Epidemiological and Diagnostic Studies on *Mycoplasma gallisepticum* and *Mycoplasma synoviae* Originating from Poultry and Non-poultry Birds

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INAUGURAL DISSERTATION
for the acquisition of the doctoral degree
at the Faculty of Veterinary Medicine
Justus-Liebig-University Giessen, Germany

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List of abbreviations

°C	Grade Celsius
µg	Microgram
µl	Microliter
AFLP	Amplified fragment length polymorphism
APS	Ammonium persulphate
Bp	Base pair
BT	Blue tits
CCU	Color changing units
CFU	Colony forming unit
CK	Chicken
D-index	Discrimination index
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assays
GS	Goose
GTS	Gene-targeted sequencing
HGT	Horizontal gene transfer
HI	Hemagglutination-inhibition
HS	House sparrow
IB	*Infectious bronchitis*
IF	Immune-flourescence
IP	Immuno-peroxidase
M. gallinaceum	*Mycoplasma gallinaceum*
M. gallinarum	*Mycoplasma gallinarum*
M. imitans	*Mycoplasma imitans*

M. iowae	*Mycoplasma iowae*
M. meleagridis	*Mycoplasma meleagridis*
MG	*Mycoplasma gallisepticum*
MG IGSR	*Mycoplasma gallisepticum*- 16S-23S inter genic spacer region
MG IGSR-PCR	Polymerase chain reaction of *Mycoplasma gallisepticum*- 16S-23S inter genic spacer region
Mgc2	*Mycoplasma gallisepticum* cytadhesin 2
Mgc2-PCR	Polymerase chain reaction of *Mycoplasma gallisepticum* cytadhesin 2
Ml	Milliliter
Mbp	Million base pairs
MS	*Mycoplasma synoviae*
Multiple GTS	Multiple gene-targeted sequencing
NAD	Nicotinamide adenine dinucleotide
ND	*New castle disease*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
Pg	Pico gram
PPLO	Pleuropneumonia-like organism
pvpA	Phase-variable putative adhesin
pvpA-PCR	Polymerase chain reaction of phase-variable putative adhesin
pvpA-RFLP	Phase-variable putative adhesin- restriction fragment length polymorphism
RAPD	Random amplified polymorphic DNA
RE	Restriction endonucleases
REA	Restriction endonuclease analysis
RT-PCR	Real-time- Polymerase chain reaction
SLS	Single locus sequencing
SN	Swan
SPA	Serum plate agglutination
TEMED	Tetra methyl ethylene diamine

TK	Turkey
U	Units
V	Volt
vlhA	Variable lipoprotein and haemagglutinin
VlhA-PCR	Polymerase chain reaction of variable lipoprotein and haemagglutinin
ZF	Zebra finches

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1. Introduction

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are worldwide avian pathogens of significant economic importance (Raviv and Ley, 2013). MG and MS are known to be associated with respiratory and synovial infections like chronic respiratory disease (CRD) in chicken, infectious sinusitis in turkeys and infectious synovitis (Ferguson and Noormohammadi, 2013; Raviv and Ley, 2013). Lately, MS has also been reported to be associated with new syndromes like eggshell apex abnormalities (EAA) (Feberwee et al., 2009) as well as the layer *E. coli* peritonitis syndrome (EPS) in layers (Raviv et al., 2007b).

Definite MG/MS diagnosis with genotype identification represents the first step for subsequent successful control measurements. Many techniques have been described for MG and MS diagnosis that is based on detection of the pathogen (through pathogen cultivation or molecular detection) and or specific antibodies (through serological tests) (OIE, 2008).

Cultivation of MG and MS is laborious, expensive, time consuming and can be unsuccessful (Kleven, 2008a). On the other hand, the commonly used serological tests are usually hindered by lack of specificity and/or sensitivity (OIE, 2008). Molecular detection of MG and MS represented rapid, accurate, complementary or even alternative diagnostic tool for cultivation and serological tests.

For MG and MS typing, methods like random amplified polymorphic DNA (RAPD), pulsed field gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP) and DNA sequencing have been described. However, some problems associated with low reproducibility and inability for inter laboratory data exchange have been reported with RAPD (Hong et al., 2005b). PFGE and AFLP proved good discriminatory power but they are time consuming, laborious and require purified genomic DNA (Champion et al., 2002). Sequencing of single and multiple gene portions known as gene-targeted sequencing (GTS) showed robust typing capability for MG and MS (El-Gazzar et al., 2012; Ferguson et al., 2005). Sequencing offered also the possibility for typing of uncultivable pathogens, as well as inter laboratory data exchange (Armour et al., 2013; Bayatzadeh et al., 2014; Ferguson et al., 2005; Gharaibeh et al., 2011).

