995). A single Cys residue (Cys-8) in the short N-terminal ectodomain of the M-Protein ectodomain is involved in the formation of an intermolecular disulfide bridge with a Cys-34 residue in the $G_L$ ectodomain (Snijder et al., 2003).

The $G_L$-M heterodimers are essential for infectivity and are transported to the Golgi complex (de Vries et al., 1995) and incorporated into the virus particle at some point during this process. Disulfide-linked M protein homodimers were observed in cells infected with EAV, but these were not incorporated into virions. The M and N proteins of EAV are highly conserved (Balasuriya et al., 1995; Chirnside et al., 1994; Glaser et al., 1995; Hedges et al., 1996).

The nucleocapsid protein (N; 14kDa) is a small, phosphorylated, basic protein of 110 amino acids, and is also referred to Gp7. It is expressed abundantly in infected cells and constitutes about 20-40% of the protein content of the virion. The N protein interacts with genomic RNA during assembly of the nucleocapsid, although the important regions have not yet been defined. Sera from some EAV-infected horses contain high titres of anti-N protein antibodies. The N protein therefore, might provide a suitable antigen for EAV-specific serological tests (Chirnside et al., 1995b; Kehyar et al., 1997).

The EAV Gp4 protein is a class I integral membrane protein of 28 kDa with three functional N-glycosylation sites and with little of its carboxy terminus exposed. Gp3, on the other hand, is a heavily glycosylated protein whose hydrophobic amino terminus is not cleaved off. It is an integral membrane protein anchored by either or both of its hydrophobic terminal domains and with no parts detectably exposed cytoplasmically. Both proteins localize in the ER when expressed independently and in EAV infected cells. But only a small fraction of the synthesized Gp3 and Gp4 proteins end up in virions. Most of the oligosaccharides of these virion glycoproteins are biochemically mature (Snijder et al., 2003; Wieringa et al., 1985).

The specific functions of the envelope proteins of EAV and the other arteriviruses have yet to be fully characterized, but it has been shown by reverse genetics that E, GP2, GP3, GP4, GP5 and the M-protein are all essential for the production of infectious progeny virus (Meulenberg and Petersen-den Besten, 1996; Molenkamp et al., 2000a; Snijder et al., 2003).

### 3.1.6 Neutralization determinants of the virus

A variety of EAV-specific neutralizing monoclonal antibodies and peptide-specific antisera have recently been developed (Balasuriya et al., 1993; Balasuriya et al., 2001; Chirnside et al., 1988; Cho et al., 2000; Deregt et al., 1994; Glaser et al., 1995; Kondo et al., 1994; Tobiasch et al., 2001; Weiland et al., 2000; Yamaguchi et al., 1997). All neutralizing MAbs consistently recognize the major envelope glycoprotein ($G_L$), as determined by western immunoblotting, immunoprecipitation and ELISA assays. Amino acid residues 19 through 116 of the $G_L$ envelope glycoprotein are predicted to constitute the hydrophilic ectodomain (de Vries et al., 1992; de Vries, 1994). By subcloning and

expression of the $G_L$ envelope glycoprotein, Chirnside et al. (1995) mapped an immuno-dominant epitope between amino acids 55 through 98. In addition, they also demonstrated that a bacterial fusion protein covering this region and a $G_L$ specific synthetic peptide (residues 75 through 97) induced EAV-neutralizing antibodies in horses (Chirnside et al., 1995a). Based on these findings, it was concluded that amino acids 54 through 99 in the putative hydrophilic ectodomain of the $G_L$ protein were responsible for the virus neutralizing response in horses. Balasuriya et al. (1995) showed that amino acids 99 to 104 were critical to expression of three interactive neutralization epitopes on the $G_L$ protein. Furthermore mice immunized with a 20-amino-acid $G_L$ -specific synthetic peptide, which spans amino acids 93 to 112 of the $G_L$ protein, developed antibodies that neutralized EAV, confirming that this region is an important neutralization determinant of EAV (Balasuriya et al., 1997). Glaser et al. (1995) demonstrated that amino acid residues 96, 99, 100 and 113 are critical for virus neutralization. Most of the major neutralization epitopes recognized by different laboratories fall within the V1 variable region (amino acids 61 to 121) of the $G_L$ protein (see below).

### 3.1.7 Genetic and phenotypic variation

Many RNA viruses do not exist as a single genotype of defined sequence; rather they are genetically heterogeneous and exist as a population of related genomes (Domingo et al., 1980; Domingo, 1989; Domingo et al., 1993; Fukunaga e al., 1984; Westkott et al., 1995). In such populations, even under natural conditions, a particular genome may be selected over others altering the phenotype of the virus, the so-called virus “quasi-species” (Baranowski et al., 1999; Domingo et al., 1985; Domingo and Holland, 1988; Domingo, 1992; Domingo et al., 1992; Holland et al., 1992; Labrie and Moineau, 2002).

Although there is only one serotype of EAV, genetic variation amongst field strains of the virus has been demonstrated by RNase T1 oligonucleotide fingerprinting (Murphy et al., 1988; Murphy et al., 1992; Stadejek et al., 1999). Furthermore, oligo-nucleotide fingerprinting of sequential isolates from the same persistently infected stallion revealed an ongoing variation of 6 to >20% (Murphy et al., 1992a). Hedges et al. (1999) amplified EAV genomes from semen of two Thoroughbred stallions that were

infected during the 1984 outbreak of EVA and subsequently became long-term carriers. Sequence analysis of ORFs 2 through 7 showed a variation of approximately 1% per year. Nucleotide sequence homologies and phylogenetic analysis of the M and N protein genes (ORFs 6 and 7) of ten EAV isolates demonstrated strain variation and possible RNA recombination between EAV isolates (Chirnside et al., 1994). Sugita et al. (1994) showed the existence of distinct geographic groupings of EAV isolates by sequence analysis of the M protein gene of viruses from Europe and the United States. A difference in restriction fragment length polymorphism (RFLP) pattern of ORF 6 of seven EAV isolates and the modified live vaccine virus has been described (Sekiguchi et al., 1995). The genetic variation and phylogenetic relationship of ORF 5 of 3 different laboratory strains of the original Bucyrus strain of EAV, the modified live virus vaccine (ARVAC®, Fort Dodge Laboratories), and 18 field isolates of EAV from North America and Europe have been reported (Balasuriya et al., 1995a). The field isolates and laboratory strains of EAV compared in this study segregated into 4 phylogenetic groups that include two North American ($NA_1$ and $NA_2$) and two European ($E_1$ and $E_2$) groups. In addition, sequence data from individual European and North American isolates indicate movement of viruses between the two continents. Comparative analysis of deduced amino acid sequences of the $G_L$ protein of the viruses identified three distinct variable regions (V1 [aa 61-121], V2 [aa 141-178], and V3 [aa 202-222]), a putative signal sequence (S[aa 1-18]), and four conserved regions (C1[aa 19-60], C2 [aa 122-140], C3 [aa 179-201], and C4 [aa 223-255]; Balasuriya et al., 1995a). The same viruses were used by Hedges et al. (1996) to study the genetic variation of ORF 2 of EAV, which encodes the minor envelope glycoprotein ($G_S$) of the virus. The data indicated that the $G_S$ protein was highly conserved between isolates, considerably more so than the $G_L$ protein encoded by ORF 5. Phylogenetic analysis of ORF 2 and ORF 5 gave similar but not identical results indicating that different selective pressures are exerted on the two proteins. Sequence analysis of ORF 2 and 5 also suggest that EAV behaves as a "quasi-species" in nature (Hedges et al., 1999; Magnusson et al., 1970; Stadejek et al., 1999). There does appear to be minor antigenic variation amongst field strains of EAV, based on complement fixation test results and the requirement for complement for effective virus neutralization (Fukunaga and McCollum, 1977; Fukunaga et al., 1993; Fukunaga et al.,

1994; Golnik et al., 1986; McCollum, 1976). All field strains are neutralized by equine polyclonal antiserum to the virulent Bucyrus strain of EAV. Comparison of structural protein sequences has shown that the M and N proteins are more conserved than the glycoproteins (Balasuriya et al., 1993; Balasuriya et al., 1995; Glaser et al., 1995). In addition, the ORF 5 protein sequence is more variable (89.8-99.6% identity) than the ORF 2 protein sequence (Stadejek et al., 1999). Geographically and temporally distinct EAV isolates also vary in their virulence and in their abortigenic potential (Balasuriya et al., 1999; Burki, 1970; McCollum and Swerczek, 1978; McCollum, 1996; McCollum and Timoney, 1996a). Timoney et al. (1987a) reported in 1987 that virus strains shed in the semen of long-term carrier stallions are of low virulence and cause only asymptomatic infection in mares bred to these stallions. The clinical severity of EVA differs between outbreaks, and it can be difficult to isolate viruses from nasal swabs and blood of febrile horses. Intravenous inoculation of the blood of affected horses into susceptible animals may be required for virus isolation, all of which suggests that there is strain variation between EAV isolates (Clayton, 1987; Doll et al., 1957; McCollum and Bryans, 1973).

### 3.2 Clinical signs of an EAV infection

The clinical signs associated with an EAV infection vary widely from inapparent (or subclinical) to severe clinical infection. The signs displayed by EAV infected horses depend on a variety of factors including the age and physical condition of the horse(s), challenge dose and route of infection, strain of virus and environmental conditions (Timoney et al., 1987; Timoney and McCollum, 1993). The most common symptom of EVA is an upper respiratory tract infection but it can also result in abortion of pregnant mares, fulminant congenital infection in neonates, and persistent infection of the stallion with shedding of EAV in semen.

Subclinical or mild infections are very common, especially in mares bred to persistently infected stallions. Epidemiological investigations of the 1984 outbreak of EVA in Kentucky revealed considerable variation in the incidence of clinical disease in naturally infected horses. The ratio of clinical disease to inapparent infection in mares bred to a group of stallions with clinical EVA in this outbreak was 1.4:1. In contrast, the corresponding ratio of a group of mares bred to a persistently infected carrier stallion was

1:6 (Timoney and McCollum, 1988). During this epizootic 57% of affected farms had only 1 or 2 cases of EVA and just 14% of premises had 5 or more cases of the disease. The reason for this variation in the clinical severity of EVA is not clear. However, although there is only one serotype of EAV there is evidence of limited antigenic variation amongst EAV isolates (Murphy et al., 1988; Murphy et al., 1992) as well as variation in the pathogenicity of different isolates (McCollum and Timoney, 1996; Timoney and McCollum, 1988). The clinical disease produced by different isolates can range from the severe and lethal experimental infections caused by the Bucyrus strain of the virus, to clinically inapparent infections transmitted by many long-term carrier stallions (MacLachlan et al., 1996; McCollum and Swerczek, 1978; Timoney et al., 1988; Timoney and McCollum, 1993).

The clinical signs observed in natural cases of EVA vary considerably (Mumford, 1985; Timoney and McCollum, 1987). The incubation period of 3 to 14 days, usually 6 to 8 days following venereal exposure, is followed by pyrexia of up to 41ºC that may persist for 2 to 9 days. In addition to pyrexia, typical cases may have any combination of the following signs and symptoms: depression and anorexia; nasal and ocular discharge; conjunctivitis and rhinitis; leukopenia; periorbital and supraorbital edema; edema of the limbs, especially of the hind limbs; mid-ventral edema involving the scrotum and prepuce of the stallion and mammary glands of the mare; urticaria that may be localized to sides of the neck or face, or be generalized over most of the body; abortion of pregnant mares. Less frequently observed signs are icterus; photophobia; corneal opacity; coughing and dyspnea; abdominal pain and diarrhea; ataxia; petechiation of the nasal mucosa, conjunctiva and mucous membrane inside the upper lip; submaxillary and submandibular lymphadenopathy and adventitious edema in the intermandibular space, beneath the sternum or in the shoulder region (Burki and Gerber, 1966; Clayton, 1987; Collins et al., 1987 Doll et al., 1957; Doll et al., 1957a; Gerber et al., 1978; Jaksch et al., 1973; Jones, 1969; McCollum and Swercezk, 1978; Timoney, 1984; Timoney and McCollum, 1993). The most consistent clinical features of EVA are pyrexia and leukopenia. Regardless of the clinical severity, most naturally infected horses recover from EVA. In contrast, experimental inoculation with the virulent Bucyrus strain of EAV causes 60% mortality in adult horses (MacLachlan et al., 1996; Timoney and McCollum, 1993).

Neonatal foals can develop severe fulminating interstitial pneumonia (Carman et al., 1988; Vaala et al., 1992). Foals up to few months of age can develop a rapidly progressive pneumo-enteric syndrome (Golnik et al., 1981) and deaths of foals following natural infections have been reported.

Abortion in pregnant mares is often not foreshadowed by premonitory signs, and may occur late in the acute phase or early in the convalescent phase of the EAV infection (Bryans et al., 1957; Clayton, 1987; 1984; Doll et al., 1957; Timoney and McCollum, 1993). Abortions occur 10 to 33 days after EAV infection and are recorded between the $160^{th}$ and $300^{th}$ day of pregnancy following natural or experimental infection (Cole et al., 1986; Doll et al., 1957). In natural outbreaks of EVA, abortion rates have varied from less than 10% to between 50 to 60% (Timoney and McCollum, 1993). Infections with the strain of EAV that caused the 1984 Kentucky outbreak resulted in an abortion rate of 71% (Cole et al., 1986). There are indications that different EAV isolates have different virulence characteristics and abortigenic potential.

In the acute phase of EAV infection, stallions may undergo a period of temporary subfertility associated with reduced libido, decreased sperm motility, concentration, and percentage of morphologically normal sperm in ejaculates (Neu et al., 1992). In experimental infections, these changes persisted for up to 6 or 7 weeks after infection (Neu et al., 1988; Timoney et al., 1993). These changes were believed to be due to increased testicular temperature rather than a specific pathologic effect of the virus. Transmission of EAV from persistently infected carrier stallions to mares at the time of breeding does not appear to cause any short- or long-term virus-related fertility problems (Timoney et al., 1993). Some 30 to 60% of mature infected stallions become carriers of the virus and shed virus in their reproductive tract for periods that range from several months to lifelong infection.

Apart from abortion in pregnant mares and fulminant respiratory disease in young foals, mortality does not occur in natural outbreaks of EAV (Timoney et al., 1993).

### 3.3 Pathogenesis and pathology of EAV infection of horses

Information pertaining to the pathogenesis and pathology of EVA is mostly derived from experimental studies in horses inoculated with the virulent Bucyrus strain of EAV and by monitoring of natural outbreaks of EVA (Breese and McCollum, 1973; Cole et al., 1986; Crawford et al., 1973; Doll et al., 1957a; Estes et al., 1970; Fukunaga et al., 1981; Fukunaga et al., 1982; Henson and Crawford, 1974; Jones et al., 1957; MacLachlan et al., 1996; McCollum et al., 1962; McCollum et al., 1971; McCollum, 1976; McCollum, 1981; McCollum and Timoney, 1996; Prickett et al., 1973). After nasal infection, the virus rapidly invades the respiratory endothelium and alveolar macrophages. By day two it is present in the bronchial lymph nodes (McCollum et al., 1971). Replication occurs in the pulmonary lymph nodes, vascular endothelium and circulating monocytes. Virus then is disseminated throughout the body where it predominantly is localised in macrophages and dendritic cells in lymphoid tissues. The virus then further disseminates to a wide variety of tissues between 3 to 8 days after infection. Vascular injury is most severe at approximately 10 days, although it can persist for up to 14 days in the renal tubular epithelium. Infectious EAV is not detected after 28 days except in the reproductive tract of persistently infected carrier stallions.

The pathogenesis of abortion induced by EAV was controversial for a long time. Fetal infection and maternal factors, such as myometris and a reduced placental blood supply, originally were implicated (Coignoul and Cheville, 1984; Doll et al., 1957). A more recent study by MacLachlan et al. (1996), suggests that fetal stress, caused by in utero EAV infection, and subsequent activation of the fetal hypothalamic-pituitary axis more likely contribute to the pathogenesis of EAV induced abortion.

The most common gross lesions in horses infected with the Bucyrus strain of EAV are severe panvasculitis that results from replication of the virus in vessels. Vascular injury leads to edema, congestion, and hemorrhage of the subcutaneous tissue, lymph nodes and viscera (Doll et al., 1957; Jones, 1969; MacLachlan et al., 1996). The body cavities may contain moderate to abundant amounts of yellowish clear fluid. Lymphadenomegaly, edema, hemorrhage can occur along the course of the colonic and ceceal vessels but are also evident throughout the body (Jones, 1969). The lungs,

especially those of infected neonates, are wet and increased in weight, with a prominent lobular pattern. The trachea may contain froth.

Histopathological changes occur in various organs, but the blood vessels in all organs are the principal target. The vascular damage can vary from very mild lesions to severe endothelial and even transmural necrosis, leading to increased vascular permeability that results in the characteristic gross lesions. It is likely that the virus has a selective affinity for small arteries, specifically those of approximately 0.5 mm in diameter. These arteries are the smallest that have well developed muscular coats. Reports vary on the effects on arterioles, larger muscular and elastic arteries and veins (Del Piero et al., 1995; Estes et al., 1970; Jones, 1957). In general these vessels are less or not inflamed or necrotic. The lesions in the small arteries begin in the arterial media as necrosis of muscle cells, with loss of nuclei and replacement of cytoplasma with homogenous eosinophilic material (firbrinoid). Leukocytosis and edema of the adventitia are followed by edema of the media and infiltration with lymphocytes. The changes are often limited to small segments of affected arteries. Thrombosis can occur. The lungs show mild to severe interstitial pneumonia characterized by alveolar infiltration with macrophages and neutrophils, hyaline membrane formation, and fluid-filled alveoli. Follicle necrosis, edema and hemorrhage occur within the lymphoid tissues. EAV antigen is contained within the stromal dendritic cells and within the macrophages of lymph node sinuses and spleen. In adult horses lesions in the digestive system are most frequently observed in small intestine, large colon and cecum as edematous thickening of the submucosa along with engorgement of capillaries of submucosa and mucosa. The arterial lesions can range from simple necrosis and hyaline replacement of the media to thrombosis of the submucosal arteries with resultant infarction, mainly of the cecum and colon. Renal lesions occur in the terminal stages of infection and consist of tubular necrosis, lymphocytic interstitial nephritis and glomerular tuft disorganization.

In pregnant mares experiencing abortion the uterine epithelial cells are swollen with accompanying edema and infiltration of neutrophils. The myometrium may contain necrotic myocytes, macrophages and swollen endothelial cells within affected areas.

Transplacental infection of the fetus occurs during EAV infection of pregnant mares, although histological lesions in aborted fetuses and the placenta are either

relatively subtle or non-specific (Balasuriya et al., 1997; MacLachlan et al., 1996). The changes are represented by mild perivascular lymphocytic and mild interstitial pneumonia. More severe lesions are rare and consist of vasculitis involving the allantochorion, brain, liver, spleen and lung.

Foals that died a week after artificial inoculation showed more severe lesions. Infarction of the intestine was found more often than in the lung (Johnson et al., 1991; Jones et al., 1957).

Lesions in the male reproduction tract have been studied in experimentally infected prepubertal and peripubertal colts (Holyoak et al., 1993). During the acute phase of the infection, 7-14 days post- infection, acute necrotizing vasculitis involving the testis, epididymides, vas deferens, ampulae, prostate, vesicular glands and bulbourethral glands was present. Multifocal lympho-plasmocytic infiltrate in the ampulae and parachyma of the reproductive tract occurred in the chronic phase of infection.

### 3.4 Equine immune response to an infection with EAV

Immunity is prolonged after natural or experimental infection of horses with EAV (Doll et al., 1957; Doll et al., 1968; Fukunaga et al., 1990; Gerber et al., 1978; Hullinger et al., 1998; McCollum, 1986). Immune horses were protected from clinical EVA when challenged with the virulent strain of EAV, and the humoral immune response was characterized by the development of both complement-fixing and neutralizing antibodies to EAV (Fukunaga and McCollum, 1977; McCollum, 1970) that are detected 1 to 2 weeks after infection. The complement-fixing antibodies peaked at 2 to 3 weeks and steadily declined to disappear by 8 months post infection, whereas neutralizing antibodies peaked between 2 to 4 months and persisted for 3 years or more. The serum neutralization titers can increase with advancing age, likely as a consequence of reinfection (Burki et al., 1992). Mares with anti-EAV antibodies prior to being bred to carrier stallions also had an obvious anamnestic humoral immune response.