On the other hand, the role of free-ranging birds in MG/MS transmission and epidemiology has not been extensively studied except through relatively few studies. Some of these studies focused mainly on MG with less attention toward MS (Gharaibeh and Hailat, 2011). Others were experimentally applied and lacked the natural infection conditions (Dhondt et al., 2008; Gharaibeh and Hailat, 2011; Kleven and Fletcher, 1983). As far as our knowledge, none of these studies investigated infecting MG/MS strains originating from free-ranging birds down to the subspecies level (Farmer et al., 2005; Luttrell et al., 2001; Stallknecht et al., 1998).

Herein during the first part of study, we investigated MG and MS infection in 104 poultry flocks by isolation as well as MG and MS species-specific PCR. The genetic profile of occurring MG and MS strains was investigated and compared by genotyping. MG genotyping was carried out through restriction fragment length polymorphism of phase-variable putative adhesin (*pvpA*-RFLP) and multiple gene-targeted sequencing (GTS) analysis of portions of the MG cytadhesin 2 (*mgc2)* gene, *pvpA* gene and MG 16S-23S intergenic spacer region (IGSR). MS genotyping was carried out through sequencing of the conserved portion of variable lipoprotein and hemagglutinin A (*vlhA)* gene. AFLP technique was also applied for typing of cultivable MG and MS. The discriminatory capability of each typing method was estimated and compared to explore the most appropriate method. The relationship between German and global MG and MS strains published in GenBank was also investigated.

Through the second part of study, we investigated MG and MS originating from two naturally infected chicken farms as well as from the contacting free-ranging birds within each farm. MG and MS originating from both hosts were genotyped and compared. MG strains were compared through multiple GTS analysis of *mgc2* gene, *pvpA* gene and MG 16S-23S IGSR. For MS, the conserved portion of *vlhA* gene was sequenced and compared.

2. Literature

2.1. Historical overview

The first probable isolation of Mollicutes was in 1898 by Nocards and Roux from a cattle showing signs of contagious bovine pleuropneumonia, from which the ''Pleuropneumonia like organisms (PPLO)'' name was given. However, the first report on avian *Mycoplasma* was by Dodd in 1905 in England who described sinusitis in turkeys and termed it as "epizootic pneumoenteritis of the turkeys" (Lancaster and Fabricant, 1988).

''Infectious coryza of chickens'' was the term given in 1935 for describing a slow and long lasting coryza and this was probably the first case of Chronic respiratory disease (CRD) in chicken (Nelson, 1935; Raviv and Ley, 2013). However, the term ''chronic respiratory disease'' (CRD) was first given by Delaplane and Stuart (1943) for the clinical manifestations of the disease in chicken. Dickinson and Hinshaw (1938) introduced the term "infectious sinusitis" to describe the disease in turkeys (Lancaster and Fabricant, 1988).

The first description of infectious synovitis associated with Mycoplasma was in 1954 and 1956 (Olson et al., 1954; Olson et al., 1956). The respiratory form and air sac infection has been reported in cases of *M. synoviae* combination with ND and IB vaccines (Olson et al., 1964a; Olson et al., 1964b).

2.2. Biology and taxonomy of *Mycoplasma*

The genus *Mycoplasma* belongs to the class Mollicutes (in Latin, mollis, soft; cutis, skin). The name ''Mollicutes'' refers to the nature of these organisms that lack the bacterial cell wall known in other bacteria. Mycoplasmas are surrounded by just a trilaminar membrane (Bradbury, 2005; Razin et al., 1998). Mycoplasmas are the smallest free living eubacteria possessing the smallest genome (0.58 – 2.2 Mbp) with low G+C content (23 – 40 mol %). The genome of *Mycoplasma*s lacks even the genetic data required for a cell wall synthesis. Lack of cell wall renders *Mycoplasma* resistant to antibiotics affecting cell wall synthesis like penicillin which is usually incorporated in cultivation medium to control other bacterial growth (Razin and Herrmann, 2002).

The genus *Mycoplasma* contains more than 200 identified species infecting a wide range of hosts including human, animals, plants and insects (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). From animal *Mycoplasma*, more than 20 species are known to infect avian hosts. *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) are the most clinically relevant avian Mycoplasmas (Raviv and Ley, 2013).

2.3. *Mycoplasma gallisepticum*

MG is the most pathogenic avian *Mycoplasma* species with significant economic impact as one of the costliest diseases confronting poultry industry worldwide (Raviv and Ley, 2013). Infection with MG is commonly known as chronic respiratory disease (CRD) in chickens and infectious sinusitis (IS) in turkeys. MG infection is characterized by respiratory manifestations and frequently swelling in infraorbital sinuses in turkeys (Raviv and Ley, 2013). Another form of MG infection was observed in American house finches that experienced severe conjunctivitis (Delaney et al., 2012; Ley et al., 1996).

The impact of MG on egg production was studied, where MG-infected flock produced 12 fewer eggs per hen than uninfected flock. MG F-strain-vaccinated hens produced 6 eggs more than unvaccinated infected hens. Losses in commercial layer flocks in Southern California were estimated to be approximately 127 million eggs due to MG. Egg production losses in association with costs of MG control programs were approximately $7 million (Mohammed et al., 1987).

Pathogenesis starts with the attachment of MG to host epithelial cells. This attachment has been shown to be mediated through capsular structures (blebs or tip structures) on the outer surface of the *Mycoplasma* membrane responsible for cytadsorption to chicken tracheal epithelium (Bencina, 2002; Tajima et al., 1982). Like for many other Mycoplasmas, MG epithelial colonization is followed by epithelial invasion and dysfunction. Complications due to infection with other pathogens or due to environmental factors exaggerate the MG infection (Kleven, 2008a).

The genome of several MG strains including virulent and attenuated strains is about 1 Mbp (Papazisi et al., 2003; Szczepanek et al., 2010). The first published complete MG genome sequence was from a MG R_low passage reference strain that revealed 996 422 bp with 31 mol% G+C content and demonstrated two copies of the rRNA and 33 tRNA genes (Papazisi et al., 2003). Genomes of the attenuated high-passage MG R strain, as well as of MG F, ts-11 and 6/85 vaccine strains, were also published (Szczepanek et al., 2010).

In addition to killed vaccine, live MG vaccines like F, ts-11 and 6/85 vaccine strains have been used for MG control programs in different countries (Jacob et al., 2014). Differentiation between MG field and vaccine strains has always been a matter of challenge for many researchers, especially with the increasing usage of live MG strains for vaccination nowadays (Ghorashi et al., 2015; Raviv et al., 2008).

2.4. *Mycoplasma synoviae*

Exudative synovitis, tendovaginitis, or bursitis are different forms of MS infection due to involvement of synovial membranes of joints and tendon sheaths preceded by systemic MS infection (Ferguson and Noormohammadi, 2013). Infections with MS are mostly subclinical and not clinically apparent until complicated with other pathogens like Newcastle disease virus (NDV) and or infectious bronchitis virus (IBV) causing air sac lesions.

Lately, MS has been associated with a new form of infection known as eggshell apex abnormalities (EAA). Alteration in shell surface, shell thinning with cracks and breaks occurrence are the characteristics of the new infection form (Feberwee et al., 2009). Also, involvement of MS in development of *E. coli* peritonitis syndrome (EPS) in layers has been reported (Raviv et al., 2007b).

The complete genome sequence of MS has been published (May et al., 2015; Vasconcelos et al., 2005). Analysis of MS genomes (strain 53) demonstrated the identity of several genome segments between *M. synoviae* and *M. gallisepticum* and the possibility for horizontal gene transfer between both pathogens *(Papazisi et al., 2003; Vasconcelos et al., 2005)*.

2.5. Diagnosis

Diagnosis of MG and MS is based on the detection of the pathogen and/or of specific antibodies. Detection of MG and MS pathogens is carried out through cultivation and or species-specific nucleic acid detection using molecular tools like PCR. Serological tests like serum plate agglutination tests (SPA), hemagglutination inhibition (HI) or enzyme-linked immunosorbent assay (ELISA) are tests used for the detection of MG and MS specific antibodies (Kleven, 2008b; OIE, 2008).