Colostrum from immune mares contains EAV- neutralizing antibodies that protect foals against clinical EVA (Hullinger et al., 1998; McCollum, 1976). The neutralizing antibodies appear in the serum a few hours after feeding of colostrum, peak at 1 week of

age, and gradually decline to disappear between 2nd and 6th month of age. Passive immunization studies have shown that the transfer of maternal antibodies is highly effective in preventing EVA, even following intranasal inoculation of highly virulent EAV strains (McCollum, 1976).

The serologic responses of EAV infected horses to the four known EAV structural proteins have recently been characterized (Hedges et al., 1998; Kondo et al., 1998). Differences in humoral immune responses of 34 horse sera were apparent in Western immunoblotting studies utilizing the M, N, $G_L$ and $G_S$ proteins expressed by recombinant baculoviruses and in purified virions (MacLachlan et al., 1996). The data show that the immune response of individual horses is dependent on the infecting EAV strain and interval after infection. The conserved M protein was most consistently recognized by sera from horses naturally and experimentally infected with EAV (MacLachlan et al., 1996). Another Western immunoblotting assay that utilized N and M proteins also identified the M protein as a major target of the equine humoral immune response (Kheyar et al., 1997). Recent immunisation studies using alphavirus-based expression vectors have shown that the co-expression of $G_L$ and M proteins is required for the induction of neutralizing antibodies in mice (Balasuriya et al., 2000). This result was then confirmed by a study, in which only horses that received a vector that co-expresses both the $G_L$ and M proteins were protected against challenge with a virulent strain of EAV (Balasuriya et al., 2001).

The response of horses to the non-structural proteins of EAV has not been determined yet. Recent testing of different equine sera by immonoprecipitation assay suggests that equine antisera recognize both the nsp1 and 2 proteins of EAV (Snijder, unpublished data).

### 3.5 Epidemiology

In 1953 a severe outbreak of EVA occurred on a standardbred stud farm in Bucyrus, Ohio, that ultimately resulted in the identification of EVA as a distinct virus disease of horses (Doll et al., 1957; Doll et al., 1957a). Since the original discovery several outbreaks have been reported from Germany (Burki and Gerber, 1966; Herbst and Danner, 1985; Kaaden et al., 1990; Liebermann, 1988; Nowotny and Burki, 1992; Pesch

et al., 1998; Schneller et al., 1996), Switzerland (Burki, 1970; Burki and Gerber, 1966; Golnik et al., 1981; Weiss et al., 1994), Austria (Jaksch et al., 1973; Kolbl et al., 1991; Nowotny, 1992; Nowotny and Burki, 1992), Poland (Golnik et al., 1981; Golnik et al., 1986), Italy (Autorino et al., 1992; Cancellotti and Renzi, 1992; Cecarelli et al., 1996), the United Kingdom (Anonymous, 1988; Anonymous, 1993; Camm and Thursby-Pelham, 1993; Higgins, 1993; Higgins, 1993a; Newton et al., 1999; Wood et al., 1995), Spain (Monreal et al., 1995), the Netherlands (van Gorkom et al., 1994), Canada (Clayton, 1987; Klavano, 1987) and the United States (Dwyer et al., 1993; McCollum et al., 1962; Mumford, 1985; Timoney, 1984; Timoney and McCollum, 1993). The first recorded outbreak in the United Kingdom followed the importation of an Anglo-Arab stallion from Poland (Higgins, 1993). The three major outbreaks in the United States were reported on a racetrack in Kentucky in 1977 (McCollum and Swerczek, 1978), in Thoroughbred horses during breeding season in 1984 in central Kentucky (McCollum and Timoney, 1996; Timoney et al., 1988) and on the Arlington racetrack in Chicago in 1993 (Dwyer et al., 1993). This last outbreak also affected horses at Churchill Downs, Prairie Meadows and Ak-Sar-Ben (McCollum et al., 1994). Today EAV is distributed throughout the world. Serological surveys have shown that EAV infection occurs in North America (Chirnside, 1992; Lang and Mitchell, 1984; McCue et al., 1991), Europe (Akashi et al., 1976; Akashi et al., 1975; Burki et al., 1992; de Boer et al., 1979; de Boer et al., 1978), South America (Nosetto et al., 1984), Australia (Anonymous, 1988; Huntington et al., 1990; McKenzie, 1988; McKenzie, 1990; McKenzie, 1996), Africa (Moraillon and Moraillon, 1978; Moraillon et al., 1978; Paweska, 1994; Paweska and Barnard, 1993) and Asia (Akashi et al., 1975; de Vries, 1994). Infection is especially prevalent in standardbred horses (McCollum and Bryans, 1973; McCue et al., 1991; Timoney et al., 1988). Serological surveillance in South Africa has shown that EAV infection also occurs in donkeys (Paweska, 1994; Paweska et al., 1997).

EAV can be spread both vertically (congenital infection of foals) and horizontally (respiratory and venereal transmission). It has been reported that exposure of pregnant mares in very late gestation to EAV can lead to transplacental transmission of the virus and congenital infection of the fetus (Balasuriya et al., 1999; Balasuriya et al., 1998; Vaala et al., 1992). EAV is not teratogenic, but congenitally infected foals develop a

rapidly progressive, fulminating interstitial pneumonia and/or fibronecrotic enteritis (Carman et al., 1988; Golnik et al., 1981; Lopez et al., 1994).

Horizontal transmission is of greater significance to the international spread of EAV than vertical transmission (Burki, 1965; Burki et al., 1992; Cole et al., 1986; Doll et et al., 1957; McCollum et al., 1961; Timoney et al., 1987a; Timoney et al., 1992; Timoney and McCollum, 1993). Horizontal transmission via the respiratory route occurs in acute infections following aerosolization of respiratory tract secretions from acutely infected horses. This has been demonstrated during the course of natural and experimental infections (Doll et al., 1957; McCollum et al., 1971; McCollum and Swerczek, 1978). Aborted materials such as fetuses, placenta and amniotic fluid and materials from infected animals, such as urine, vaginal and lacrimal secretions, feces and blood can also contribute to horizontal transmission (Cole et al., 1986; Fukunaga et al., 1981; McCollum and Timoney, 1996; Neu et al., 1988; Timoney and McCollum, 1993). Masturbates of acutely and/or chronically infected stallions have been discussed as another potential source for aerosol transmission (Burki et al., 1992). Considering that direct and close contact is necessary for aerosol transmission of EAV (Collins et al., 1987; Timoney and McCollum, 1988), the most important and primary route of dissemination of EAV during outbreaks is the aerosolization of infectious particles (McCollum et al., 1971; McCollum et al., 1971). The virus is shed in high concentrations in respiratory secretions for up to 7 or 14 days during acute infection (McCollum et al., 1971). However it has been demonstrated that direct and close contact is necessary for aerosol transmission of EAV (Collins et al., 1987; Timoney and McCollum, 1987). The second important mode of transmission is the venereal transmission, from acutely or chronically infected stallions that shed the virus in their semen (Kaaden et al., 1990; Timoney, 1986; Timoney et al., 1993; Timoney and McCollum, 1987). This was first demonstrated after the 1984 epidemic in Kentucky (Kaaden et al., 1990; Timoney et al., 1987; Timoney and McCollum, 1993; Timoney et al., 1987a). The venereal transmission by long-term carrier stallions is important for perpetuation and maintenance of the virus in equine populations throughout the world. They appear to shed virus solely by the venereal route. Some 85 to 100% of seronegative mares bred to long-term carrier stallion's seroconvert to EAV within 28 days after breeding. Mares are readily infected

following artificial insemination with semen from a long-term carrier stallion (McCollum et al., 1988).

Transmission also may occur through indirect contact (e.g. personnel, vehicles, twitches, apparels and shanks) and congenital infected foals (Collins et al., 1987; Timoney et al., 1988). Other potential modes of transmission of EAV appear to be teaser stallions and nurse mares (Timoney et al., 1993). EAV has been experimentally transmitted by the intravenous, subcutaneous, intranasal and intratracheal inoculation of lung or spleen suspensions from acutely infected horses (Doll et al., 1957; McCollum et al., 1971; Timoney and McCollum, 1993). EAV also has been successfully transmitted by inoculating tissue culture supernatants from infected cell lines (Fukunaga et al., 1982; MacLachlan et al., 1996). Burki et al. (1972) failed to propagate EAV in Aedine and Anopheline mosquitoes, and concluded that arthropod transmission is not involved in virus dissemination.

Until the 1984 epizootic in Kentucky there was very little information available regarding the carrier state of EAV. However it was noted in the nineteenth century that healthy stallions transmitted epizootic cellulitis-pink-eye and influenza to mares at the time of breeding, which very likely was EVA (Clark, 1892; Plataeu and Hacquet, 1888). It was postulated, based on clinical observation and transmission studies, that the causative agent of EVA is shed in the semen of certain convalescence stallions (Clark, 1892; Plataeu et al., 1888). In 1913 Bergmann et al. reported that a persistently infected stallion continued to transmit the disease to mares for almost seven years. He also reproduced the disease by inoculating susceptible horses with seminal fluid from a persistently infected stallion. Schofield (Bishop, 1989) described in 1937 two outbreaks of “equine influenza” that were attributed to carrier stallions. Investigations of the 1984 EVA outbreak on Thoroughbred farms in Kentucky reconfirmed the importance of the carrier stallion in the dissemination and perpetuation EAV infection (Timoney et al., 1987; Timoney and McCollum, 1992). Timoney et al. (1986) confirmed the chronic carrier state in Thoroughbred stallions naturally infected with EAV using test mating and/or isolation of virus from semen. Both short term and long term convalescent carriers were identified (Timoney et al., 1986; Timoney and McCollum, 1992). One of the possible factors that may influence the carrier state is the period of sexual rest given to

stallions following exposure to EAV. An insufficient period of sexual rest following exposure to the virus may predispose to establishment of persistent infection. Extensive epidemiological studies involving both, Standardbred and Thoroughbred horses, failed to demonstrate any breed predilection for establishment of persistent infections, with 30 to 35% of naturally infected stallions becoming long-term carriers (Timoney et al., 1992).

Persistently infected stallions can be divided into three groups based on their duration of virus shedding in semen (Timoney et al., 1987; Timoney et al., 1992). The short-term or convalescent carrier state lasts only a few weeks after clinical recovery. The intermediate carrier state lasts for 3 to 7 months in both naturally and experimentally infected animals (Timoney et al., 1992). The long-term or chronic carrier state can last for several years. Persistently infected long-term carrier stallions spontaneously may cease virus shedding after intervals of 1 to 10 years, with no apparent later reversion to a shedding state. The mechanism responsible for this spontaneous clearance of EAV from persistently infected stallions has not been discovered yet, but studies have shown that persistence of EAV in stallion is testosterone-dependent (Little et al., 1992; McCollum et al., 1994). When persistently infected stallions were castrated and treated with testosterone they continued to shed the virus in semen, whereas saline treated animals ceased shedding virus. The immunosuppressive effects of high levels of circulating testosterone also might allow the virus to persist in the male reproductive tract.

Studies of the persistence of EAV in prepubertal and peripubertal colts (Holyoak et al., 1993a) indicated that EAV could replicate in the male reproductive tract of a significant proportion of colts for a variable period of time after clinical recovery (up to six months). The absence of circulating concentrations of testosterone, equivalent to those found in sexually mature stallions, prevents long-term persistent infection with EAV and colts exposed to the virus before the onset of puberty did not become persistently infected. There is no evidence that carrier stallions are or can become intermittent shedders of the virus or have latent infection. The virus is contained within the sperm-rich fraction and not within the pre-ejaculatory fluid of semen, and there is little variation in virus titers contained in sequential ejaculates from the same stallion.

All carrier stallions have moderate to high titers of serum neutralizing antibody to EAV and shed the virus constantly in the semen but not in urine or in respiratory

secretions (Timoney et al., 1987). The ability of the virus to persist in the face of moderate to high titers of neutralizing antibodies clearly indicates that humoral immunity does not prevent viral replication within the male reproductive tract. EAV has not been isolated from the buffy coat of persistently infected stallions. EAV is localized in different parts of the male reproductive tract and highest titers of virus consistently have been demonstrated in the ampulla of the vas deference and the bulbourethral glands with $> 10^5$ PFU per gram of tissue (Fukanaga et al., 1992; Neu et al., 1988).

There is no evidence to indicate that mares or congenitally infected foals become persistently infected following infection with EAV (Timoney et al., 1988; Timoney and McCollum, 1993). EAV was not isolated from the reproductive tract of seropositive mares one month after infection (McCollum and Timoney, 1996) and the consistent failure to transmit the virus to susceptible horses during mating or at pasture has lead to the conclusion that the carrier state does not exist in the mare (Burki et al., 1992; Chirnside, 1992).

### 3.6 Diagnostics

The clinical signs of EVA vary widely and can occur in different combinations. A number of infectious and non-infectious diseases of horses show similar clinical signs, and therefore a presumptive diagnosis cannot be based only on the presence of characteristic signs (McCollum et al., 1970; Timoney and McCollum,1993). The list of differential diagnosis of EVA includes other viral respiratory tract infections of horses, such as equine herpesvirus 1 and 4, equine influenza, equine rhinovirus, equine adenovirus, equine infectious anemia and African horse sickness. Non-viral infections include leptospirosis, purpura hemorrhagica, urticaria and toxicosis due to hoary alyssum (Berteroa incana). The characteristic lesions of arteritis may help to differentiate EVA from some of the other diseases, but they are not pathognomic for EVA. After performing an immunoperoxidase histochemistry staining of skin biopsies from two EAV infected horses, Del Piero (2000) suggested that this test may be used as a supporting test for the clinical diagnosis, especially when a cutaneous macropapular rash is evident. In cases of abortion the primary differential diagnosis should include herpesvirus 1 and rarely herpesvirus 4. Herpesvirus-infected fetuses are expelled without any premonitory

signs; they are mostly fresh and have characteristic gross lesions. On the contrary fetuses aborted after EAV infections are often partly autolysed and lack pathognomic lesions.

The currently used, officially recommended, method for detection of EAV in semen and tissue samples is virus isolation in susceptible cell culture, although modern molecular methods are also available today (Belak et al., 1994; Gilbert et al., 1997; Herbst and Danner, 1985; Herbst et al., 1996; Klug and Sieme, 1999; Starik, 1999; St-Laurent et al., 1994).

The isolation and propagation of EAV from equine tissue was originally carried out on primary equine kidney cell cultures (McCollum et al., 1961), whereas the RK-13 continuous cell line is now more commonly used. Any viruses isolated in RK-13 cells are confirmed to be EAV by immunohistochemical staining with EAV-specific antisera (immunofluorescence, immunoperoxidase) or microneutralization assay with EAV specific antibodies. Virulent EAV strains, such as the Bucyrus strain, are not difficult to isolate, whereas other strains can be problematic. During the Kentucky outbreak in 1977 (McCollum 1981), viruses could not be isolated from nasal swabs or buffy coat of febrile horses. The same observation was made during the outbreak in Switzerland (Gerber et al., 1978). In addition there are reports that viruses that cause subclinical infections in Standardbred nurseries (McCollum ad Bryans, 1973) and the modified Bucyrus strain of EAV (vaccine) may be difficult to recover from affected horses (McCollum, 1969; McCollum et al., 1970).

Nasopharyngeal swabs, conjunctival swabs and citrated, heparinized EDTA blood samples for the separation of buffy coat are the usual samples for virus isolation from live horses. Semen is used for the detection of persistently infected stallions, with virus being detected by either isolation or RT-PCR assay. The specimens for the virus isolation should be collected as soon as possible after the onset of clinical signs of EVA. The nasopharyngeal and conjunctival swabs should be immediately placed in transport medium (any cell culture medium or balanced salt solution containing 2 to 5% antibody free serum) and either refrigerated or, preferably, frozen at $-20^0$C or lower (Timoney et al., 1988). For serological diagnosis acute and convalescent sera (paired serum samples) at a 21 to 28 day interval should be collected. In the case of abortion the placenta, fetal fluid, lung, spleen and lymphoid tissue should be collected for virus isolation and antigen

demonstration. In young foals that show signs of pneumonia and enteritis, a wide variety of tissues should be collected.

The continued movement of persistently infected stallions and infected semen emphasizes the need for a rapid, sensitive and specific test to detect EAV. Virus isolation in cell culture or test breeding of stallions to mares are both time consuming and expensive, thus reverse transcription-polymerase chain reaction (RT-PCR) based methods have been developed although they are not yet in widespread routine use (Belak et al., 1994; Gilbert et al., 1997; Ramina et al., 1999; Sekiguchi et al., 1995; Starik, 1998; St-Laurent et al., 1994). Detection of EAV by real-time PCR has been described recently by Balasuriya et al. (2002). TaqMan ® RT-PCR minimizes the risk of cross determination and provides an accurate and quantitative test for detection of EAV in clinical specimens.

Serological tests to determine previous exposure of horses to EAV include the serum neutralization (SN) test, complement fixation (CF) test and enzyme-linked immunosorbent assay (ELISA; Senne et al., 1985; Fukunaga and McCollum, 1977; Fukunaga et al., 1994; Fukunaga, 1994) The complement fixation test is simple and rapid, but it is rather insensitive and can only detect horses that were recently infected.

The current standard test for the serological diagnosis of EAV infection is the SN test (Senne et al., 1985). Although highly sensitive and specific, there are several inherent disadvantages to this test. The assay is expensive and time consuming to perform, and it requires the growth and maintenance of cell cultures as well as infectious virus. In addition, results tend to vary between laboratories when the reagents are not standardized. Addition of 10% guinea pig complement to the assay increases reproducibility and sensitivity (Fukunaga et al., 1993; Hyllseth and Pettersson, 1970; Maess, 1971; Radwan and Crawford, 1974; Radwan and Burger, 1973). The SN assay putatively detects antibodies to the variable $G_L$ protein, which includes the known neutralization determinants of EAV (Balasuriya et al., 1993; Balasuriya et al., 1995; Balasuriya et al., 1995a; Chirnside et al., 1995; Deregt et al., 1994).

Several different EAV-specific ELISAs have recently been developed in an effort to develop one that is sufficiently specific and sensitive to replace the SN test. These ELISAs include a variety of different antigen preparations, all based on EAV proteins.

Several ELISAs that incorporate whole virus or recombinant N, M and $G_L$ proteins have been described for the serological diagnosis of EAV infection (Chirnside et al., 1995; Chirnside et al., 1995a; Chirnside et al., 1995b; Kondo et al., 1998a, Starik et al., 2001). Iniguez et al. (1998) used a random peptide library displayed on phage as antigen in an ELISA. Assays that utilized whole virus antigen preparations produced an unacceptable number of apparent false positive results as horses vaccinated with tissue culture-derived vaccines can develop antibodies to cell culture proteins remaining in the EAV whole virus antigen preparation (Weiland et al., 2000). ELISA and Western immunoblotting assays that utilize the N, M and $G_L$ proteins of EAV, and peptides expressed as bacterial fusion proteins, all provide potentially sensitive and specific antigens for ELISAs for the serological diagnosis of EAV infection, however, the bacterial fusion partner may contribute significantly to background absorbance, potentially compromising specificity (Chirnside et al., 1995; Chirnside et al., 1995a; Chirnside et al., 1995b; Kheyar et al., 1997). An ELISA assay that incorporated individual baculovirus expressed M, N and $G_L$ proteins confirmed that the M protein is most consistently recognized by convalescent equine sera (Hedges et al., 1998). The responses of individual animals varied and ELISAs that utilized individual EAV structural proteins were not consistently reliable in the detection of sera that contained neutralizing antibodies to EAV. An ELISA based on a cocktail of all three EAV structural proteins, however, was used successfully to detect antibodies in most equine sera that were positive in the standard serum neutralization assay following natural or experimental EAV infection (100% specificity, 92.3% sensitivity). All sera from carrier stallions and annually vaccinated horses evaluated in this study had obvious reactivity with the N protein, whereas seropositive non-carrier stallions, mares, and geldings did not consistently respond to the N protein. This finding may be useful in screening stallions for persistent EAV infection, however it does not reliably distinguish persistently infected stallions from vaccinated stallions.

### 3.7 Prevention and control

The prevention and the control of EVA outbreaks are important because there is no treatment for infected animals, only supportive care.

For quarantine management it is important to recognize that mares who become infected shed the virus from the respiratory tract only for 14 days and from the vaginal tract for approximately eight days. Thus, mares isolated for three weeks after exposure pose no significant risk for the spread of disease. There is no evidence that foals infected in uteri either develop malformations or become carriers of EAV.