2.5.1. Cultivation and identification

M. gallisepticum and *M. synoviae* are relatively fastidious micro-organisms that require protein rich medium usually enriched with serum or serum factors (Kleven, 2008a). MG and MS ferment glucose which is also added to the growth medium. MS requires extra addition of Nicotinamide adenine dinucleotide (NAD) (Kleven, 2008b).

Similar to other Mycoplasmas, MG and MS are resistant to antibiotics affecting cell wall synthesis and are partially resistant to thallium acetate. Penicillin (2,000 IU/ml) and thallium acetate (up to 1:2.000) are added to the growth medium to control other bacterial and fungal contamination. Phenol red is added to the broth medium as indicator for growth and pH changes, e.g. MG and MS ferment glucose leading to acid formation that lowers the pH and the colour of the phenol red indicator changes from red to orange/yellow (Bradbury, 1998; OIE, 2008).

Several laboratory and commercial liquid and agar media are known to be used for MG/MS isolation, e.g. Frey medium (Frey et al., 1968), SP-4 medium (Bradbury, 1977; Bradbury, 1998), PPLO medium (Kleven, 2008b) and medium offered by *Mycoplasma* Experience, Reigate, Surrey, United Kingdom.

Optimal growth of MG and MS is usually achieved at 37°C with 5% CO_2 and high humidity. MG and MS colonies are usually seen after 3–5 days post incubation, however some isolates may take longer. Growth of fastidious isolates may require 2 or 3 serial passages with 5-7 days intervals. Direct plating of exudates or tissue swabs onto *Mycoplasma* agar may result

in colonies after 4–5 days of incubation, but initial culture in broth followed by agar plates inoculation has been reported to be more sensitive for isolation (Kleven, 2008b).

Typical *Mycoplasma* colonies are small (about 0.1—1.0 mm), circular with elevated centre giving the 'fried egg' appearance on solid medium, however it might not be seen with some *Mycoplasmas*. Overgrowth of non-pathogenic *Mycoplasma* like *M. gallinarum* and *M. gallinaceum* on MG or MS is known, especially in samples of multiage layers (Kleven, 2008b).

MG and MS colonies could be identified by direct or indirect immune-fluorescence (IF), immuno-peroxidase (IP), immuno-binding assay or growth inhibition test using species-specific antibodies (OIE, 2008; Rosendal and Black, 1972; Talkington and Kleven, 1983).

In spite of considering cultivation as a standard for infection diagnosis, slow growth and the fastidious nature of MG and MS that might take up to 3-4 weeks as well as the frequent overgrowth by non-pathogenic *Mycoplasma*, represent critical obstacles hindering the wide applicability MG and MS cultivation in practice.

2.5.2. Serological tests

Many serological tests have been described for the detection of MG and MS specific antibodies. From these tests, serum plate agglutination (SPA), the hemagglutination-inhibition (HI) and Enzyme-linked immunosorbent assays (ELISA) are the mostly used (OIE, 2008).

The SPA test is a simple, quick, and inexpensive test for the detection of MG and MS antibodies. It is carried out by mixing equal amounts of tested serum sample and stained MG or MS antigen. Antigens for MG and MS are commercially available. The SPA test has efficient sensitivity because it detects mainly the early produced immunoglobulin after infection, the IgM antibodies (Kleven, 1975) however, its main disadvantage is the low specificity. Cross reactions and false positive results are known to occur with test application. Cross reactions between MG and MS, recently vaccinated birds with oil-emulsion vaccines as well as media components included through antigen preparation are causes for SPA false positive reactions (Glisson et al., 1984; Kleven, 2008b; Yoder, 1989).

On the other hand, the hemagglutination-inhibition (HI) is more specific compared to the SPA test, but some obstacles hinder its wide usage like the laboriousness, time consumption, and unavailability of reagents needed on the commercial scale (Kleven, 2008b; Kleven et al., 1988). The main disadvantages of the HI test are the inability to detect antibodies of variant MG strains and the low sensitivity as it might not detect antibodies before 3 weeks post infection. This is because the HI test detects the lately produced immunoglobulins, the IgG antibodies (Talkington and Kleven, 1983).

Enzyme-linked immunosorbent assays (ELISA) were developed to improve the sensitivity of the hemagglutination-inhibition test and the specificity of serum plate agglutination tests. Beside many available commercial ELISA kits for the detection of MG and MS, laboratory developed ELISA assays have also been described (Higgins and Whithear, 1986; Opitz et al., 1983).

In order to improve specificity of ELISA, MG and MS species specific proteins were purified and cloned to be used for coating ELISA plates (Buyuktanir et al., 2008; Noormohammadi et al., 2002a; Noormohammadi et al., 2002b; Noormohammadi et al., 1999). Multiplex ELISAs detecting MG, MS and *M. meleagridis* have also been described (Ben Abdelmoumen Mardassi et al., 2008).

The efficiency of culture and PCR was compared with serological tests (SPA, HI and ELISA) for the detection of MG. PCR and culture showed comparable results and were better than serological tests. Different degrees of false positive results with most of the serological tests were demonstrated. A combination of more than one diagnostic tool for definite MG diagnosis was recommended (Feberwee et al., 2005b).

Generally, high frequency of antigenic variability experienced by MG and MS isolates, cross reaction between MG and MS and other pathogens, interference with oil-emulsion vaccines and medications have been reported as reasons for false positive results achieved by serological tests (Kleven, 2008b). Therefore, MG and MS serological tests are recommended as screening tests rather than being definite diagnostic tests. Flocks with positive serological results should be confirmed by other methods like pathogen isolation and molecular detection of the infecting pathogen (Raviv and Ley, 2013).

2.5.3. Molecular diagnosis and identification

Introduction of molecular techniques to the MG and MS diagnostic represented an important shift. Accuracy, time saving, and cost effectively of molecular techniques made them to complementary or even alternative for conventional diagnostic methods.

In early research works, DNA probes were widely used for MG and MS diagnosis (Dohms et al., 1993; Fernandez et al., 1993; Geary, 1987; Geary et al., 1988; Khan et al., 1987; Santha et al., 1987; Zhao and Yamamoto, 1990). Afterwards, PCR represented a safer, more accurate and applicable alternative that avoids the harmful effect of radiolabeled probes with better sensitivity and easier applicability. Improvements for sensitivity and specificity of conventional PCR took place with the introduction of new modifications like semi-nested, nested, multiplex, real-time and TaqMan probe PCRs.

2.5.3.1. PCR for MG diagnosis

Species-specific PCRs for MG were firstly developed in the nineties of the last century. The earlier PCR trial used Amp-L and Amp-R primers and produced a 732 bp amplicon. This PCR showed positive results with nucleic acid of 16 MG strains and isolates, while negative results were shown with DNA of 16 avian *Mycoplasma*s other than MG as well as with *E. coli*, lambda phage, pUC8 plasmid and calf thymus (Nascimento et al., 1991). Two years later, the MGF-PCR that differentiated MG F vaccine strain from other MG strains was developed. MGF-PCR used MGF-P1 L and MGF-P1R primers and amplified a 524 bp product with MG F-strain only (Nascimento et al., 1993).

By the middle of 1990s and with the advances in sequencing and utilization of 16S rRNA nucleotide sequence as a basis for bacterial taxonomy, other MG species-specific PCR assays based on MG 16S rRNA gene sequence were developed (Kempf et al., 1993; Lauerman, 1998). The new PCR assays proved to be sensitive and specific for MG detection, where MG species-specific PCR tested positive with 70 out of 72 tracheal swabs collected from MG experimentally infected specific pathogen free (SPF) chickens (Kempf et al., 1993).

Combination of PCR with restriction fragment length polymorphism (RFLP) analysis (PCR-RFLP) was used to detect and differentiate MG, MS, *M. iowae* and *M. meleagridis* , however it did not differentiate between MG and *M. imitans* (Fan et al., 1995b). Another PCR-RFLP

amplified the *Mycoplasma* specific 16S rRNA sequence by PCR using common primers to avian *Mycoplasma*s followed by restriction of the amplified product using restriction enzymes. Species identification is based on the restriction pattern. The new PCR was specific (could differentiate between MG, MS and *M. imitans*) and sensitive (100 Mycoplasmas detection capability).

With the identification of the MG specific cytadhesin-like protein MGC2, a PCR based on the *mgc2* gene was developed (Hnatow et al., 1998). Combination of PCR targeting the *mgc2* gene with restriction of the amplified product (PCR-RFLP) was able to differentiate between some MG strains like MG 6/85 and ts-11 vaccine strains (Lysnyansky et al., 2005).