The approach to prevention and control of EVA has been dramatically changed since the 1984 outbreak in Kentucky, and several management procedures have been proposed (Anonymous, 1993; Fukunaga, 1994; Glaser et al., 1996; Lasalle, 1969; Mumford, 1985; Timoney et al., 1988). These programs are primarily directed at controlling the spread of EAV amongst breeding Thoroughbred populations through carrier stallions. Sensitive breeding management based on the different status of individual stallions and mares appear to effectively control the spread of the virus. Only seronegative stallions should be bred to negative mares. Stallions can be infected at any time after puberty and are then likely to become carriers. Therefore, all breeding stallions should be closely monitored and the semen of carrier stallions should be regularly tested for the presence of virus. Stallions can reclaim their virus free status if minimums of two semen samples, collected with an interval of at least one week, are negative for the virus (Klug, 1999). In controversial cases stallions can be test bred to two seronegative mares, following the Codes of Practice of the Horse-Race Betting Levy Board. A persistently infected stallion can be used for breeding, but should only be bred to seropositive mares or mares that have been vaccinated at least six weeks prior to the breeding.

The most effective method of prevention is vaccination (Anonymous, 1993b; Cook et al., 1989; Fukunaga et al., 1984; Fukunaga et al., 1990; Fukunaga et al., 1997; Harry and McCollum, 1981; McCollum, 1996; McCollum, 1986; McKinnon et al., 1986). Currently two vaccines are available for the control of EAV infection, a modified live virus (MLV) vaccine (ARVAC $^{TM}$, Fort Dodge Laboratory) and a killed whole virus adjuvanted vaccine (Artevac, Ford Dodge Laboratories, Sligo, Ireland). The MLV was obtained by passing the Bucyrus strain of EAV 131 times in horse kidney cells, 111 times

in rabbit kidney cells and 24 times in equine dermal cells (Doll et al., 1968; McCollum, 1970; McCollum et al., 1986). The vaccine has been used in the United States of America since 1985, but it is not licensed worldwide. The ARVAC $^{TM}$ vaccine is freeze-dried (lyophilized) and highly stable at -20°C for at least a year, relatively stable at 4°C and has a short life at 37°C. The vaccine is delivered with its own dilution solution and is delivered intramuscularly (dosage 1ml per animal). Neither persistent infection nor clinical signs occur after vaccination and protective immunity lasts for approximately a year. Although vaccination does not protect vaccinated horses from reinfection, the vaccine virus was isolated from an aborted fetus (Cole et al., 1986), thus it is only recommended for use in stallions and non-pregnant mares. Its use is especially discouraged in pregnant mares in the last two months of gestation (Hyllseth et al., 1970; Timoney et al., 1988a; Timoney and McCollum, 1993). The Artevac vaccine was licensed in the United Kingdom in 1993 under the Animal Test Certificate and has been widely used since then. May 2002 the vaccine was licensed in Germany, and Fort Dodge released the product to the market in autum 2003.

Many countries prohibit widespread use of the currently available vaccines because the vaccines do not permit serological discrimination between naturally infected and vaccinated animals. Thus, vaccinated animals may suffer movement restrictions due to their serological status. Considerable effort has recently been expended to develop a subunit vaccine, which offers considerable theoretical advantage, including lack of replication and the ability to distinguish vaccinated and naturally infected horses (Anonymous, 1993; Balasuriya et al., 2001; Castillo-Olivares et al., 2001; Fukunaga et al., 1992; Fukunaga et al., 1997; Giese at al., 2002; Tobiasch et al., 2001). Boehringer Ingelheim Animal Health shows further approaches to a vaccine in a recent study. Using a DNA vaccine containing EAV ORF 2, 5 and 7 in combination with equine interleukin 2, all horses developed a high titer of neutralizing antibodies, which where still detectable after 12 months (Giese et al., 2002).

# 4. Materials

All materials and chemicals used for this study are standard items and have been acquired from either Sigma-Aldrich, Inc. or Fisher Scientific, International Inc., unless noted otherwise.

### 4.1 Cell lines

| | |
|---|---|
| BHK-21 | Baby Hamster Kidney-21; (ATCC #CCL-10) |
| P3x | P3x63Ag.8.653 cells (ATCC #CRL-1850); |
| RK13 | Rabbit Kidney-13 (ATCC #CCL-37); |

### 4.2 Media and media supplement for cell culture

- Carboxymethyl Cellulose Sodium Salt (CMC) Solution

Dilute 3 g CMC in 70 ml $dH_2O$, stir until 90% are resolved. Autoclave, add 330 ml of cell line specific growth media before use, stir overnight at 4°C.

- Hypoxanthine-Aminopterin-Thymidine (HAT) medium

| | |
|---|---|
| RPMI-1640 (Gibco) | 800 ml |
| HAT media supplement | 1 vial |
| Fetal bovine serum (FBS; HyClone) | 100 ml |
| Origen Hybridoma cloning factor | 100 ml |
| Non–essential amino acids (100x; Gibco) | 10 ml |
| Sodium pyruvate (100x; Gibco) | 10 ml |
| Gentamycin (50 mg/ml; BioWhittacker) | 1.0 ml |

- Hypoxanthine-Thymidine (HT) medium

| | |
|---|---|
| RPMI-1640 | 800 ml |
| HT media supplement | 1 vial |
| FBS | 100 ml |
| Origen Hybridoma cloning factor | 100 ml |
| Non–essential amino acids (100x) | 10 ml |
| Sodium Pyruvate (100x) | 10 ml |
| Gentamycin (50 mg/ml) | 1.0 ml |

| | |
|---|---|
| - Freezing medium | |
| FBS | 50% |
| Cell line specific growth media | 40% |
| Dimethyl Sulfoxide, sterile filtered (DMSO) | 10% |
| - Nutrient media for BHK-21 | |
| Minimum essential media (MEM; Gibco) | 400 ml |
| Tryptose phosphate broth (Difco) | 50 ml |
| FBS | 50 ml |
| Penicillin and Streptomycin (100x; BioWhittaker) | 5 ml |
| - Nutrient RPMI Media for P3X63Ag8.653 | |
| RPMI-1640 | 450 ml |
| FBS | 50 ml |
| Non-essential amino acids (100x) | 5 ml |
| Sodium Pyruvate (100x) | 5 ml |
| - Nutrient media for RK-13 | |
| MEM | 400 ml |
| Calf serum (CS; HyClone) | 50 ml |
| Penicillin and Streptomycin (100x) | 5 ml |
| Polyethylene glycol solution 40% | |
| PEG 1450 (50x; ATCC) | 1 vial |
| RPMI-1640 | 3 ml |
| - STV-solution | |
| Sterile water | 400 ml |
| PBS (20x, sterile) | 21 ml |
| Trypsin-Ethylin-diamin-tetracidic-acid (EDTA; Gibco) | 47 ml |
| Phenol red (Sigma) | 1 ml |

### 4.3 Buffers and solutions

| | |
|---|---|
| - Acetic acid 3% solution | |
| Acetic acid, glacial | 3 ml |
| $dH_2O$ | 100 ml |

- Citrate acid solution`

| | |
|---|---|
| 0.1M Citrate acid | 21.01 g |
| $dH_2O$, sterile filtered | 1000 ml |

Adjust the pH to 4.5

- ELISA blocking buffer

| | |
|---|---|
| 3% Non-fat dry milk powder (Safeway brand) | 3 g |
| Elisa wash buffer | 100 ml |

Stir for 1h and afterwards centrifuge at 3000 rpm (max) for 30 min. Decant the supernatant into a clean beaker before use.

- ELISA coating buffer

| | |
|---|---|
| 3.5% Triton-X-100 Stock | 3.5 ml |
| PBS; sterile filtered | 100 ml |

To make working solution

| | |
|---|---|
| 0.0035% Triton-X-100 | 1 ml of stock |
| PBS; sterile filtered | 1000 ml |

- ELISA HRP substrate solution

| | |
|---|---|
| 0.1 M Citrate acid solution, pH 4.5 | 10 ml |
| Hydrogen Peroxidase (30% soln.) | 3.3 µl |
| O-Phenylenediamine Dihydrochloride (OPD) | 10 mg |

- ELISA stopping solution

| | |
|---|---|
| 1% Sodium Dodecyl Sulfate (SDS) | 1 ml |
| $dH_20$ | 100 ml |

- ELISA Wash buffer

| | |
|---|---|
| 0.05 % Triton-X-100 | 1 ml |
| PBS | 2 l |

- IFA Antibody dilution solution

| | |
|---|---|
| FBS | 5 ml |
| PBS | 95 ml |

- IFA Fixative

| | |
|---|---|
| 3 % Paraformaldehyde | 3 ml |
| PBS | 97 ml |

| | |
|---|---|
| - IFA Permibalization solution | |
| Trition-X-100 | 1 ml |
| PBS | 99 ml |
| - IFA wash solution | |
| 10mM Glycine | 0.76 g |
| PBS Qs to | 1000 ml |
| - Phosphate Buffered Saline (PBS) | |
| NaCl | 8.0 g |
| KCl | 0.2 g |
| $Na_2HPO_4$ | 1.15 g |
| $KH_2PO_4$ | 0.2 g |
| $H_2O$ fill up to | 1000 ml |
| - Solubilization buffer | |
| 20mM Tris HCl | 3.152 g/l $dH_2O$ |
| 20mM Tris Base | 2.43 g/l $dH_2O$ |
| -> Mix both until a pH of 7.6 is reached now add: | |
| 1% Igepal CA-630 (NP-40) | 10 ml/l |
| 0.5% Sodium deoxycholate | 5 g/l |
| 0.1% Sodium dodecylsulfate (SDS) | 1 g/l |
| Phenylmethyl-Sulfonyl Flouride (PMSF) | 17 µg/ml |
| (1ul of 0.1M PMSF/ml, | |
| 0.1M Stock solution [0.1742 g in 10 ml of 2-propanol]) | |
| Aprotinin (Roche) | 1 µg/ml |
| Leupepton (Roche) | 1 µg/ml |
| Pepstatin (Roche) | 1µg/ml |
| - Trypan blue solution | |
| 0.2% Trypan blue | 0.2 ml |
| Sterile filtered PBS | 100 ml |

| | |
|---|---|
| - Western immunoblot washing buffer (TBS-T), pH 7.6 | |
| 20mM Tris Base | 2.43 g |
| 137mM NaCl | 7.73 g |
| HCl (6N) | 3.8 ml |
| 0.05 % Tween 20 | 0.5 ml |
| $dH_2O$ qs to | 1000 ml |
| - Western immunoblot antibody dilution buffer (ADB) | |
| Nonfat dry milk | 10 g |
| $NaN_3$ | 0.1 g |
| NaCl | 9 g |
| $Na_2H_2PO_4$ dibasic | 4.5 g |
| $NaH_2PO_4$ monobasic | 0.5 g |
| PBS | 100 ml |
| - Western immunoblot protein dilution buffer (ADB): | |
| Laemelin sample buffer (BioRad) | 2 ml |
| 50mM Dithiothreitol (DTT) | 16 mg |
| - Western immunoblot blocking buffer | |
| 5% nonfat dry milk (Safeway) | 5 g |
| TBS-T | 100 ml |
| - Western immunoblot conjugate dilution buffer (CDB) | |
| 1% nonfat dry milk | 1 g |
| 0.05% Tween-20 | 0.5 ml |
| PBS | 1000 ml |
| - Western immunoblot SDS-PAGE running buffer | |
| Glycine | 58 g |
| Tris base | 12 g |
| SDS | 4 g |
| $dH_2O$ qs to | 400 ml |

- Western immunoblot transfer buffer (proteins <80.000MW)

| | |
|---|---|
| 25mM Tris base | 3.08 g |
| 190mM Glycine | 15.24 g |
| 20% Methanol | 20 ml |
| $dH_2O$ qs to | 1000 ml |

### 4.4 Enzymes and other proteins

| | |
|---|---|
| Biotinylated Protein Marker Detection Pack | Cell Signaling Technology |
| Guinea pig complement C300 | Gibco |
| M88 ($NH_2$-Met-Pro-Arg-Leu-Arg-Ser-Ile-Phe-Asn-His-Arg-Gln-Leu-COOH) | Balasuriya et al. (unpublished data) |
| M148 ($NH_2$- Tyr-Ala-Gly-Arg-Leu-Phe-Ser-Lys-Arg-Thr-Ala-Ala-Thr-Ala-Tyr-Lys-Leu-Glu-COOH | deVries et al., 1992 |
| nsp1, 2 and 4 | recombinant fusion proteins; Snijder (unpublished data) |
| EAV.030H | van Dinten et al., 1997 |

### 4.5 Antibodies and antisera

| | |
|---|---|
| 12a4a11b3f2 IgG | MAb anti-NSP1; Wagner et al., 2003 |
| 1H7, 1H9, 2B3, 3F4, 5E8, 5F3, 5G11, 6A2, 6D10, 7C5, 7E5, 7D4, 8D4, 9F2, 10B4, 10H4.F9, 10F11 | MAbs anti-GL; Balasuriya et al., 1995 |
| MAb anti-N | 3E2, Rabbit #11428, ascites; MacLachlan et al., 1996 |
| polyclonal anti-M | Rabbit #887 serum; MacLachlan et al., 1998 |
| polyclonal anti-nsp1 | Rabbit anti-nsp1serum; Snijder et al., 1994 |

| | |
|---|---|
| O16 IgG | MAb anti-BTV; Heidner et al., 1990 |
| Biotinylated-Goat Anti-Mouse IgG | Zymed Laboratories Inc.; SF |
| Biotinylated-Goat Anti-Rabbit IgG (H+L) | Zymed Laboratories Inc.; SF |
| HRP-Streptavidin conjugate | Zymed Laboratories Inc.; SF |
| FITC Conjugates-Goat Anti-Mouse IgG | Sigma, MO |
| FITC Conjugates-Goat Anti-Rabbit IgG | Sigma, MO |

### 4.6 Equine sera

Fifty horse sera that were confirmed to be seronegative to EAV by serum neutralization (SN) test and 50 SN-positive sera were evaluated. The latter included 11 sera from experimentally EAV-infected horses, 19 sera from naturally EAV-infected horses, and 20 from horses vaccinated with the modified live virus (MLV) vaccine (ARVAC, Fort Dodge Laboratories, Ames, IA). Many of these sera had been previously evaluated by Western immunoblotting assay and direct ELISA using various recombinant EAV proteins as antigen (Hedges et al., 1998; McLachlan et al., 1998), and the SN titers of all sera also had been determined as previously described (Senne et al., 1985). The SN titers of the various equine sera ranged from <4 to 5125 (reported as the reciprocal of the highest dilution providing 50% protection of the EAV-infected cell monolayer).

### 4.7 Chemicals

| | |
|---|---|
| Acrylamide/Bis, 30%/0.8% | Bio Rad |
| Ammonium Persulfate (APS) | Bio Rad |
| DAKO Fluorescent Mounting Medium | DAKO |
| Dithiothreitol (DDT) | |
| Enhanced Cemiluminenscene (ECEL) | |
| Western blotting detection reagent | Amersham Pharmacia Biotech Inc |
| HydrogenPeroxide 30% solution | |
| Pristaine (2,6,10,14-Tera-Methyl-Pentadecane) | |
| Sodium Dodecyl Sulfate (SDS) | USB |
| TEMED (N,N,N,N'-Tetra-methyl-ethylenediamine) | BioRad Labaratories |

### 4.8 Equipment

| | |
|---|---|
| Eppendorf centrifuge 5415C | |
| Isotemp water bath | |
| Kodak Pro XO-Mat Processor | Eatman Kodak Company |
| Mercury-100, UV source | Chia Technical Corperation |
| Mini-Protean 3 Electrophoresis Cell System | BIORAD Laboratories |
| Olympus BH-2 Electronmicroscope | Olympus |
| PowerPac™ 200 | Bio Rad |
| PowerPac™ 300 | Bio Rad |
| Trans-Blot Cell System | Bio Rad |
| UV 160U Spectometer | Shimadzu |

### 4.9 Other materials

| | |
|---|---|
| Balb/c mice, female, 5 weeks old | The Jackson Laboratories |
| BIO-RAD minigel apparatus with | Bio Rad |
| Spacer, 1.5 mm | |
| Comb, 1.5 mm, 10 lanes | |
| Biomax ML autoradiography film | Eatman Kodak Company |
| Bright line haemocytometer | American optical |
| Freund's complete adjuvant | |
| Freund's incomplete adjuvant | |
| ImmunoPure ® IgG Protein G Purification | Pierce |
| Lab–Tek Chamber Slide System | NalgeNunc° International Corp |
| Milipore transfer membranes | Milipore |
| Mouse Immunoglobulin Isotyping Kit | BD PharMingen |
| Nunc-Immuno™plate, MaxiSorp™surface | NalgeNunc° International Corp |
| SDS-PAGE gel, 12% | Bio Rad |
| Trans Blot Fiber Pads | Bio Rad |
| Trans Blot Cell Gel Holder | Bio Rad |
| Trans Blot Apparatus | Bio Rad |
| Whatman 3 mm filter paper | |

# 5. Methods

## 5.1 Cell culture maintenance

### 5.1.1 Propagation of continuous cell lines (RK-13, BHK-21)

Use a T-75 flask with a 95% confluent cell monolayer. Discard the old media and add 4 ml of STV-solution and rinse the cells well. This will remove the FBS that will de-activate the trypsin. Decant the STV-solution before adding another 4 ml of fresh STV-solution and incubate for 1-4 min at 37°C. Bang on the flask to detach the cells from the surface. Add 10 ml of nutrient medium, the FBS will inhibit the trypsin, mix well and pour into a 50 ml conical tube. Centrifuge at 1300 rpm (200 g) for 5 min at 25°C. Discard the supernatant and suspend the cells in 10 ml of nutrient medium. Mix well by pipetting up and down to get an equal distribution of cells throughout the suspension. Transfer 1 ml of cell suspension into a new T-75 flask and add 20 ml of nutrient medium into the flask (1:10 split). Incubate the flask at 37°C for 3 days, feed on the 3$^{rd}$ day and split on the 7$^{th}$ day.

Note: To get a confluent monolayer within 36-48h transfer 2 ml of cell suspension into a T-75 flask and add 20 ml of complete medium to the flask (1:5 split). Generally one T-75 flask is sufficient to seed five 96 well plates for neutralization or micro titration assays. For specific cell lines:

One T-75 flask of BHK-21 cells (confluent) is sufficient for 5-10, 96 well plates.

One T-75 flask of RK-13 cells (confluent) is sufficient for 3-4, 96 well plates.

For chamber slides ready to infect in 24h, take 50 µl cells from the 10 ml suspension and add 350 µl media per well.

### 5.1.2 Counting cells

Trypsinize the cells and add 10 ml of complete medium, as for propagation protocol. Centrifuge and suspend the cells in 10 ml of nutrient medium. Make a 1:10 dilution of cell suspension (100 µl in 900 µl of complete medium). Take 0.5 ml of the diluted cell suspension into a snap cap tube and add 0.5 ml of 0.2% Trypan blue (1:2 dilution). Load the hemocytometer and count the cells in each of the four large squares.

Live cells do not take Trypan blue into cell; therefore count only the transparent cells. Some cells will be touching the outside borders. Count only those cells touching two of the outside borders (e.g., the upper and left). Use the following formula to calculate the number of vital cells/ml.:
Cells/ml = average number of cells per large square x $10^4$/ml x dilution factor

### 5.1.3 Freezing cells

Take a T-75 flask and prepare the cells as if to pass them. Count the cells. Calculate the needed amount of freezing medium to get approximately $10^7$ cells/ml. Carefully remove the supernatant and add the calculated amount of freezing medium. Mix the cells well. Aliquot the cells into 2 ml vials (1 ml per vial) and immediately store the vials in a –70°C freezer. Transfer the vials into liquid nitrogen after 2 days.

### 5.1.4 Recovery of frozen cells

Rapid thawing of the cells is necessary, to ensure the survival of the frozen cells during the recovery. Thaw the cells in the water bath at 37°C, by swirling the tube. Do not submerge the lid to avoid contamination of the cells, rinse the outside of the tube with EtOH before opening. Transfer the thawed cells immediately into a 50 ml conical with 15 ml of nutrient medium. Centrifuge at 1300 rpm (1200 g) for 5 min at 25°C, to rinse the DMSO out. Aspirate the supernatant and suspend the cells in 10 ml of growth medium. Transfer the cell suspension into a T-75 flask and add 10 ml of medium. Incubate at 37°C for 2-3 days. Feed the cells with nutrient medium and split the flask when the cells are about 95% confluent.

## 5.2 Cell culture and virology techniques

### 5.2.1 Infection of RK-13 and BHK-21 cells with EAV

Decant the media from two T-150 flasks of freshly confluent RK-13 cells and then wash the cells two times with MEM. Infect with equine arteritis virus at a M.O I. 10 (see titration). Suspend the virus in 20 ml (total volume) MEM. Inoculate with 10 ml of the virus suspension per flask and adsorb for 60 min at 37°C.