The specificity and sensitivity for four MG-PCRs (16S rRNA PCR, *mgc2*-PCR, the nested LP-PCR and *gapA*-PCR) were compared. The lowest specificity was shown with the 16S rRNA PCR that amplified DNA from both MG and *M. imitans*, whereas *mgc2-*, nested *LP-*, and nested *gapA* PCRs amplified DNA from MG only. The sensitivity of the PCRs of 16S rRNA and *mgc2*, nested LP, and nested *gapA* were estimated at 40, 40, 400 and 4 color-changing units (CCU)/amplification reaction, respectively. Based on the sensitivity and specificity results as well as the time turnaround, the *mgc2*-PCR seemed to be the most promising among the PCR methods investigated (Garcia et al., 2005).

Lierz and coauthors developed and evaluated species-specific PCR for detection of MG. The newly developed MG-PCR together with other species–specific assays for the differentiation of MS, *M. meleagridis*, *M. imitans* and *M. iowae* were used and evaluated for the detection of the respective pathogens in birds of prey (Lierz et al., 2008b). PCR sensitivity for MG, MS, *M. meleagridis*, *M. imitans* and *M. iowae* were high and were 0.1, 1, 10, 0.1 and 0.1 pg of genomic DNA, respectively.

Sensitivity of a new qualitative real-time polymerase chain reaction (Q-PCR) for *M. gallisepticum* detection was evaluated. The Q-PCR showed 10 to 1000 times better sensitivity than conventional PCR and culture. The Q-PCR showed good specificity with most important avian *Mycoplasma*s like MS and *M. meleagridis*, however, it could not distinguish between MG and *Mycoplasma imitans* (Mekkes and Feberwee, 2005).

Another real-time PCR assay differentiating between MG strains including the F, ts-11, and 6/85 vaccine strains has been described (Raviv et al., 2008). Also a combination of the TaqMan probes technology with real-time PCR for identification of the four common poultry *Mycoplasma*s, MG, MS, *M. meleagridis* and *M. iowae* has been reported. Sensitivity of the new PCR was 1 copy for MG and 10 copies for MS, *M. meleagridis* and *M. iowae* (Raviv and Kleven, 2009).

2.5.3.2. PCR for MS diagnosis

Similar to MG and based on the availability of 16S rRNA sequence of the M. *synoviae*, species-specific MS-1 (5'-GAAGCAAAATAGTGATATCA-3') and MS-2 (5'-GTCGTCTCCGAAGTTAACAA-3') primers were selected and utilized for MS species-specific PCR. This PCR showed 100% specificity with an estimated sensitivity of 100 colony-forming units (Lauerman et al., 1993). Afterwards, this MS-PCR design was modified by addition of two nucleotides at the 5’ end of the primer to improve specificity. Through clinical samples, this modified MS-PCR showed higher sensitivity than culture and detected 17 MS samples. However, only 7 samples out of 27 total samples were detected by culture (Marois et al., 2000).

Another PCR for MS was developed by pairing a primer complementary with the 16S-23S intergenic spacer region with a primer from the 23S rRNA gene (Ramirez et al., 2006). This PCR was sensitive (1.15 pg DNA detection) and tested positive with 21 MS field isolates, while negative with 22 other avian mycoplasmas.

A new PCR assay using primers complementary to the single-copy end of the variable lipoprotein and haemagglutinin gene (*vlhA*) of MS was developed. The new PCR (*vlhA*-PCR) amplified approximately 400 bp and showed good degree of MS intra-species discrimination (Jeffery et al., 2007). Due to high degree of sequence variation in the *vlhA* gene, new revised degenerating primers were designed to improve sensitivity of the *vlhA*-PCR. This revised *vlhA*-PCR amplified approximately 300 bp of the single-copy end of the *vlhA* gene and showed better sensitivity (Wetzel et al., 2010).

A duplex PCR assay based on the pMGA and *vlhA* haemagglutinins for the detection of MG and MS, respectively, in one reaction was described (Mardassi et al., 2005; Pflaum et al.,

2016). The assay showed enough specificity and sensitivity for simultaneous detection of MG and MS field isolates (Mardassi et al., 2005). Also a duplex real-time PCR assay using TaqMan probes and including internal controls (duplex MGMS PCR) for MG and MS detection has been described (Sprygin et al., 2010b). The new duplex MG-MS PCR showed high specificity and a sensitivity of 1 and 7 cfu/ml for MS and MG, respectively.

2.6. MG and MS typing

2.6.1. MG typing by Sequencing of partial genes

MG has surface-exposed cytadhesins known as haemagglutinins. These haemagglutinins express phase and size variability, and they are immunogenic as well (Bencina, 2002; Razin et al., 1998). MG haemagglutinins are variable lipoproteins encoded by up to 70 genes. Other cytadhesins like PvpA, Mgc2 and Mgc1 proteins are also expressed by MG but they are encoded as single copy in the MG genome. High degree of phase variation in gene and proteins of the surface integral membrane protein of MG (the *pvpA)* gene has also been documented (Yogev et al., 1994).

Molecular characterization of the *Mycoplasma gallisepticum* cytadhesin 2 (*mgc2*) gene and protein revealed differences between MG strains (Hnatow et al., 1998). Amplification of the *mgc2* gene of the MG 6/85 vaccine strain by PCR produced a characteristic amplicon that differed from other MG strains (Garcia et al., 2005; Kleven et al., 2004). Moreover, restriction of the *mgc2*-PCR amplicon with *Hae*II and *Sfa*N1 restriction enzymes known as the restriction fragment length polymorphism (*mgc2*-PCR-RFLP) technique could additionally differentiate MG ts-11 vaccine strains from other MG strains (Lysnyansky et al., 2005).

Boguslavsky and coauthors characterized an integral surface expressed membrane protein, the PvpA protein, that included high proline content in the C-terminus of the amino acid sequence with two identical copies of 52 amino acids sequences designated direct repeat 1 (DR1) and direct repeat 2 (DR2) sequences (Boguslavsky et al., 2000).

Differences in *pvpA* gene sequences between MG strains were the basis for the usage and development of restriction fragment length polymorphism of *pvpA* gene (*pvpA*-RFLP) as well

as *pvpA* sequences alignment and comparison (Ferguson et al., 2005; Liu et al., 2001; Pillai et al., 2003).

PvpA-RFLP included amplification of the variable putative cytadhesin protein *pvpA* gene by PCR followed by restriction of the amplicon using restriction endonucleases (Liu et al., 2001). *PvpA*-RFLP could distinguish 7 different genogroups within 66 MG isolates (Pillai et al., 2003). *PvpA*-RFLP together with other MG genome fragments have been also used to differentiate the MG live vaccine strain F from other MG strains like MK-7, MS-16, R , S6 and FS-9 strains (Biro et al., 2006).

Sequencing of other MG gene segments like the MG 16S-23S intergenic spacer region (IGSR) was also evaluated as a tool for MG genotyping. Some reports described that the 16S-23S intergenic spacer region is a helpful tool for *Mycoplasma* inter species differentiation (Ramirez et al., 2008; Volokhov et al., 2006). However, other studies reported a good discriminatory power of 16S-23S intergenic spacer region as a tool for MG intra-species differentiation and considered it as a promising tool for epizootiological studies (Gharaibeh et al., 2011; Raviv et al., 2007a).

On the other hand, sequencing of single target gene segments like the *pvpA, mgc2,* and *gapA* genes has been used as a basis for MG strains typing and discrimination (Hong et al., 2005b; Khalifa et al., 2014; Pillai et al., 2003; Raviv et al., 2007a; Sprygin et al., 2010a).

Combination of sequencing results of multiple segments of the MG genome like *pvpA, mgc2, gapA* and MG 16S-23S IGSR known as multiple gene-targeted sequencing (multiple GTS) proved high discriminatory capability between MG strains (Ferguson et al., 2005; Gharaibeh et al., 2011; Ghorashi et al., 2013). Multiple GTS proved also comparable typing results to those of whole genome finger printing techniques like RAPD and AFLP. Extra advantages of multiple GTS like higher reproducibility, typing of uncultivable *Mycoplasma* isolates and the ability of inter-laboratory data exchange have been reported (Ferguson et al., 2005; Gharaibeh et al., 2011). Trials to create a database for MG genotyping on the basis of multiple GTS have been described (Armour et al., 2013; Ghorashi et al., 2013).

2.6.2. MS typing by Sequencing of partial genes

Genomes of MS encode some proteins known as haemagglutinins that show high degree of variation between strains and isolates. The variable lipoprotein haemagglutinin A gene (*vlhA*) is one of those haemagglutinins. VlhA of MS are lipoproteins encoded by *vlhA* gene and pseudogene (Bencina, 2002; Noormohammadi, 2007).The *VlhA* of MS reveals a high degree of variability between strains (Noormohammadi et al., 2000). Nucleotide sequence variation of the *vlhA* of MS has been the basis for MS genotyping (Bayatzadeh et al., 2014; Dijkman et al., 2014; El-Gazzar et al., 2012; Hong et al., 2004; Jeffery et al., 2007; Ramirez et al., 2011; Wetzel et al., 2010).