Add 35 ml of complete RK-13 medium per flask and incubate at 37°C for 36-48h. Freeze the flask in –70ºC when about 90% of the cells are dead.

### 5.2.2 Production of working virus stock

Take a T-75 tissue culture flask with BHK cells, which are about 95% confluent and split into 5x T-150 tissue culture flasks. When the T-150 flasks are about 80% confluent rinse them 2x with 5 ml of MEM. Inoculate the monolayers with first passage of EAV 030H (van Dinten, 1997). Suspend 1 ml of virus stock in 10 ml of serum free MEM and add 10 ml in each T-150 flask. At the same time mock infect one T-75 flask of BHK cells. Let the virus adsorb for 1h at 37°C. Add 40 ml of complete BHK-21 medium / T-150 flask. Incubate at 37°C for 48h or until 95% - 100% CPE. Centrifuge at 3000 rpm (2800 g) in the tabletop centrifuge at 4°C for 15 min. Make 1 ml aliquots of supernatant and store at -70°C. Use one 0.5 ml frozen vial for titration of the virus.

### 5.2.3 Plaque purification of EAV

Make a confluent monolayer of RK-13 cells in a T-75 flask. Trypsinize the cells and suspend in 10 ml of RK-13 medium. A 1:5 dilution takes 24h to form a monolayer, 1:10 dilution takes 3 days. Add 1 ml of diluted cell suspension into each well of a 6 well tissue culture plate and incubate at 37°C for the appropriate time. Remove the medium from the wells and rinse plates 2 times with 3 ml MEM. Add 3 ml of MEM to each well for the time of the virus dilution preparation. Thaw the virus and make ten-fold serial dilution in MEM ($10^{-1}$ to $10^{-6}$). Use 200 µl of virus and 1800 µl of MEM. Make duplicates for each dilution and allow two wells of cell control per titration.
Add 100 µl of diluted virus to each well and slowly rotate for equal distribution and incubate for 1h at 37°C, do not move during incubation. During the 1h incubation period boil 1% agarose in Earls BSS and leave it at 45°C in a water bath. Pre-warm the RK-13 medium to 45°C in a water bath. After the 1h adsorption period, mix 1% agarose with pre-warmed RK-13 medium at a 1:1 ratio for 30 ml (15 ml media + 15 ml 1% agarose in Earls BSS). Add 4 ml of agarose and RK-13 medium mix per well and incubate at 37°C. After 4 days dump the media and add 2 ml of crystal violet per well. Incubate for 4h up to 12h and remove the crystal violet by carefully rinsing the plates under running tab water.

Let the plates dry upside down for about an hour before calculating the plaque forming units (PFU). Use the following formula:

PFU = amount of PFU x dilution factor / infectious volume

Example $10^{-7}$ -> 21 & 34 plaques => average of 28 plaques

$28x10^7$ plaque forming units / well = $2.8x10^8$ PFU/well

$2.8x10^8$ PFU/ 100 µl (infection volume)

$2.8x10^9$/ 1000 µl

### 5.2.4 Serum neutralisation

Use 96 well tissue culture plates for the test. First calculate how many plates are needed for each test. The test should be run with the following controls: pure virus, $10^{-2}$,$10^{-3}$ and pure cells as negative control. All samples should be run in doubles. Make a 10% solution of guinea pig serum in MEM. Take the tissue culture plates and put 50 µl of the serum solution in row B – H. Dilute all samples at 1:4, mix well, and put 100 µl in row A. Make a 2-fold serial dilution from 1:4 up to 1:512 by taking 50 µl from row A into row B, mix 10x by pipetting up and down. Continue up to the last row; row H, which will have the 1:512 dilutions. Add 100 µl of virus diluted in MEM to each well, except the control wells, treat them as described above. Incubate the plates for 2h. Add RK-13 cells, one confluent T-75 should be diluted in 30 ml of complete media. Ideally the wells should be confluent after 24h. Incubate at 37°C overnight and add 50 µl of medium next morning. Monitor the cell growth. Stop the test when the negative control wells are confluent. Carefully dump the media and add crystal violet to each well. Incubate for 4h or overnight. Rinse the wells under slow running tab water. Let the plates dry for at least 1h upside down. The dilution where only one or two plaques are recognizable is the cutting point.

## 5.3 Production and characterization of monoclonal antibodies

### 5.3.1 Collection of blood and separation of serum from blood

Bleed mice by cutting the tip of the tail and collect the blood in Eppendorf centrifuge tubes. Let the blood coagulate for 1 h at 25°C. Centrifuge for 10 min at 4°C at

1200 g in an Eppendorf centrifuge. Transfer the serum under sterile conditions into a new centrifuge tube and centrifuge again for 10 min at 4°C at 1200 g. For finale storage at –20°C transfer the serum sterile into a new centrifuge tube.

### 5.3.2 Immunization of mice

Before the first immunization collect blood as described above from each mouse as negative control serum. Inject 0.2 ml of a 1:2 emulsion of the according antigen in Freund's complete adjuvant i.p.. Bleed the mice after three weeks and immunize the following day with a 1:2 emulsion of the according antigen in Freund's incomplete adjuvant i.p.. Repeat this one more time and collect blood for a last time three weeks after the last immunization.

For nsp1 immunization use 50 µg of nsp1 bacterial fusion protein.

For nsp2 immunization use 10 µg of nsp2 bacterial fusion protein.

For nsp4 immunization use 10 µg of nsp4 bacterial fusion protein.

For M145 immunization use 150 µg of M145 peptide.

For M88 immunization use 150 µg of M88 peptide.

### 5.3.3 Thawing of P3x63Ag.8.653 cells

Recover frozen cells from liquid $N_2$ and thaw the cells in a 37 °C water bath, by swirling the tube. Suspend the thawed cells in 15 ml complete RPMI-1640 medium and centrifuge at 25 °C for 5 min at 1300 rpm (1200 g) in a tabletop centrifuge. Aspirate the supernatant and suspend the cells in 5 ml complete RPMI-1640. Transfer the cell suspension into a T-25 flask and add 5 ml of medium. Incubate at 37 °C for 2-3 days.

### 5.3.4 Propagation of P3x63Ag.8.653 cells

When the cells are 80-90% confluent they are ready to be passed. Bang the flask hard a few times and split the media into two bigger flasks add complete RPMI-160 media, according to maximum media capacity of the flask. Incubate at 37 °C with 5% $CO_2$.

### 5.3.5 Counting of P3x63Ag.8.653 cells

Bang the flask to detach all the cells and to equally distribute the cells throughout the medium. Take 0.5 ml and make a 1:50 dilution in PBS. Take 0.5 ml of diluted cell suspension into a snap cap tube and add 0.5 ml of 0.2% Trypan blue, 1:2 dilution. Load the hemocytometer and count the live cells in each of the four large squares. Calculate the cells/ml. For the fusion $2x10^8$ live cells are needed, $10^7$ cells/ml for freezing.

### 5.3.6 Freezing of P3x63Ag.8.653 cells

Prepare the cells as if to pass them and count the cells. Calculate the needed amount of freezing medium to get approximately $1x10^7$ cells/ml. Carefully remove the supernatant and add the calculated amount of freezing medium. Mix the cells well. Aliquot the cells into 2 ml vials (1 ml per vial) and immediately store the vials in a –70 °C freezer. Transfer the vials into liquid nitrogen after 2 days.

### 5.3.7 Harvesting of mouse spleen cells and fusion protocol

Ensure that the mouse is healthy and bleed it a final time. Now pool all available T-150 tissue culture flasks with P3x cells, which should be in the log phase of growth. Take 2 ml and make a 1:5 or 1:10 dilution in PBS for the cell count. $1.5 – 2.0x10^8$ viable cells are needed for a fusion. Split the cell suspension in 50 ml conical tubes and centrifuge them at 1300 rpm (1200 g) for 5 min at 25 °C in a tabletop centrifuge. Decant the supernatant and suspend the pellets in 5 ml RPMI-1640. Pool the suspensions in one 50 ml conical tube and bring the volume up to 40 ml. Centrifuge, as described before. Wash the cells two more times. Euthanise the mouse using carbon dioxide and dip the body in 70% alcohol. All proceeding steps should be under sterile conditions. Let the alcohol dry of by evaporation and then collect the spleen aseptically. Put the spleen in a tissue grinder and add 10 ml of RPMI-160, grind the spleen with 10 strokes. Add 35 ml of RPMI-1640 in 50 ml conical tube and transfer the spleen cells into the tube. Centrifuge the spleen cells at 1300 rpm (1200 g) for 5 min at 25 °C in a tabletop centrifuge. Decant the supernatant and suspend the cells in 10 ml of RPMI-1640. Make a 1:100 dilution of cell suspension in 3% acetic acid and count the cells in a hemocytometer. $5x10^7$ spleen

cells are needed for a fusion. Centrifuge the cell suspension as above and suspend cells using the calculated amount of RPMI-1640. Use additional spleen cells to freeze a minimum of 1 vial for back up. Add $5x10^7$ spleen cells to the P3x cells, mix them well and centrifuge them at 1300 rpm (1200 g) for 5 min at 25 °C. Suspend and centrifuge the cell mixture one more time. Aspirate the supernatant and put conical tube in a warm water bath. Add 1 ml of PEG over 1 min of time. Use the 1ml glass pipette and carefully stir the cells for a minute. Now add 2 times 1 ml RPMI over 1 min and then 7 ml RPMI over 3 min. After the fusion immediately centrifuge as before. Slowly suspend the cells in 130 ml HAT medium. Transfer 5 ml of the cell suspension into a T-25 flask and incubate at 37 °C. Plate the cells in 12x 96 well tissue culture plates by adding 0.1 ml to each well. Incubate the plates at 37 °C for a week without disturbing the cells. Use the T-25 flask to check on the cells every other day.

### 5.3.8 Propagation of hybrid cell line

After a week add 100 µl HAT medium in each well. From now on check the plates every other day. Depending on the rate of cell death of the P3x cells and the growth of hybrid cells feed the plates after 3 days by aspirating half of the medium and adding 100 µl/well HAT medium. Feed them depending on their growth form now on every 2 to 3 days. When the hybrid cells cover about 50% of the wells start screening, under optimal condition this will be around day 21. After the first screening, when there are no other cells alive, start to wean the cells of the HAT medium. After the aspiration of 100 µl medium add 75 µl HAT and 25 µl of HT medium. Increase the amount of HT over 4 steps until the cells receive 100% HT.

### 5.3.9 Screening of hybrids for positive cells

Screen the wells with a microscope for hybrid growth. If 50% or more of the well is covered with hybrid cells collect 50 µl of supernatant. Use the IFA for a first screening and the Western immunoblotting assay to confirm positive results.

### 5.3.10 Expansion of hybridoma cells

Name the hybridoma secreting specific antibodies according to their destination, for example 10H6. Now label 24 well tissue culture plates and add 1 ml of HT. Remove 100 µl of supernatant from the wells, which are to be expanded, put them with 100 µl of HT in a new well. Mix the cells by pipetting up and down for 10 times. Transfer 100 µl of the suspended cells into their new destination in the 24 well plates. Incubate at 37 °C and feed them every 2-3 days. When the well is 50% confluent, screen the hybridoma a second time. Transfer the rapidly growing cells into a T-25 flask, containing 7 ml of HT medium. Put 200 µl of cell suspension into original well and add 1 ml HT to each well. Incubate plate and flask at 37°C. Feed the flask after 24-48h of incubation with 10 ml HT medium. Count the cells to determine the appropriate dilution for freezing. Cells in log phase of growth should be frozen at 1.1 x $10^5$ cells/ml.

Calculation:
a = required amount of freezing medium
b = volume of hybridoma suspension / T-25 flask
c = dilution factor
d = average of cells per square
$10^7$ live cells/ml = 100 x $10^5$cells/ml = required final cell concentration
$(100x10^5 / d) \times c \times a = b$ (ml)
Suspend the hybridoma cells in appropriate volume of freezing medium and transfer into a minimum of two aliquots and store at –70 °C overnight. Transfer to liquid nitrogen for long-term storage after 2 days.

### 5.3.11 Cloning of hybridoma cell lines by limiting dilution

Take a T-25 tissue culture flask with hybridoma cells. Count the cells and calculate the number of viable cells/ml. Dilute the cells to get 16 cells/µl, 1600 µl are needed for cloning in duplicates. To be safe make more volume, optimal are 5 ml, therefore 800 cells are needed.

Calculation:

Number of viable cells/ml = number of viable cells / square x $10^4$ x dilution factor

Number of viable cells/µl = number of viable cells / square x 10 x dilution factor

Label the 96 well tissue culture plates and add 100 µl of HT-medium to each well. Now add 200 µl of the prepared cell suspension into each well in column #1 and #2. To make a serial dilution take 100 µl from column #1 into column #3 and from column #2 into column #4. Repeat this scheme 4 more times. This should lead to the following amount of cells in each well:

16 cells / 100 µl in column 1 + 2

8 cells / 100 µl in column 3 + 4

4 cells / 100 µl in column 5 + 6

2 cells / 100 µl in column 7 + 8

1 cells / 100 µl in column 9 + 10

0 cells / 100 µl in column 11 + 12

Incubate the plate at 37 °C for 7 – 14 days. Monitor the plates under the microscope for cell growth. Screen all wells when the new colonies cover 50% of the well. Expand only confirmed positive colonies, which were started by a single cell. Expand the selected clones and repeat the cloning procedure two more times. Make sure that between each cloning back up vials are stored in liquid nitrogen.

### 5.3.12 Production of ascitic fluid

Use 5-week-old female BALB/c mice for the ascitic fluid production. Inject 0.5 ml pristane i.p into each mouse and repeat this procedure after 5 weeks. The mice are ready for the infection with the hybridoma cells one week after the second injection. For the cell preparation use one T-75 flask with wanted hybridoma cell line. Bang the flask and collect the cell suspension in a 50 ml conical. Centrifuge 1300 rpm (1200 g) for 5 min at 25°C, carefully aspirate the supernatant and suspend the cells in 20 ml of PBS. Repeat this procedure two more times. Collect 0.5 ml of cell suspension before the final spin and count the viable cells. For one infection 2 x $10^6$ cells are needed, which should

be suspended in 0.5 ml PBS. After the cells are suspended draw 0.5 ml of the suspension into a 3ml syringe. Inject the whole volume i.p., using a 22 gauge 11/2 needle. After the infection check the mice daily. The first sing of tumor and ascitic fluid production is a slowly increasing belly volume. Monitor the mice carefully and kill them when they start to show signs of being uncomfortable. After the mouse is killed dip it in 90% alcohol for 10 seconds and let the alcohol dry off. The following steps should be performed under sterile conditions. Separate the skin from the abdomen muscles and carefully insert a 22 gauge 11/2 needle into the abdomen cavity. If the tension on the belly is too high, the tissue can rupture. Aspirate the ascitic fluid and collect it in a 50 ml conical. Spin at 2600g for 10 min at 4°C. This will separate the blood cells from the ascitic fluid. Aspirate the supernatant and centrifuge it at 1400 rpm (1600g) for 20 min at 4°C. Remaining pristane should be floating on top of the ascitic liquid, carefully collect the ascitic fluid leaving the pristane behind. For long-term storage at –20°C degree aliquot the ascitic fluid into 2 ml vials.

### 5.3.13 Purification of MAb

Allow protein G column and ImmunoPure ® (G) Buffers to warm to room temperature. Open a protein G column by carefully removing the top cap first. This prevents air bubbles from being drawn into gel. Pour off the storage solution and equilibrate the protein G column with 5 ml of ImmunoPure ® (G) Binding Buffer. If the samples are serum, ascitic fluid or tissue culture supernatant, it is necessary to dilute samples at least 1:1 with ImmunoPure ® (G) Binding Buffer. This is necessary to ensure that the proper ionic strength and pH are maintained for optimal binding. Apply up to 4 ml of the diluted sample to the protein G column and allow the sample to flow completely into the gel. The flow of buffer will stop automatically when the liquid level reaches the top frit. Wash the protein G column with 10 ml of the ImmunoPure ® (G) Binding Buffer. Take 2 ml fractions by applying 5 x 2 ml aliquots of binding buffer. Elute the bound IgG with 6 ml of ImmunoPure ® (G) Elution Buffer. Collect the elute in 1 ml fractions in 2 ml glass tubes which contain 100 µl of 500mM Tris buffer. This raises the pH of the protein fractions immediately to neutral. Take 10 µl from each of the

collected samples and make a 1:10 dilution. Monitor the elution of bound proteins by absorbance in a photo-spectrometer at 280 nm. The eluted immunoglobulin fractions can now be desalted over an Excellulose™ Desalting Column. Apply up to 1.25 ml of purified immunoglobulin to the equilibrated column. By applying the immunoglobulin fractions to the Excellulose™ column, desalting and/or buffer exchange can be accomplished. For desalting, use a buffer such as PBS in the equilibration and washing steps. Apply 10 x 1 ml aliquots of the equilibration buffer to the column and collect 1 ml fractions. Protein concentration can be monitored by absorbance at 280 nm. Use the following formula to calculate the Ab concentration per ml:

Ab mg / ml= (spec. reading x dilution) / 1.4

Regenerate Excellulose™ Columns by passing 20 ml of PBS through the column. For storage, the column should be washed with 10 ml of water containing 0.02% sodium azide and then capped when approximately 2 ml remain. Regenerate the Protein G Column by washing with 5 ml of ImmunoPure ® (G) Elution Buffer. If the column is to be stored, wash the column with 0.02% sodium azide in water and make sure that about 2 ml of solution remain when column is sealed. Store columns and buffers refrigerated.

### 5.3.14 Mouse immunoglobulin isotyping ELISA

Bring all reagents of the mouse immunoglobulin isotyping ELISA Kit to room temperature before use. Prepare the buffers and Antigen Mixture as followed:

- Coating Buffer: Dilute required quantity of 10x PBS with distilled water (50 ml/plate)
- Blocking Buffer: Dilute required quantity of 10% BSA 1:10 with 1x PBS (35 ml/plate).
- Dilute Positive Reference Antigen Mixture 1:50 with Blocking Buffer (1ml/plate).
- Dilute HRP-labeled rat anti-mouse Ig MAb 1:100 with Blocking Buffer (10 ml/plate).
- Substrate Solution: Within 15 min prior to use, mix equal volumes of Substrate Reagent A and B (5 ml of each solution for each plate) in a clean glass tube or flask.

The required amounts of the purified coating antibodies should be diluted immediately before use. Dilute an appropriate amount of each isotype-specific rat anti-mouse purified MAb in Coating Buffer and deliver 50 µl of each reagent to applicable rows. Tap the plate gently to ensure even distribution of antibody solution on the bottom of wells. Incubate the covered plate, at 36°C for 1h or at 4°C overnight. Use washing solution

(0.05% Tween-20 in PBS) to wash out plate contents. Then shake out remaining contents and blot excess on a clean paper towel. Repeat the wash 3 times. Add 200 µl of blocking buffer to each well and incubate at room temperature for 30 min. Wash 3 times, shake out the blocking buffer, and blot dry. Pipette 100 µl of each hybridoma culture supernatant to be tested to appropriate plate columns and incubate for 1 hour at room temperature. Positive controls should be included as desired; negative controls generally consist of parent myeloma culture supernatant. After 1h wash 3x, shake out remaining contents, and blot dry.

Add 100 µl of HRP-labeled rat anti-mouse Ig MAb solution to each well, and incubate at room temperature for 1h. Wash 6 times, soaking the wells for 30 seconds to 1 min on each wash. Thorough washing at this step is very important. Add 100 µl of prepared Substrate Solution to each well and incubate plate for 3 – 10 min at room temperature. Positive reaction wells will develop a greenish-blue color. Negative wells will be colorless. Pipette 50 µl of stop solution to each well. Positive wells will become yellow. Read visually.

### 5.4 Molecular Biology

#### 5.4.1 Characterization of EAV expressed proteins

Prepare 6 T-150 tissue culture flasks by seeding BHK cells. When the monolayer is about 90% confluent rinse each flask 3 times with MEM. Infect each flask with 1 ml of EAV.030H working stock. Incubate for 1h at 37°C. After feeding each flask with 40 ml of nutrient media incubate them for 48h at 37°C. Aspirate the supernatant and add 15 ml of the solubilization buffer. After an incubation period of 15 min at 4°C the monolayer should start to detach from the flask surface. Use a 10 ml pipette to rinse the liquid up and down to ensure that all cells are collected. Pour the buffer into a 50 ml conical and centrifuge at 1400 rpm (1600 g) for 15 min at 4°C. Aliquot the supernatant in 1 ml portions in Eppendorf tubes and store them at –80°C. Control antigen was prepared as described above, except that the flask was mock infected with MEM.

### 5.4.2 Immunofluorescence assay

Prepare a T-75 tissue culture flask with a 90 % confluent BHK-21 monolayer as to pass. After the resuspension in 10 ml of growth medium take 1 ml and dilute it further in 10 ml of growth medium. Seed 200 µl of the cell suspension in each chamber of the Lab–Tek Chamber Slide System. Incubate the slides at 37°C for 12h. Carefully aspirate the medium out of each well. Add 100 µl of fresh medium to the bottom row of each slide; these are the negative control chambers. Infect the 4 chambers of the top row with 100 µl of final virus dilution in MEM. Incubate for 1h at 37°C and ad 200 µl of complete BHK medium to all chambers. Incubate the slides for 12h. The following steps can be proceded at the workbench. Aspirate the medium and wash each well 1 time with PBS. Handle the uninfected chambers first. Add fixative 200 µl per chamber and incubate for 1h at 4°C. Rinse all chambers 3 times with wash solution and afterwards permeabilize with 200 µl of permeabilization solution for 5 min at 25°C. Thoroughly rinse the slides 3 times with wash solution. The slides are now ready for the assay.
Add 50 µl of the Primary antibody diluted in antibody dilution solution. After the incubation for 1h at 25°C rinse the slides 3 times with wash solution. Add 50 µl of secondary Ab diluted in antibody dilution solution. Repeat the incubation and wash step. Let the slides dry and remove the chambers. Place 3 drops of the DAKO Fluorescent Mounting Medium on the glass slide and cover it with a matching cover slip. For the best results the slides should be examined right away under a fluorescent microscope. Kept in the dark at 4°C the fluorescence will be visible for about a week.

### 5.4.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of proteins

Assemble the minigel apparatus: Wash glass plates and spacers with distilled water. Air dry in a rack. The glass plates must be absolutely clean. Just before assembly, wipe the glass plates with 70 % ethanol. Assemble glass plates with 1.5 mm spacers in between on to the Plexiglas holders (large glass plate should be against the Plexiglas). The screws must be tight enough to prevent leaks. Mount the Plexiglas holders on to the gel-casting stand (small glass should face out). Snug the Plexiglas holder tightly into rubber pad in the bottom of the gel-casting stand. Use a 12 % resolving

gel for viral proteins and a 4 % stacking gel. The total volume is 50 ml for resolving gel, and 25 ml for stacking gel (sufficient for 4 minigels).

| Ingredients | 12 % Resolving Gel | 4% Stack. Gel |
|---|---|---|
| 30% Acrylamide/Bis solution 37.5:1 ratio | 20 ml | 3.3 ml |
| 0.5M Tris HCl, pH 6.8 | - | 6.3 ml |
| 1.5 M Tris HCl, pH 8.8 | 12.5 ml | - |
| 10% SDS | 500 µl | 250 µl |
| $dH_2O$ | 16.75 ml | 15 ml |
| TEMED | 25 µl | 25 µl |
| 10% Ammonium persulfate | 250 µl | 125 µl |
| TOTAL | 50ml | 25ml |

First make up gel solutions without 10 % ammonium persulfate (10 % APS) and TEMED and degas for 5 min (air inhibits polymerization). Keep the gel solutions in vacuum chamber until needed. Add fresh 10 % APS and TEMED to the resolving gel solution to initiate polymerization, mix well. Pour about 7.5 ml of resolving gel into the glass mold up to 1 cm below the top of the smaller glass plate (this 1 cm is to pour the stacking gel). Carefully add distilled water over the resolving gel with a Pasteur pipette to avoid contact with air. Allow one hour for polymerization at room temperature. The resolving gel can be poured the day before if overlaid with distilled water and covered with plastic wrap. When gels are polymerized, pour off water. Add 10 % APS to stacking gel, mix and immediately pour over the top of the resolving gel. Add until close to the top. Place combs (before placing the combs should be washed with warm water and dried) in to the stacking gel (stop 3-5 mm above the inter phase of stacking and resolving gels). Avoid air bubbles. Allow 15 min for polymerization (during this time get the samples ready). Remove combs. Air bubbles should run in to replace combs. Alternatively use pre-poured gels, acquired from a commercial source (BioRad).

Make up one liter of 1x SDS-PAGE running buffer and pour 500 ml into the buffer tank. Remove the gels (molds with the Plexiglas holders) from the casting stand and lock into the central unit with thumbs pushing from the bottom. The small glass plate goes against the rubber gasket. Bottom clicks into place on base plate of the central unit. Drop the assembly unit into the buffer tank. Add running buffer to the center compartment to the

maximum. Do not allow inside buffer and buffer in the tank to communicate (not imperative). Flush out each of the wells with pipette before loading the samples. Preparation of Samples and Running the Gel: Denature protein in sample buffer (with the appropriate concentrations of DTT) by boiling for 1-3 min. The samples can be stored at –20°C. If samples were frozen, bring them to room temperature and boil again to get SDS to come into solution. The loading volume for a 10-lane comb is 30-35 µl per lane. Use long slender pipette tips and gently eject the sample into the well made by the comb. Load sample buffer into lane 1 and 10 to ensure a nice clean gel. Load appropriate molecular weight markers to the second lane. Proteins are negatively charged and they migrate to the anode at the bottom. Run at 100 V, 30 mA (For two minigel units use 60 mA). Running time is about 45-60 min. Stop when the dye front reaches the bottom. Mark the left side of the glass plates with a "Sharpie" pen to indicate lane one. Take the plates apart and cut the corner of the gel with a blade for identification. Gels are now ready for western immunoblotting transfer.

### 5.4.4 Western immunoblotting assay

Separate the proteins according to their molecular weight by running on a SDS-PAGE gel. Cool the cooling unit to 4°C (put it in the refrigerator 2-3h before use). Remove the gels from the glass plates. Before removing mark the top and bottom left hand corners (top - gel one, top and bottom - gel two). Immerse the gels in transfer buffer until they are laid on to the Millipore transfer membrane. Cut the Millipore transfer membranes to the required size. Mark the right hand top corner with the gel number using a dull pencil. The Millipore membranes are hydrophobic. Soak first in methanol for 2-3 seconds, transfer to distilled $H_20$ and allow the paper to wet by capillary action, transfer to transfer buffer. Cut the filter paper, Whatman 3 mm, to the required size. Just before use, dip the filter paper in transfer buffer.
Assemble the transfer sandwich fiber pads / gel / membrane / fiber pads, keep all the components wet and make sure the sandwich is tightly assembled. Place the complete sandwich in the transfer buffer tank with the membrane closest to the positive electrode, the anode. Make sure hat the stir bar still is able to operate.

Transfer for 45 min at 0.66 amps (80/100V) or overnight at 0.22 amps at 4ºC. Keep a magnetic stirrer in the transfer buffer tank and leave on a stir plate. After transfer disconnect the power supply. Disassemble the sandwich and mark the membrane by clipping one corner or by marking wholes using a pin. Rinse the membranes several times with TBS-T (2-3 quick rinses) and wash for 10 min on the rocker. Add the blocking buffer and incubate at room temperature for 1h, the membranes can be left in the blocking solution overnight, rocking at 4ºC. Remove the blot membrane from the blocking solution and wash with TBS-T washing buffer. Briefly rinse the membranes with three changes of washing buffer, then wash once for 5 min with fresh changes of the washing buffer at room temperature.

During washing dilute the primary Ab in Western Immunoblot Protein dilution buffer (ADB). Use tissue culture supernatants either undiluted or at a 1:10 dilution. Serum should be diluted at 1:200, ascitic fluid 1:1000. Purified IgG should have a concentration of 10 μg/ml. Incubate the blot in diluted primary antibody for 1 hour at room temperature. Wash as before. During the washing step dilute the biotinylated secondary antibody (biotinylated goat anti-rabbit or biotinylated goat anti-horse IgG (H+L) immunoglobulin) 1:10,000 in ADB. Incubate the membrane in the diluted secondary antibody for 1h at room temperature. Wash with TBS-T washing buffer. Briefly rinse the membrane in three changes of washing buffer, then wash three times for 10 minutes with fresh changes of the washing buffer at room temperature. In general, Avidin-HRP conjugates should be used at concentrations of 0.5-5 μg/ml which in this case is a 1:2000 dilution of the commercial stocks. Reagents labeled with horseradish peroxidase should be diluted with conjugate dilution buffer (CDB). Add conjugates to the membrane and incubate at room temperature for 45-60 min. Wash the membrane as directed before. During these washes, make sure the developer is warmed up.

Take the ECL (Enhanced Chemiluminescence) detection reagents supplied and mix an equal volume of detection solution 1 with detection solution 2 to give sufficient to cover the membranes (final volume required is 0.125 ml/square cm membrane). Drain the excess buffer from the washed blots and place them in fresh containers. Add the blots to the detection reagent directly; do not let the blots dry out. Incubate for precisely 1 min at room temperature. Drain off excess detection reagent by holding the blot vertically and

touching the surfaces with a disposable pipette and wrap blots in Saran Wrap. Avoid air pockets.

Place the blots, protein side up, in the film cassette, and tape them in. For good results minimize the delay between incubating the blots in detection reagent and exposing them to the film. Take the film and cassette to the dark room and carefully place a sheet of autoradiography film on top of the blots, close the cassette and expose for 15 seconds. Do not move the film whilst it is being exposed. Try a very brief exposure as well as one up to 1- 15 min. ECL is very sensitive, longer exposures should not be necessary.

### 5.4.5 d-ELISA

Number the needed amount of Nunc Nalge° 96 well certified Maxisorp ELISA plates. Each sample should be tested in doubles on positive and negative antigen. The row H will be used for the positive control, nsp1, the negative control, O16 MAb BTV, and a blank. Make a 1:9 dilution of the antigen in coating buffer and coat the plates by adding 100 µl of the antigen dilution. Tap the plate gently to ensure even distribution of antibody solution on the bottom of wells. Cover the plate with plate sealer and incubate overnight at 4°C. Then shake out remaining contents, and blot excess on a clean paper towel. Block unbound sites by adding 200 µl of blocking buffer per well and incubate for 1h at 37°C. Swiftly dump the blocking buffer and wash each well with wash buffer from a squirt bottle three times. Before adding the primary antibody at 50 µl per well blot excess on a clean paper towel.

Use the following dilution in blocking buffer for the different primary Antibodies:

BTV O16 MAb with 4 µg/ml,

nsp1 MAb 12A4 with 4 µg/ml

3E2 ascitic fluid 1:8000

M antipeptide sera 1:600.

Now incubate for 1h at 37°C. Repeat the above described washing step every time before adding new solution to the plates. Dilute the secondary antibody, biotinylated-Goat Anti-Mouse IgG or biotinylated-Goat Anti-Rabbit IgG (H+L), 1:10000 in blocking buffer. Add 50 µl/well and incubate for 1h at 37°C. Add 50 µl/well of Streptavidin-HRP

conjugate, 1:2000 in blocking buffer, and incubate for 1h at 37°C. Prepare the substrate solution immediately before the use. Add 100 µl/well of substrate solution. Stop the reaction with 50 µl of 1 % SDS solution after 15 min. The optimal signal reading of Streptavidin-HRP is at 480nm. Read the optical density (OD) of the plates at the UV max Kinetic microplate reader.

### 5.4.6 c-ELISA

Prepare the plates up to the first wash step as described for the d-ELISA. Add 50 µl of the equine serum to be tested in each well and incubation of 1.5h at 37°C. Wash carefully and thoroughly after the incubation time. Add 50 µl of MAb 12A4 (nsp-1), MAb 3E2 (N), or rabbit anti-peptide sera (M), as the competing antibody to determine the ability of the test horse serum (inhibitory antibody) to block the binding of each of the various EAV protein-specific antibodies. For the dilution of each Ab see results of the d-ELISA. The protocol of the c-ELISA is otherwise identical to that for d-ELISA. The correlation between OD values and log SN titers were determined by linear regression and correlation analysis using the SPSS 11.0 statistical analyze program.

# 6. Results

## 6.1. Production of antibodies to nsp1, 2, 4 and the M protein of EAV

### 6.1.1 Production of MAbs to nsp1 of EAV

3 four-week-old, female, BALB/c mice were injected i.p. 3-times at a 21-day interval with 50 μg of non-structural protein 1 (nsp1) recombinant bacterial fusion protein. Before each injection the mice were bled and the serum was tested by Western immunoblotting assay. Mouse C10M1 showed a positive reaction on day 21- after the first immunization. Mouse C10M2 and C10M3 seroconverted to nsp1 after the first boost, on day 42. After the final second boost, all 3 mice were confirmed positive for antibody to nsp1 by both, IFA staining (Figure 2) and Western immunoblotting assay (Table 1).

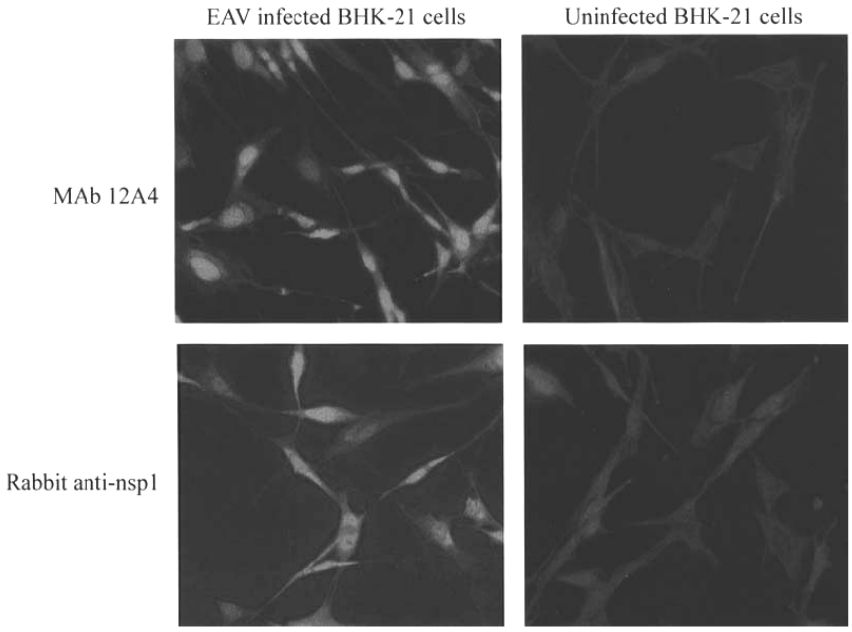
**Table 1:** Western immunoblotting assay of the collected serum samples from 3 mice, C10M1, C10M2, and C10M3, immunized with nsp1 recombinant bacterial fusion protein. The intensity of the band is represented by the symbols: - negative; + weak positive; ++ positive; +++ strongly positive

| Time point of blood collection | Mouse C10M1 | Mouse C10M2 | Mouse C10M3 |
|---|---|---|---|
| Day 0 | - | - | - |
| Day 21 | + | - | - |
| Day 42 | ++ | + | + |
| Day 63 | +++ | ++ | ++ |

The mouse with the strongest reaction, C10M1, was killed and its spleen harvested. Fusion of splenic lymphocytes with P3x murine myeloma cells was done in order to create hybridoma cell lines secreting MAbs against nsp1. At day 24 after the fusion all 12 96 well tissue culture plates were visually checked for hybridoma growth and the wells

with hybridoma growth identified. Tissue culture fluid supernatant was collected from the 164 wells that showed hybridoma growth and tested by IFA. Of the 164 selected wells 23 were positive by IFA staining and thus selected for expansion. Throughout the 3 cloning steps all but three wells lost their capability of antibody production. Those three wells, 12A4a11b3f2 (12A4), 7G9f11g5 and 7G9f11g7 (7G9), were confirmed to produce MAbs to the nsp1 protein of EAV by IFA and Western immunoblotting assay after each cloning step.

**Figure 2:** Immunofluorescence staining assays of EAV-infected and mock-infected BHK-21 cells. The infection was stopped after 12 hours and MAb 12A4 (nsp1-specific) and a rabbit anti-nsp1 serum were used as antibodies. Both show a nuclear staining pattern typical for the localization of nsp1 in the infected BHK-21 cells. The mock-infected BHK-21 cells show no staining.



To determine the immunoglobulin isotype of the MAbs 12A4 and 7G9, purified supernatant of each MAb was tested with the Mouse Immunoglobulin Isotyping kit from BD PharMingen. Each MAb was tested in duplicate. Both MAbs were identified as IgG1 kappa visually by the greenish-blue color the wells developed.

Immunofluorescence staining of EAV infected BHK-21 cells with MAb 12A4 produced strong perinuclear and nuclear staining. This finding is consistent with the localization of the nsp1 protein in EAV-infected cells (Tijams et al., 2002).

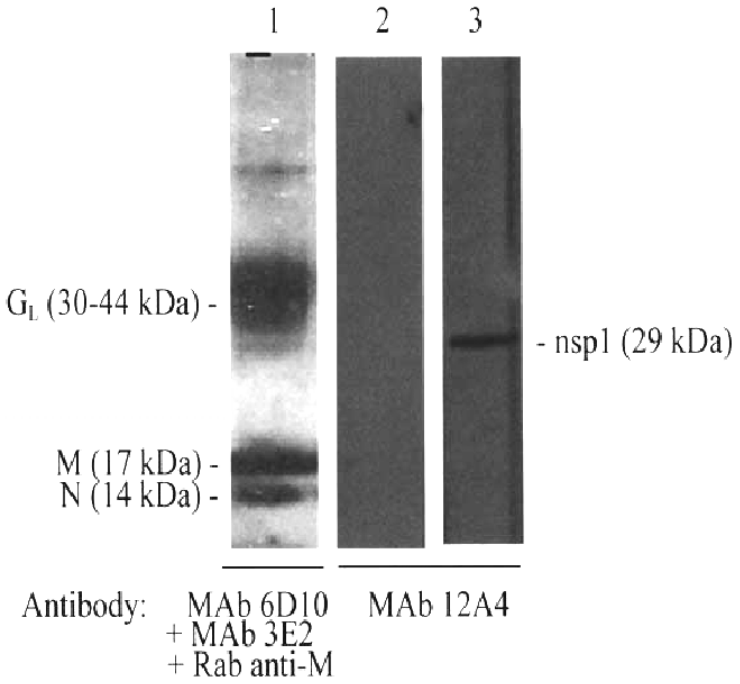
The MAb 12A4, as well as the monospecific rabbit anti-nsp1 serum, strongly reacted by Western immunoblotting assay with a 29K protein in the lysate of EAV-infected BHK-21 cells (Figure 3).

The result of the IFA and Western immunoblotting assays was confirmed by immunoprecipitation carried out in the laboratory of Dr. Eric J. Snijder, Leiden Medical Center, Netherlands.

For further studies the IFA and Western immunoblotting results of all 3 MAb's were compared and the MAb with the strongest fluorescence and the most visible band in the Western immunoblotting assay, MAb 12A4a11b3f2 (Figure 2, 3) was selected.

**Figure 3:** Western Immunoblotting assay of MAb 12A4

Immunoblotting of gradient purified EAV (1), mock infected BHK-21 cell lysate (2) and lysate of BHK-21 cells infected with EAV.030H (3). The antibodies used in each lane are indicated below the lanes. The EAV proteins and their molecular weight are indicated on either side of the panel.



### 6.1.2 Production of antibodies to nsp2 of EAV

10 µg of non-structural protein 2 (nsp2) recombinant bacterial fusion protein was injected i.p. 3-times at 21-day intervals into 3 four-week-old female BALB/c mice. The mice were bled immediately prior to each injection and their serum was tested by Western immunoblotting assay. One of the mice, C11M3, tested positive for antibodies to nsp2 on day 21after the first immunization. All mice showed a weak positive reaction after the first boost (day 42) and 3 weeks after the final boost (day 63). Mouse C11M3 showed a strong positive reaction, whereas mice C11M1 and C11M2 displayed weak reactions (Table 2). Serum obtained after the third immunization of all 3 mice was confirmed positive for antibody production by IFA assay.

**Table 2:** Western immunoblotting of the serum samples collected from mice C11M1, C11M2 and C11M3, which were immunized with nsp2 recombinant bacterial fusion protein. The intensity of the band is represented by the symbols: - negative, + weak positive, ++ positive, +++ strongly positive

| Time point of blood collection | Mouse C11M1 | Mouse C11M2 | Mouse C11M3 |
|---|---|---|---|
| Day 0 | - | - | - |
| Day 21 | - | - | + |
| Day 42 | + | + | + |
| Day 63 | + | + | +++ |

### 6.1.3 Production of antibodies to nsp4 of EAV

In order to produce antibodies against non-structural protein 4 (nsp4) 3 four-week-old, female, BALB/c mice were injected i.p. 3 times with 10 µg of non-structural protein 4 (nsp4) recombinant bacterial fusion protein with an interval of 21 days. Serum collected immediately prior to each injection was tested by Western immunoblotting

assay, and all mice developed antibodies to nsp4 by day 42. After the second boost (day 63) the mice C12M2 and C12M3 had a stronger reaction in the Western immunoblotting assay than did mouse C12M1. Serum collected from the mice on day 63 was tested by IFA to confirm the results of the Western immunoblotting assay. The staining of the infected BHK-21 cells in the IFA assay confirmed all 3 mice had developed antibodies to nsp4 (Table 3).

**Table 3:** Western immunoblotting of serum collected from mice C12M1, C12M2, C12M3 that were immunized with nsp4 recombinant bacterial fusion protein. The intensity of the band is represented by the symbols: - negative, + weak positive, ++ positive, +++ strongly positive.

| Time point of blood collection | Mouse C12M1 | Mouse C12M2 | Mouse C12M3 |
|---|---|---|---|
| Day 0 | – | – | – |
| Day 21 | – | – | – |
| Day 42 | + | + | + |
| Day 63 | + | ++ | ++ |

### 6.1.4 Production of MAbs to the M protein of EAV

Two M-protein specific synthetic peptides, M88 (antigenic region) and M145 (carboxyterminal region), were selected as antigen for the immunization of mice in an attempt to create MAbs to the M-protein of EAV. A regime of 3 immunizations, with an interval of 21 days, of 4 four-week-old BALB/c mice was carried out for each peptide. Initial immunization consisted of 150µg of each synthetic peptide that was diluted in Freund's complete adjuvant, and subsequent immunizations included similar amounts of each peptide in Freund's incomplete adjuvant that was injected i.p. into the mice. Blood was drawn prior to the first injection and before each boost, and at day 63 after initial

immunization. All sera were tested by Western immunoblotting assay. None of the serum samples contained antibodies to the M protein, thus both approaches failed to induce antibody production to the M protein in any of the mice. Thus, MAbs were not produced to the M protein and an M protein-specific polyclonal rabbit antipeptide serum was used in all subsequent assays.

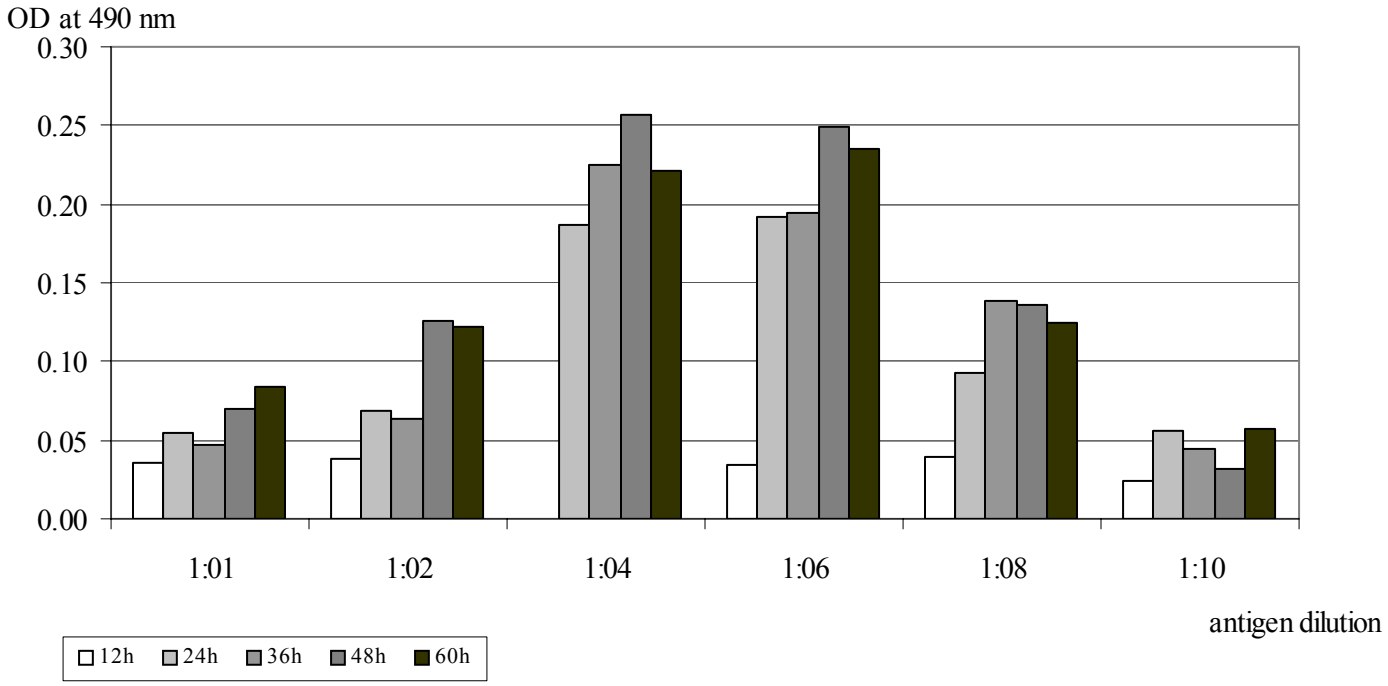
### 6.2 Development of a d-ELISA using the nsp1 MAb

To identify the optimal dilution of the ELISA antigen a preliminary screening titration was performed. Briefly, infection of BHK-21 cells with EAV.030H was terminated at 12, 24, 36, 48, and 60 hours post infection by solubilization. The protein solution was clarified by centrifugation at 1600g for 15 min at 4ºC. Serial dilutions, ranging from 1:1 to 1:10, of the antigen preparations collected at the 5 different time points were tested in a d-ELISA with MAb 12A4 (Graph 1).

The lowest readings of all samples were obtained from the antigen preparation that was harvested at 12 hours after infection. Higher values were obtained using antigen harvested at later intervals after infection, and antigen dilutions of 1:1 to 1:10 were compared to determine the optimal antigen source. The best results were obtained with antigen that was harvested at either 48 or at 60 hours after infection, and the optimal antigen dilution was either 1:4 or 1:6.

**Graph 1:** Time point titration of the ELISA antigen

The infection of BHK-21 cells with EAV.030H was terminated at 12, 24, 36, 48, and 60 hours post infection (bar lower left corner) and tested at different dilutions using MAb 12A4 as antibody. The reading of the 1:04 dilution of the 12-hour antigen preparation was 0.00.



Based on data obtained in the preliminary screening, EAV-infected BHK-21 cells that were harvested 48 hours after infection were selected as the antigen source for the ELISA. Thus BHK-21 cells were infected with EAV.030H and harvested after 48 hours, clarified by centrifugation and stored in 1 ml aliquots at -80ºC. This stock was used as an antigen source for all subsequent ELISA's.

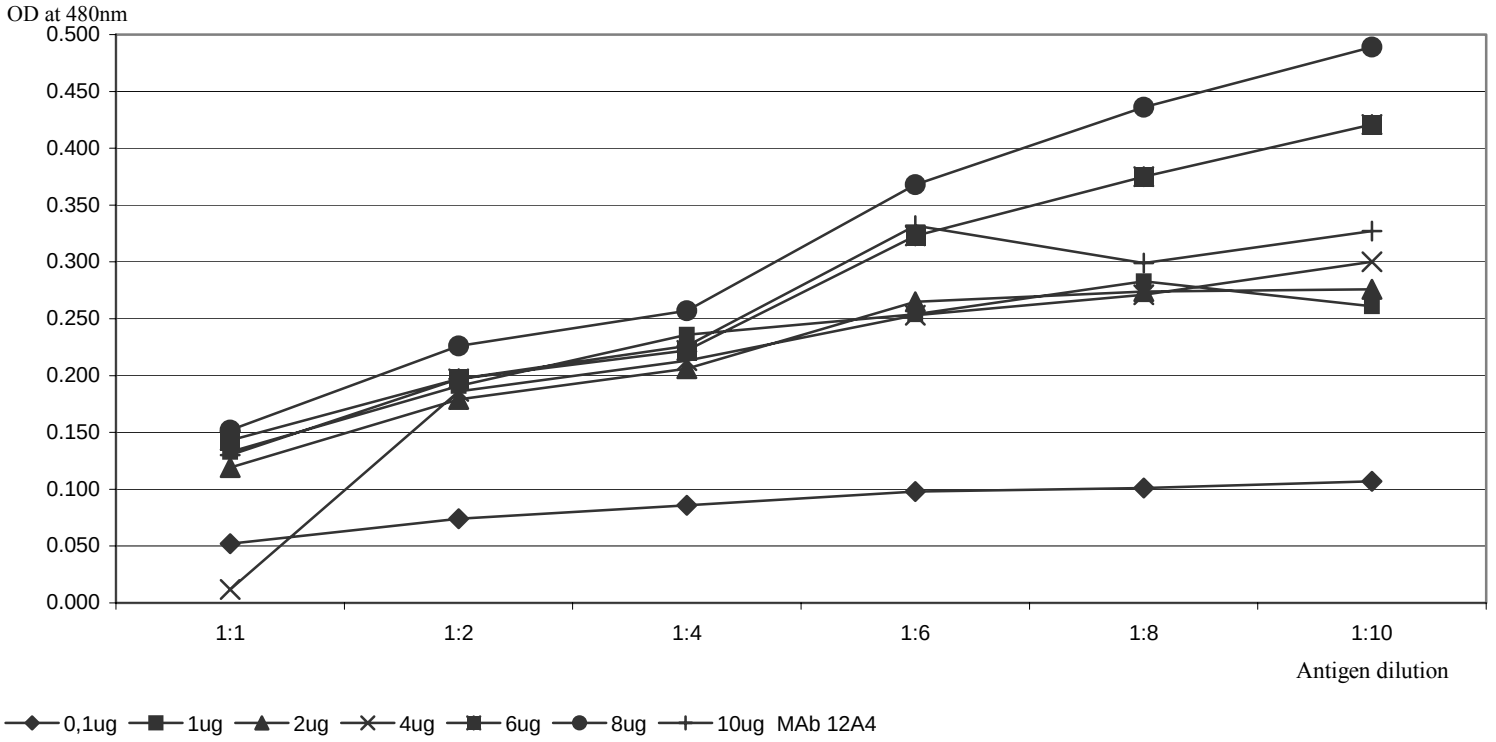
To further optimize the d-ELISA, a checkerboard titration of the antigen in combination with the MAb 12A4 was performed (Graph 2). 6 different antigen dilutions from 1:1 to 1:10 were used to determine the optimal antigen concentration. MAb 12A4 was tested at 7 different concentrations ranging from 0.1 µg to 10 µg/ml.

The highest OD readings obtained with each MAb dilution consistently were obtained at the highest (1:10) antigen dilution.

Based on these data, a 1:9 dilution of the antigen stock harvested 48 hours post infection with EAV.030H and MAb 12A4 at a concentration of 4 µg/ml was used in all assays.

**Graph 2**: Determination of the optimal coating concentration by checkerboard titration of antibody and antigen

The antibody, MAb 12A4, was used at different concentrations ranging from 0.1 µg/ml to 10 µg/ml. The antigen, BHK-21 cells harvested 48 hours after infection with EAV.030H, was tested at dilution ranging from 1:1 to 1:10.



### 6.3 Screening of other protein specific MAbs with the d-ELISA

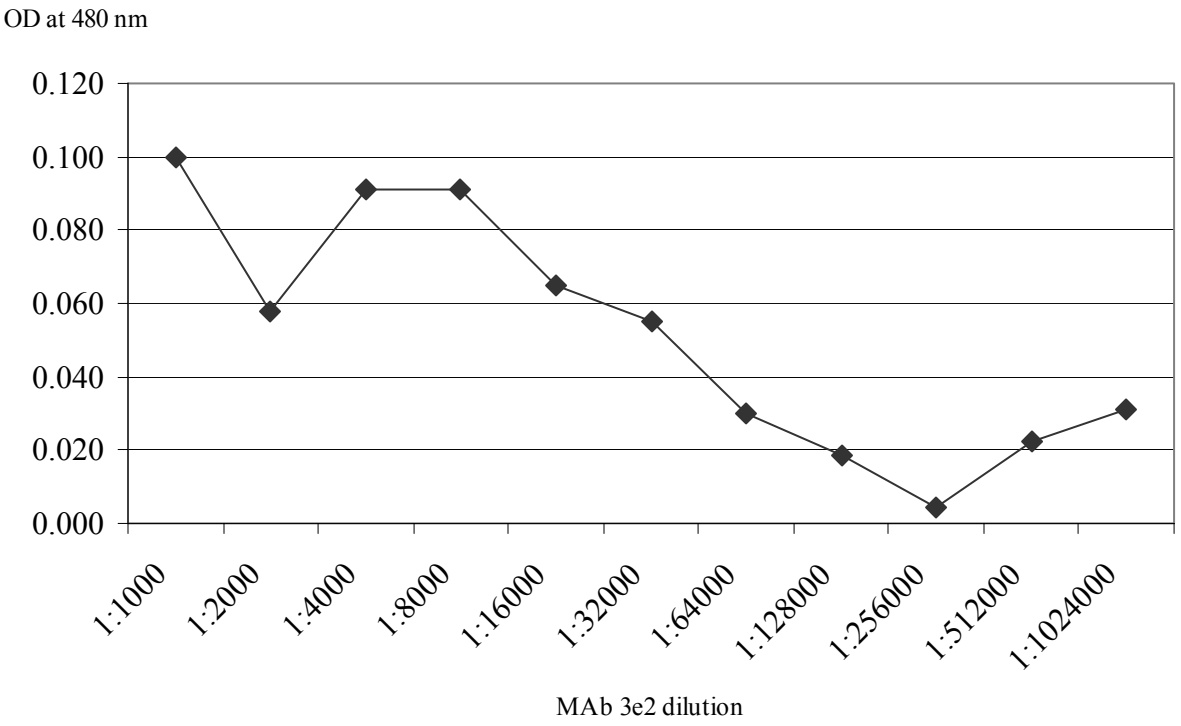
After the optimization of the d-ELISA with MAb 12A4, other available EAV-specific antibodies were tested by checkerboard titration. 17 $G_L$-protein specific MAbs (Balasuriya et al., 1995), MAb 3E2 that is specific to the N protein (MacLachlan et al., 1996), and a polyclonal M-serum specific to the M protein (MacLachlan et al., 1998) were evaluated. All MAbs tested reacted specifically with BHK-21 cells infected with EAV030H. The OD readings obtained with the 17 $G_L$-protein specific MAbs were not sufficient for their use in a c-ELISA (Table 4). In contrast, the N protein specific MAb 3E2 and the M-protein specific rabbit antipeptide serum reacted strongly with the antigen and gave high OD readings. MAb 3E2 was tested with a serial dilution ranging from 1:1000 to 1:1024000 (Graph 3) and a dilution of 1:8000 was determined to be optimal. The polyclonal M-serum was tested in a serial dilution ranging from 1:100 to 1:1000 (Graph 4) and a dilution of 1:600 was chosen for further testing.

**Table 4:** OD values of $G_L$-protein specific MAbs in a d-ELISA using the standard antigen preparation.

| $G_L$ protein specific MAb | Dilution 1:10 | Dilution 1:100 |
|---|---|---|
| 1H7 | -0.022 | -0.001 |
| 1H9 | -0.001 | 0.002 |
| 2B3 | 0.005 | 0.006 |
| 3F4 | 0.029 | 0.012 |
| 5E8 | -0.015 | -0.003 |
| 5F3 | 0.053 | 0.066 |
| 5G11 | 0.061 | 0.041 |
| 6A2 | -0.001 | -0.002 |
| 6D10 | 0.002 | 0.000 |
| 7C5 | 0.029 | 0.049 |
| 7E5 | 0.017 | -0.012 |
| 7D4 | -0.009 | -0.011 |
| 8D4 | -0.040 | -0.038 |
| 9F2 | -0.008 | 0.001 |
| 10B4 | 0.011 | -0.003 |
| 10H4.F9 | 0.011 | -0.003 |
| 10F11 | 0.000 | -0.005 |

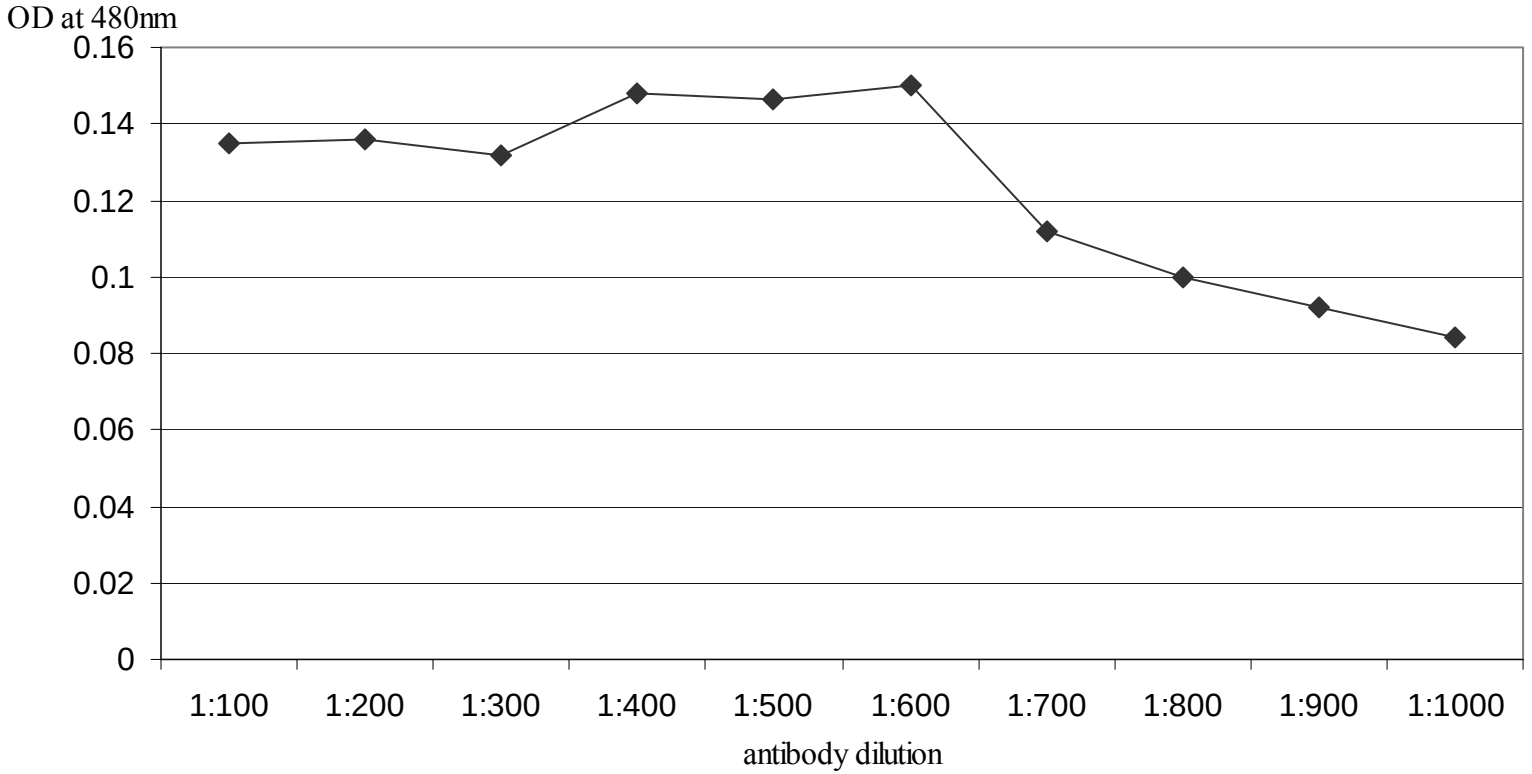
**Graph 3:** Checkerboard titration of the N-protein specific MAb 3E2

The N-protein specific MAb 3E2 was tested in a serial dilution ranging from 1:1000 to 1:1024000. The standard antigen preparation, BHK-21 cells solubilized 48 hours post infection with EAV.030, was used at a dilution of 1:09.



**Graph 4:** Checkerboard titration of the rabbit anti-M serum

A serial dilution of the rabbit anti-M serum, ranging from 1:100 to 1:1000, was tested in a checkerboard titration with the standard antigen preparation; BHK-21 cells solubilized 48 hours post infection with EAV.030H, at a dilution of 1:09.



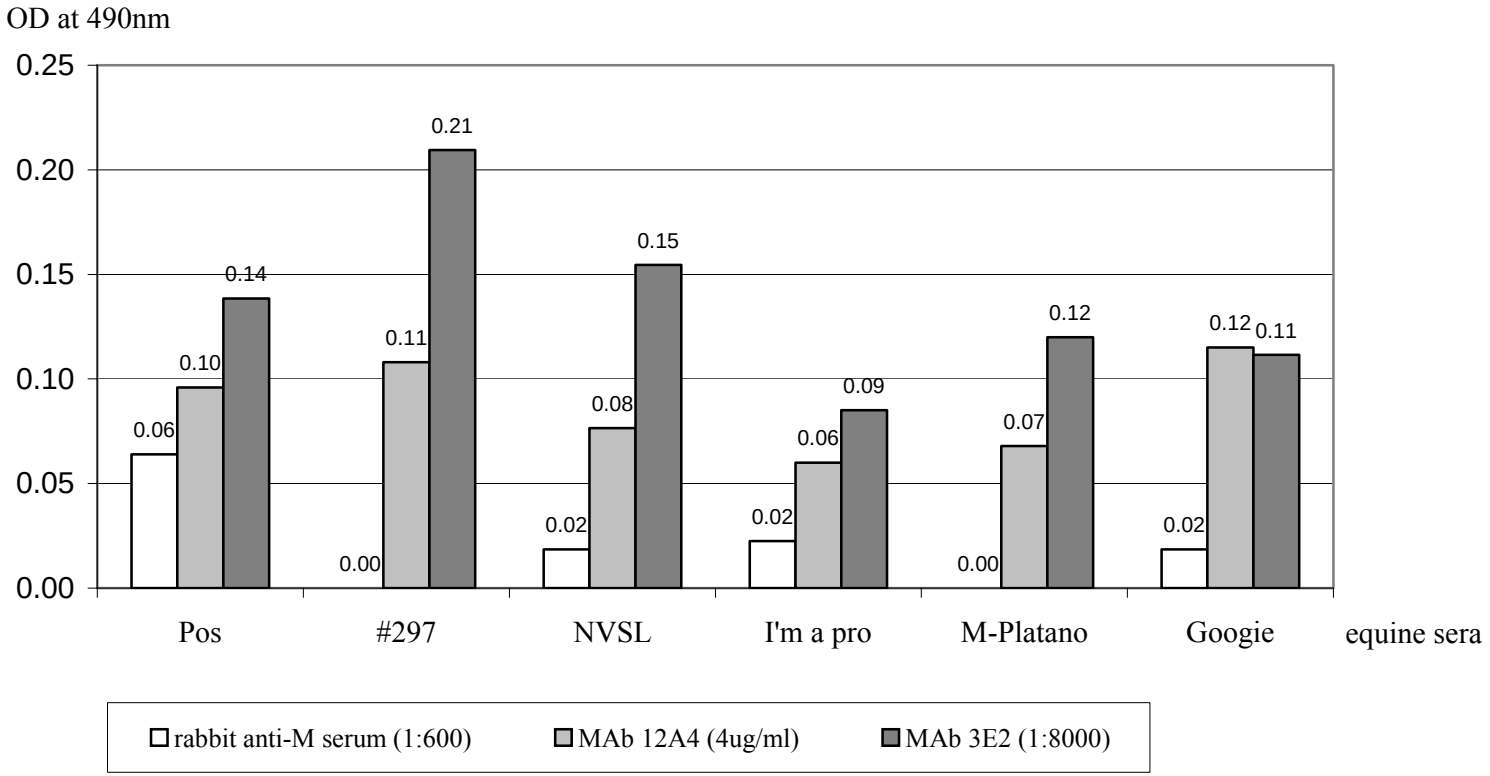
### 6.4 Development of a c-ELISA

A panel of six equine sera with high SN titers to EAV (EAV SN-positive) as well as six SN-negative equine sera were evaluated for their ability to block the binding of EAV protein–specific MAbs 12A4 (nsp1), 3E2 (N) and rabbit anti-M serum (Table 5). All 3 MAbs were used at the concentrations determined in the previous d-ELISAs. The six EAV SN-positive equine sera did not consistently inhibit the binding of MAb 3E2 in the respective c-ELISA, whereas 3 of the six positive equine sera showed an inhibition of MAB 12A4. The inhibition was less then 50%, and a further testing with 6 different SN-positive equine sera verified the initial results. An inhibition of less then 50% with only half of the SN-positive equine sera was determined to be insufficient and therefore no further testing with MAb 12A4 in a c-ELISA was conducted. In contrast, all six SN-positive equine sera strongly inhibited the binding of the rabbit anti-M antiserum (Graph 5), whereas all EAV SN-negative equine sera did not. This shows that antibodies in these SN-positive equine sera either recognized the same epitopes as the rabbit anti-M antiserum or sterically interfere with the binding of the rabbit anti-M sera.

**Table 5:** Evaluation of the ability of six EAV SN-positive and six SN-negative sera to block the binding of the EAV protein–specific MAbs 12A4, 3E2 and rabbit anti-M sera. SN titers of the equine sera and OD readings of the c-ELISAs.

| Equine sera | SN titer | OD readings | | |
|---|---|---|---|---|
| | | Rabbit anti-M | 12A4 | 3E2 |
| Ajusco | 0 | 0.12 | 0.10 | 0.11 |
| Alaoin | 0 | 0.13 | 0.12 | 0.13 |
| Ragtaza | 0 | 0.12 | 0.13 | 0.09 |
| Guaira | 0 | 0.14 | 0.11 | 0.10 |
| Gibraltar | 0 | 0.13 | 0.10 | 0.13 |
| Tequilla | 0 | 0.14 | 0.12 | 0.15 |
| Pos | 1:128 | 0.06 | 0.10 | 0.14 |
| 297 | 1:512 | 0.00 | 0.11 | 0.21 |
| NVSL | 1:256 | 0.02 | 0.07 | 0.15 |
| I'm a pro | 1:256 | 0.02 | 0.08 | 0.09 |
| M-Platano | 1:256 | 0.00 | 0.07 | 0.12 |
| Googie | 1:1024 | 0.02 | 0.12 | 0.11 |

**Graph 5:** Preliminary c-ELISA screening of 6 equine sera, positive by SN, for their ability to block the binding of EAV protein–specific MAbs 12A4, 3E2 and rabbit anti-M serum. The standard antigen preparation, BHK-21 cells solubilized 48 hours post infection with EAV.030H, was used at a dilution of 1:09.



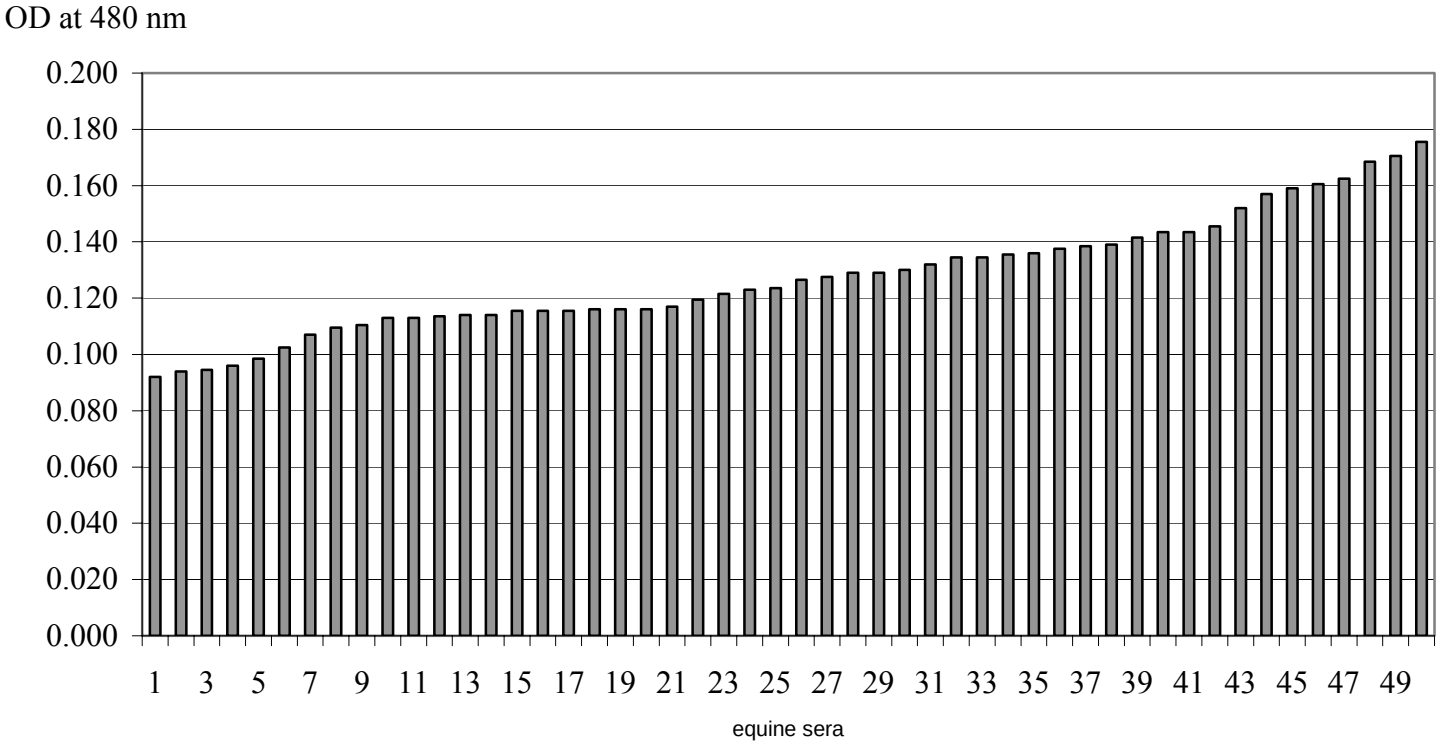
To determine the specificity and the sensitivity of the M protein-specific c-ELISA a panel of 100 equine sera was tested, including 50 SN-negative (Graph 6) and 50 SN-positive sera (Graph 7). The cut-off point for a positive equine serum is 1:4 by SN test. The SN titers of the 50 positive sera used in this experiment are shown in Table 6. None of the 50 SN-negative equine sera inhibited the binding of M protein-specific antiserum (OD > 0.09 [0.09-0.18]) in the c-ELISA (Graph 7). Forty-three (86%) of the SN-positive sera clearly inhibited the binding of rabbit anti-M serum in the assay (OD < 0.08). Seven sera (14%) gave high OD values (>0.08) and were thus classified as false negatives by this c-ELISA. At a cut off OD value of 0.08 the sensitivity and specificity of the assay were 86% and 100% respectively. Regression analysis of OD values in the ELISA demonstrated a strong and significant correlation to the SN titers of the same sera ($r_2$ = 0.82)(Figure 4).

Table 6: Number of equine sera used in the c-ELISA listed by the SN titers

| SN titer | Total number of equine sera | Numbering of equine sera |
|---|---|---|
| < 1:04 | 50 | 1-50 |
| 1:16 | 3 | 51-53 |
| 1:32 | 5 | 54-58 |
| 1:64 | 11 | 59-69 |
| 1:128 | 10 | 70-79 |
| 1:256 | 11 | 80-90 |
| 1:512 | 9 | 91-99 |
| 1:1024 | 1 | 100 |

**Graph 6:** M protein-specific c-ELISA with 50 SN-negative equine sera

The standard antigen preparation, BHK-21 cells solubilized 48 hours post infection with EAV.030H, was used at a dilution of 1:9.



**Graph 7:** M protein-specific c-ELISA with 50 SN-positive equine sera

The 50 positive sera used in this experiment had the following SN titers: #51-53 1:16; #54-58 1:32; #59-69 1: 64; #70-79 1:128; #80-90 1:256; #91-99; #100 1:1024. The standard antigen preparation, BHK-21 cells solubilized 48 hours post infection with EAV.030H, was used at a dilution of 1:9. The OD value of equine serum #54 and #91 was 0.00.

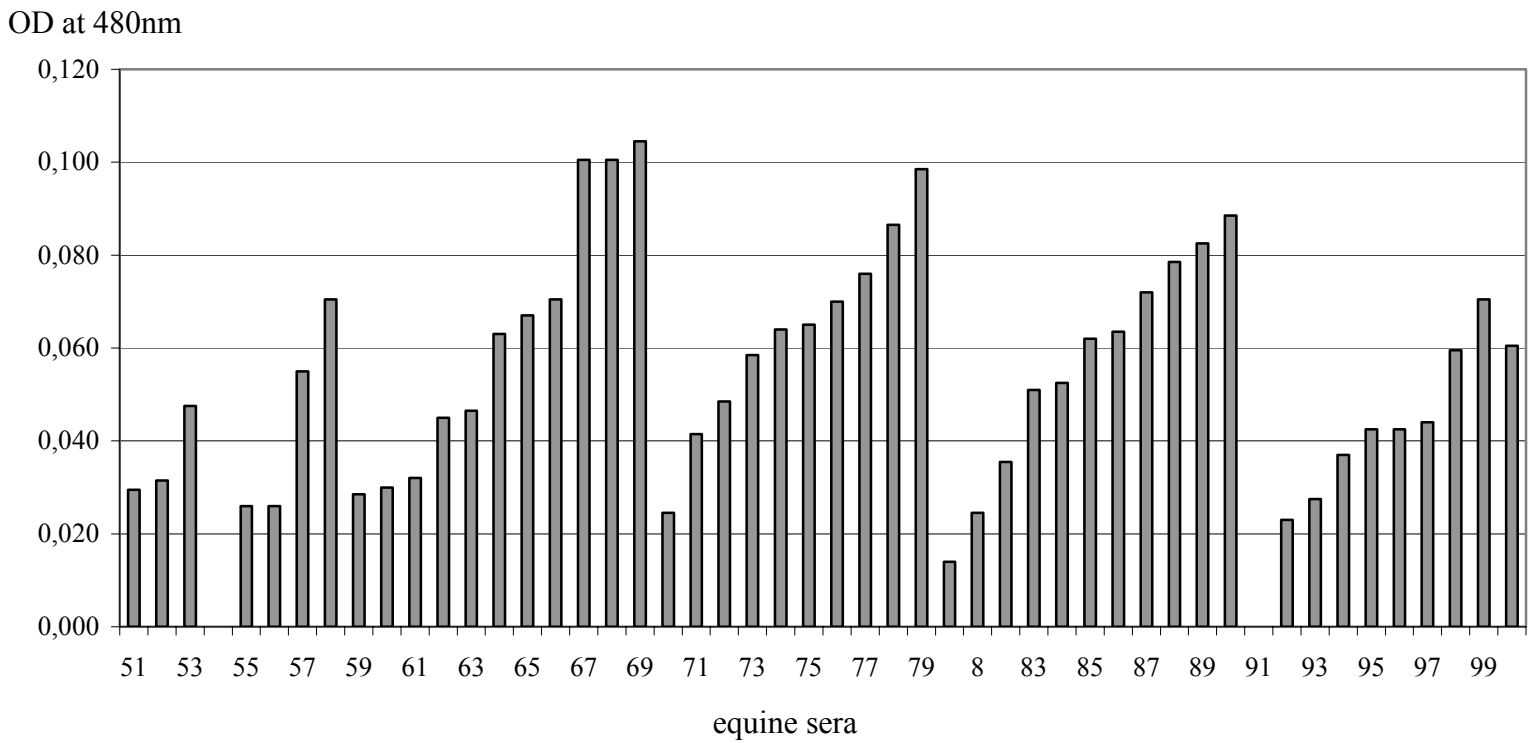
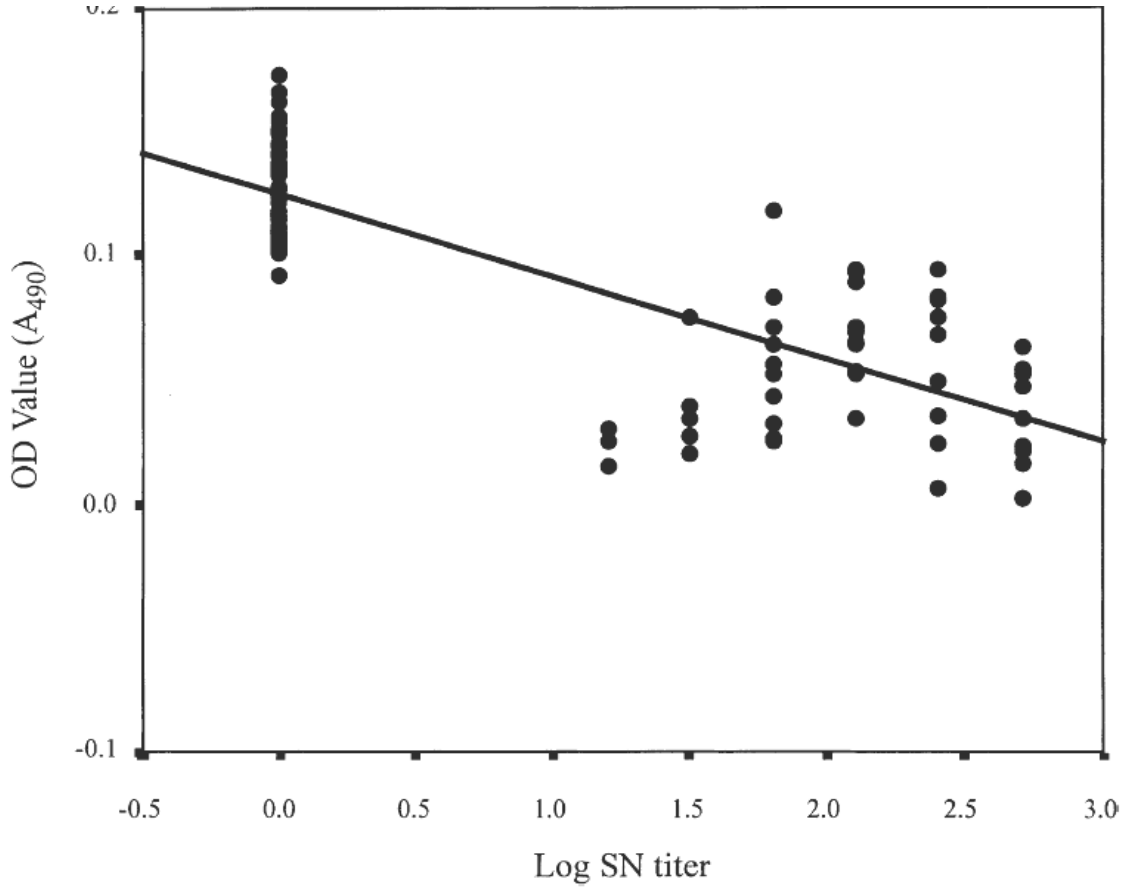


**Figure 4:** Regression analysis ($r^2$= 0.82) of the relationship between the SN titer and c-ELISA OD A490 values for 100 equine sera.



# 7. Discussion

## 7.1 Production of Mabs to nsp1 and antibodies to nsp2 and 4

MAbs have emerged as important diagnostic and research tools in modern research. The usefulness of MAbs stems from three characteristics; their highly specific binding, homogeneity, and their ability to be produced in unlimited quantities. The fact that impure antigens can be used to produce specific antibodies of such qualities makes them even more valuable.

In the 70's Koehler and Milstein (1976) showed that antibody secreting cell clones could be readily established and maintained in vitro. The two partners in a fusion are B-lymphocytes, isolated from immunized animals and myeloma cells. Hybridomas can be prepared by fusing myeloma and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore, fusions are normally done with cells from the same species. Theoretically species such as rats and rabbits could be used for fusion, but today BALB/c mice and myeloma cell lines from BALB/c mice are most commonly used. Commercial breeding facilities ensure access to animals that are disease free and genetically homogeneous. Mice are convenient to handle, inexpensive, and readily produce the requisite number of splenic lymphocytes required for a fusion. Today a broad range of myeloma cell lines is commercially available, and all commonly used mouse strains can serve as successful fusion partners with derivates of BALB/c myelomas. If the lymphocytes are obtained from a BALB/c mouse, the hybridoma will carry only histocompatibility antigens of BALB/c mice and therefore will be able to grow in the peritoneal cavity of BALB/c mice. After the immunization of the mice and testing for an immune response, the mice are sacrificed in order to use their splenic lymphocytes as one fusion partner. Theoretically any fusogen can effect this cell fusion, but in practice hybridoma fusion became routine after the introduction of PEG by Pontecorvo (1975). PEG fuses the plasma membranes of adjacent myeloma and/or antibody secreting cells, forming a single cell with two or more nuclei. The nuclei are retained until the nuclear membranes are dissolved prior to mitosis. During mitosis and further rounds of cell division a different set of chromosomes is delivered to daughter cells and chromosomes

can be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy- or light chain gene is lost, the daughter cell will be unable to produce the desired antibody. Prior to use hybridomas are cloned three times. Each cloning is done from a single cell to ensure that all progeny are descendants of one hybridoma cell and, therefore, identical so that truly monoclonal antibodies are produced.

In this study BALB/c mice were immunized with 50µg of nsp1 bacterial fusion protein in Freund's complete adjuvant and boosted with 50µg of nsp1 bacterial fusion protein in Freund's incomplete adjuvant. All 3 mice immunized with nsp1 mounted a strong and readily detected immune response after the first boost, as proven by western immunoblotting and IFA. The fact that sera from all immunized mice reacted with the nsp1 protein shows clearly that the chosen protein is a good immunogen. All mice immunized with nsp2 and nsp4 converted to seropositive after the final boost, but overall the immune response was weaker compared to nsp1. The EAV protease domains are located in nsp1, 2 and 4 and well conserved. Nsp1 and nsp2 both have been characterized as papainlike cysteine protease (Snijder and Meulenberg, 1998). Nsp2 has been proposed to belong to a new subgroup of viral cystein proteases for its number of unique properties, whereas nsp4 is a member of a rare group of proteolytic enzymes. Nsp1 seems to be the best-researched nsp of EAV (Ziebuhr et al., 2000) and well conserved (Snijder and Meulenber, 1998) and therefore was chosen for the production of MAbs and the further use in the ELISA. To evaluate the potentiale of nsp's, MAbs to nsp2 and 4, as well to the other nsp's, and testing of there possible use in a c-ELISA should be conducted in further studies.

### 7.2 Production of MAbs to the M protein

Despite the fact that similar approaches were used for the immunization of mice with the nsp1 and the M-proteins of EAV, only mice immunized with nsp1 produced detectable antibodies that led to the successful production of protein-specific MAbs. Numerous factors determine the success of MAb production. Theoretically any structure can be recognized as an antigen. But only proteins can engage the T-lymphocytes required for immunologic memory and therefore only proteins induce a fully developed adaptive immune response. Immunological memory is produced following initial contact

with an antigen, and the sensitized individual is now primed to mount a potent immune response after challenge with the same antigen. Further contact with the same antigen leads to an increasingly intense immune response. Hyperimmunisation is the repetitive injection with an antigen to achieve a heightened state of immunity. The hyperimmunisation of mice in order to receive an immune response to a specific antigen is the first of many steps in the attempt to produce murine MAbs. The immunogenicity of a substance depends on a broad range of different factors, both antigen and host factor dependent. Antigen dependent factors include the molecular size, complexity, chemical composition and the susceptibility to antigen processing and presentation (Harlow et al., 1988). In general, the larger the mass of an antigen, the more immunogenic it is. Proteins with a mass approaching 100 kDa tend to be most immonogenic. In general, substances with a molecular mass less than 5-10 kDa are poor immunogens; however a few substances with a molecular mass less than 1 kDa have proven to be immunogenic (Kuby, 2003). The genetic constitution of an immunized animal influences the degree and the type of immune response that the animal manifests. Hugh McDevitt demonstrated that inbred mouse strains differ in their response to a synthetic polypeptide immunogen (Kuby, 2003).

Many proteins are poorly or non-immunogenic when administered alone, and a variety of factors can enhance the immunogenicity of a protein. The immunogen dosage, route of administration and the presence of adjuvants can influence immunogenicity. Optimal immunization often is achieved with intermediate doses of antigen that is administered either subcutaneously or intraperitoneally in a denatured form along with adjuvant. Freund's complete and incomplete adjuvants remain popular for this purpose. Freund's complete adjuvant is an oil-water emulsion that includes non-viable mycobacterium. The adjuvant delays the release of the antigen and enhances its uptake by macrophages. In addition, the dead mycobacteria co-stimulate the macrophages. To avoid the possible risk of an allergic reaction of the immunized animal and the sudden death through shock, Freund's complete adjuvant is only used for the first immunization and incomplete Freund's adjuvant is used for all subsequent immunizations (Harlow et al., 1988; Kuby, 2003).

For the advantages of intraspecies partners for fusion, and the specific advantages of BALB/c mice and myeloma cell lines derived from BALB/c mice see 6.2 .

In this study two sets, each of three mice, were immunized with 2 different peptides, M88 and M145. None of the mice immunized with the M peptides showed a detectable reaction, whereas mice immunized with nsp1 mounted a strong immune response. This suggests that the lack of response to the M protein was not a consequence of the immunization protocol, rather that the antigen was not presented in optimal format for processing and/or presentation.

Although the mice used for production of nsp1 and M protein-specific antibodies were immunized with slightly different quantities of antigen, it is likely that differences in processing and presentation of the various antigens are responsible for differences in the immune response of mice to them. Specifically, the M protein is an unglycosylated protein that spans the membrane of the EAV three times and only a very short region (10-18 amino acid residues) is exposed on the virion surface. Mice immunized with a recombinant Venezuelan equine encephalitis virus (VEE) vector that expresses the M-protein of EAV mounted an immune response to the EAV M protein, but for uncertain reasons MAbs also were not successfully produced by fusion of splenic lymphocytes from these mice (Balasuriya et al., 2000). In contrast, rabbits were successfully immunized with 10mg of the M145 peptide (MacLachlan et al., 1998), which shows that different species can react differently to the same antigen. Efforts to develop MAbs to the M protein of other arteriviruses also have frequently been unsuccessful, whereas MAbs readily have been produced to the $G_L$ and N proteins of these same viruses (Drew, 2000,; Nelson et al., 1999; van Nieuwstadt et al., 1996; Yang et al., 2000).

Due to the lack of MAbs against the M-protein a polyclonal anti-M serum from a rabbit (MacLachlan et al., 1998) was used in the c-ELISA we developed. Polyclonal sera contain antibodies of different specificity. Antibodies generated after a natural immune response or after immunization are a mixture of molecules of different specificities and affinities. Numerous antibodies are produced to different epitopes on the immunizing antigen, but even antibodies directed at a hapten with a single antigenic determination can be heterogeneous. With the help of affinity chromatography polyclonal antisera can be purified, but they still may include minor populations of antibodies that can give cross-

reactions. Thus, each polyclonal antiserum is likely different from other antisera to the same antigen, even if it is produced the same way using the same materials.

### 7.3 Development of a d-ELISA using the nsp1 MAb

ELISA is an exquisitely sensitive and economic method for the detection of either antigens or antibodies. It is now widely used as an immunological assay for detection of protein-specific antibodies since large numbers of tests can be performed in a relatively short time. There are distinct variations of the ELISA for detection of protein-specific antibodies. D-ELISAs measure the binding of antibody directly to an antigen. Thus plates are coated with the target antigen, and the binding of the primary antibody is quantitatet usinga secondary antibody labeld with an enzyme. The enzyme coupled to the labeled secondary antibody converts a colorless substrate into colored reaction product.

Development of M and nsp1 protein-specific ELISAs required coating antigen that contained both structural and non-structural EAV proteins. Purified whole virus preparations obviously lack non-structural EAV proteins (Pesch et al, 1998). An ELISA that incorporates baculovirus expressed protein cocktails as antigen was described by Hedges et al. (1998). This cocktail contains multiple EAV structural proteins but lacks the non-structural viral proteins, thus lysates of EAV infected BHK-cells that contain all structural and non-structural EAV proteins were used as coating antigens for the ELISA.

The EAV replication cycle leads to the first release of progeny virus after 6 to 8h and peaks between 12 and 20h post infection in BHK-cells (Balasuriya, 1996). Thus EAV-infected BHK cell lysates were harvested at 12-hour intervals after infection and were evaluated by checkerboard titration to determine the optimal antigen concentration. Highest readings were obtained with antigen collected at 48 and 60 hours post infection, which represents approximately 4 replication cycles.

After checkerboard titration of MAb 12A4 against different antigen dilutions, a combination of an antigen dilution of 1:10 with MAb 12A4 (4µg/ml) was determined to be optimal for further testing. The decision was influenced by the fact that the production of antigen and antibodies is expensive and timeconsuming and therefore higher dilutions are chosen if they lead to comparable results. An OD reading of 0.300 was deemed

sufficient for interpretation of the c-ELISA, for it gives a good signal and latitude for inter-test variation.

### 7.4 Screening of other EAV protein specific MAbs with the d-ELISA

A variety of EAV-specific polyclonal and MAbs were tested by checkerboard titration in direct ELISA using the EAV-infected cell lysate. Affinity is defined as the strength of binding of one molecule to another at a single site, as occurs when an antibody binds an antigen. The increase in antibody binding affinity (affinity maturation) that occurs during immune responses reflects increased production of IgG antibody derived from mature B cells that have undergone isotype switching and somatic hypermutation. B cell maturation occurs after repeated antigen exposure, which explains why murine MAbs generally have high affinity to their particular antigen.

Eighteen murine MAbs and a single polyclonal antiserum were evaluated. Individual antisera were specific for one of 3 EAV structural proteins ($G_L$, M and N). Whereas Cho et al. (2000) recently described a $G_L$-specific c-ELISA, the panel of 17 MAbs against the $G_L$ protein we evaluated consistently failed, for undetermined reasons, to bind to the $G_L$ protein contained in lysates of EAV infected cell lysates. Balasuriya et al. characterized all MAbs against $G_L$ (Balasuriya et al., 1997), and he already reported that not all MAbs recognize the $G_L$ protein consistently in a c-ELISA using CsCl gradient purified virus as antigen. He concluded that seven of the MAbs recognized conformational-dependent neutralization determinants of the $G_L$ protein. His study shows that MAbs to the same antigen can have different affinities to their target. A low affinity to the $G_L$ protein in the virus used in this study is assumed to be the reason for the lack of signal. $G_L$ and M exist as a heterodimer in the EAV particles and infected cells (de Vries et al., 1995a; Snijder et al., 2003), thus it is likely that this heterodimer conformation influences presentation of the antigenic regions of the $G_L$ protein. Therefore the $G_L$ proteins produced through lysing infected cells may have a different antigenic confirmation that explains the low binding affinity of all MAbs against $G_L$ used in this study.

In contrast, MAb against the N protein as well as the antipeptide serum against the M protein showed good signal strength. A dilution of 1:8000 for the MAb 3E2 and a

dilution of 1:600 for the polyclonal M-serum were determined to be optimal for further testing.

### 7.5 Evaluation of the c-ELISA

The c-ELISA allows measuring antibody in a sample of unknown composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labeled specific antibody. It could also be used to measure the amount of antigen using an antibody attached to the plate. Due to this characteristic the c-ELISA was selected, because crude antigen preparations can be used in the assay as the test quantifies only the displacement of each EAV protein-specific antibody by the test serum, thus it avoids the lack of specificity inherent in d-ELISAs. Specifically, binding of antibodies contained in the test serum to proteins other then the target EAV antigen in d-ELISAs can produce false positive reactions. For example, false positive results can occur after binding of antibodies to cell culture proteins in lysates of virus infected cells or to the fusion partner in ELISAs that incorporate individual viral proteins that are expressed as bacterial fusion proteins. Crucial for the c-ELISA is the fact that the two antibodies compete for the same epitope on the target antigen, if not false negative results will occur. Even if the two antibodies from different origin bind the same epitope false results can be obtained if the laboratory antibody very strongly displaces the binding of the test sera. False positive results would occur if the known labeled antibody has a weak binding affinity and, therefore, can be displaced without the presence of competing antibodies.

The strong signal shown by MAbs 12A4, 3E2 and the polyclonal serum against M in the d-ELISA made those antibodies potential candidates for a c-ELISA. All three antibodies were tested by c-ELISA with six positive equine sera. Only the polyclonal anti-M showed an inhibition of the binding of all six equine sera. Neither the MAb against nsp1 nor the MAb against N showed any inhibition of the positive equine sera. Since it was determined that both MAbs bind to their specific EAV protein, this result clearly shows that no competition between the antibodies in the equine serum and the MAbs occurred. It previously has been shown that EAV-infected horses respond immmunologically to the N protein, and serum from a horse previously infected with

EAV immunoprecipitated the nsp1 protein (Eric J. Snijder personal communication), suggesting that the equine antibodies and the MAbs recognize different epitopes on these two proteins.

An antigen is defined as any substance, which can bind to a specific antibody. They therefore have the potential to generate specific antibodies, but some need to be attached to an immunogen in order to do so. Antigen can have more then one antigenic determinant (Gershwin, 1989), also known as epitopes. In addition different species often recognize different epitopes, and highly specific MAbs against one specific protein therefore not necessarily compete with antibodies from the natural host.

A panel of 50 positive and 50 negative equine sera was tested in the c-ELISA with the rabbit polypeptide serum against M. All 50 negative sera showed no inhibition, whereas 43 (86%) of the 50 positive sera clearly inhibited binding. Seven (14%) of the positive sera gave high OD readings and were classified as false negatives. Respectively the assay showed a specificity of 100% and a sensitivity of 86%. This result is consistent with earlier studies (MacLachlan et al., 1998), which showed that M is the most consistently recognized protein of EAV.

As previously discussed, there are inter- and intraspecies differences in the immune response to individual antigens. The neutralizing epitopes of EAV are located on the $G_L$ protein, which is subject to a greater variation than the other viral proteins, which potentially complicates diagnostic ELISAs that focus on this protein. Diagnostic tests should have high specificity and sensitivity. The goal is to create tests that give neither false positive nor false negative results, although this clearly is difficult and not always achievable. A test specificity of 100% and a sensitivity of at least 99% are considered desirable although a false-negative rate of 1% is still considerable. Based on these criteria, the M-protein specific c-ELISA for serological detection of EAV infection of horses that was developed in this study would not be useful for routine diagnostic investigation. However, it did provide a general prediction of the equine humoral response to EAV as regression analysis showed a strong and significant correlation between the SN titers and the OD values of individual equine sera.

## 7.6 Conclusions

The objective of this study was to better characterize the virus protein-specific antibody response of EAV-infected horses. The c-ELISA was selected because crude antigen preparations can be used in the assay as the test quantifies only the displacement of each EAV protein-specific antibody by the test serum, thus it avoids the lack of specificity inherent in direct ELISAs (Chirnside et al., 1995; Chirnside et al., 1995a; Cook et al., 1989; Hedges et al., 1998; Nugent et al., 2000). Despite these potential inherent advantages, none of the developed c-ELISAs to various EAV proteins had acceptable sensitivity although the M protein-specific c-ELISAs most consistently detected EAV antibodies in SN-positive sera.

Previous studies of the humoral immune response of horses to EAV have focused on detection of antibodies to structural viral proteins (Hedges et al., 1998; MacLachlan et al., 1998). Data from the present study confirm the conclusions of previous ELISA and Western immunoblotting studies that EAV SN-positive horse sera do not reliably identify the N protein. Furthermore, the data show that although nsp1 is abundantly produced in EAV-infected cells (Tijams et al., 2002), and that serum from a horse previously infected with EAV immunoprecipitated the nsp1 protein (Eric J. Snijder, personal communication), a c-ELISA utilizing the MAb we developed to nsp1 was not useful in identifying EAV SN-positive horse sera. The M protein was confirmed by c-ELISA to be most consistently recognized by SN-positive sera from naturally and experimentally infected horses, as previously shown using both Western immunoblotting and direct ELISA utilizing M protein expressed from recombinant baculovirus (Hedges et al., 1998; MacLachlan et al., 1998). Recently, Jeronimo and Archambault (2002) also demonstrated that EAV-specific horse sera readily recognize the carboxy terminal region (amino acids 88-162) of the M protein and further suggested this region might be useful for serodetection of EAV-infected horses. Although highly specific, the M protein-specific c-ELISA using rabbit anti-peptide serum to the extreme carboxy terminus (amino acids 145-162) lacks sensitivity, as does the direct ELISA which was developed using recombinant M protein (Hedges et al., 1998). Murine MAbs have yet to be produced to the M protein of EAV, despite intensive efforts by ourselves and others (unpublished), thus it is uncertain as to whether the sensitivity of the assay would be improved with a M

protein-specific MAb rather than the M protein-specific rabbit antipeptide sera that we used.

The SN test remains the gold standard for serological diagnosis of EAV infection of horses (Balasuriya et al., 1997; Balasuriya et al., 1998; Senne et al., 1985). The SN test putatively detects antibodies to the $G_L$ protein, as this protein expresses the known neutralization determinants of EAV (Balasuriya et al., 1997). The MAbs evaluated by the direct ELISA consistently failed to bind to the $G_L$ protein contained in EAV-infected cell lysates, and therefore the development of a c-ELISA incorporating a $G_L$-specific MAb failed. It was previously shown that these same $G_L$-specific MAbs bind strongly in direct ELISAs that incorporate purified EAV virions as antigen (Balasuriya et al., 1993), however purification of EAV for use as ELISA antigen is both difficult and expensive and thus not practical.

## 8. Summary

The increasing number of equine arteritis virus (EAV) outbreaks worldwide suggests that equine viral ateritis (EVA) is an emerging disease. A quick, accurate and reliable serological test would be invaluable in preventing future outbreaks of EVA, and for import/export regulation pertaining to EAV infection of horses.

The objective of this study was to further characterize the humoral immune response of horses to EAV with a long-term objective of designing an improved diagnostic serologic test. Using a crude cell lysate of BHK-21 cells infected with the EAV clone 030H as antigen, direct and competitive enzyme-linked immunosorbent assays (c-ELISAs) were developed using monoclonal and polyclonal antibodies to structural ($G_L$, N and M) and non-structural (nsp1) viral proteins. An nsp1-specific monoclonal antibody, MAb 12A4, was produced to facilitate development of a c-ELISA using this protein. All MAbs against the $G_L$ protein gave insufficient signal for further use in a c-ELISA; MAb 12A4, 3E2 and a monospecific rabbit anti-M serum were selected for use in different c-ELISAs. A panel of 50 positive and 50 negative equine sera, confirmed by serum neutralization (SN), was tested in the c-ELISA. Only the antipeptide serum against the M-protein showed inhibition of positive horse sera, respectively showing a specificity of 100% and a sensitivity of 86%. Regression analysis of OD values in the ELISA confirmed a strong and significant correlation to the SN titers of the same sera ($r^2$= 0.82).

Data obtained using the various c-ELISAs confirm that the M protein is a major target of the antibody response of horses to EAV. However, none of the c-ELISAs that were developed were as sensitive in detecting EAV-specific antibodies in horse sera as the existing SN test.

# 9. Zusammenfassung

Die global ansteigende Zahl von Infektionen mit dem Equinen Arteritis Virus (EAV) lässt darauf schliessen, dass die Equine Virus Arteritis (EVA) zu den "emerging diseases" gezählt werden kann. Ein schneller, präziser und zuverlässiger serologischer Test ist notwendig zur Einhaltung von Im- und Export-Bestimmungen und damit unerlässlich zur Prävention zukünftiger EVA-Ausbrüche.

Das Ziel der vorliegenden Arbeit war, einen verbesserten diagnostischen Test für EVA zu entwickeln, der neue Erkenntnisse bezüglich der humoralen Immunantwort von EAV infizierten Pferden berücksichtigt.

Ein unbehandeltes Zelllysat aus mit EAV Klon 030H infizierten BHK-21 Zellen wurde als Antigen für die Entwicklung von direkten ELISAs (d-ELISA) und competitiven ELISAs (c-ELISA) verwendet. Desweiteren wurden monoklonale und polyklonale Antkörper gegen die Strukturproteine $G_L$, N, M und ein Nicht-Strukturprotein, nsp1, von EAV eingesetzt.

Die Herstellung eines nsp1-spezifischen monoklonalen Antikörpers (MAb), MAb 12A4, ermöglichte die Entwicklung eines c-ELISAs. Die gegen das $G_L$ Protein gerichteten MAbs wurden aufgrund unzureichender Signalgebung für einen Einsatz in einem c-ELISA ausgeschlossen. Letztendlich wurden MAb 12A4, 3E2 (anti-N) und ein monospezifisches Kaninchen anti-M Serum für den Einsatz in den verschiedenen c-ELISA Testmethoden ausgewählt. Eine Auswahl von 50 positiven und 50 negativen Pferdeseren, alle mittels Serumneutralisationtests (SN) vorgetestet, wurde im c-ELISA eingesetzt. Eine Inhibition von positiven Pferdeseren konnte lediglich mit dem gegen das M-Protein gerichteten anti Peptid Serum erzeugt werden. Dabei wurde eine Test-Spezifizität von 100% und eine Sensivität von 86% ermittelt; eine Regressionsanalyse der OD-Werte zeigte eine starke und signifikante Korrelation ($r^2 = 0.82$) zu den Titern der Serumneutralisationstests derselben Seren. Die experimentelle Auswertung der Daten verschiedener c-ELISA Testmethoden deutet darauf hin, dass das M-Protein ein Hauptziel der Immunantwort von EAV infizierten Pferden ist. Dennoch erreichte keine der entwickelten c-ELISA Testmethoden eine mit dem SN-Test vergleichbare Sensitivität.

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