The *vlhA* gene of MS is widely used for MS typing in many countries (Bayatzadeh et al., 2014; Dijkman et al., 2014; Ogino et al., 2011). Because of the high degree of variation in the *vlhA* gene between MS strains, different primers sequences for improving the *vlhA* amplification efficiency have been described (Hammond et al., 2009; May and Brown, 2011; Ogino et al., 2011; Wetzel et al., 2010). A PCR design amplifying approximately 400 bp of the MS-*vlhA* gene was developed in 2007 (Jeffery et al., 2007). Also a revised *vlhA*-PCR using degenerating primers and amplifying approximate 300 bp of the MS-*vlhA* gene was established (Wetzel et al., 2010). The revised *vlhA*-PCR assay showed better sensitivity than other PCRs and could overcome the problem of false negative results (300 pm).

In a recent study, the discriminatory power of the *vlhA* gene and the AFLP were compared. Comparable clustering of investigated MS strains was described, in spite of detection of minor differences between both methods (Dijkman et al., 2014).

The potential capability of the MS 16S-23S intergenic spacer region (IGSR) was investigated for MS strains differentiation. The heterogeneity of the two copies of the 16S-23S IGSR of MS limited its wide use for MS typing. This heterogeneity necessitated the inclusion of a cloning system which is not available for all laboratories (Ramirez et al., 2011).

Although the *vlhA* gene is yet the only gene available for MS genotyping, trials for utilization of other MS gene segments or other techniques are research focus for many scientists. This should enable definite, robust MS strains differentiation.

2.6.3. Whole genome fingerprinting techniques

Different whole genome typing techniques like random amplified polymorphic DNA (RAPD), restriction endonuclease analysis (REA), pulsed-field gel electrophoresis (PFGE) and the amplified fragment length polymorphism (AFLP) have been described for MG and MS typing.

The RAPD technique is based on utilization of random primers for amplification of purified genomic DNA. The RAPD technique produced characteristic electrophoretic profiles with MG strains that markedly differed from those of MS, *M. gallinarum* and *M. iners* using the same arbitrary primers (Geary et al., 1994). Another RAPD assay with different arbitrarily primers was developed and could differentiate and group 25 MG strains and isolates according to the electrophoretic pattern on agarose gels (Fan et al., 1995a).

Efficiency of RAPD technique and pulsed-field gel electrophoresis (PFGE) was compared for MG and MS strains typing. The two techniques showed comparable results and could differentiate MG strains from the *M. imitans* strains. Low reproducibility of RAPD as well as the laboriousness and time demand and low typeability of PFGE were the drawbacks of these techniques (Harada et al., 2009; Marois et al., 2001; Mettifogo et al., 2006).

The AFLP is based on amplification of selective restriction fragments. AFLP assay includes digestion of genomic DNA with restriction endonucleases followed by ligation of specific adapters according to restriction sequences. Afterwards, ligated fragments are amplified by PCR using fluorescent-labeled primers and PCR amplicons are separated onto a polyacrylamide gel. The AFLP was evaluated for characterization of 50 strains of human and animal mycoplasmas. AFLP showed high discriminatory power with reproducible AFLP electrophoretic patterns that included 60 to 80 fragments ranging from 50 to 500 bp (Kokotovic et al., 1999).

The discriminatory capability of the RAPD technique and the AFLP for MG and MS differentiation was compared. AFLP showed higher discriminatory capability than the RAPD technique for MG and MS genotyping (Feberwee et al., 2005a; Hong et al., 2005a; Hong et al., 2005b). AFLP offers the advantage of combination of segmenting the genomic DNA using specific restriction enzymes together with amplification of restricted genomic DNA by

PCR. This combination allows better resolution of electrophoretic patterns and more bands allowing better discrimination between isolates (Hong et al., 2005a; Savelkoul et al., 1999).

2.7. Host range of MG and MS

MG and MS spread horizontally by direct and indirect contact with infected birds. Vertical or trans-ovarian transmission through eggs plays a significant role in the epidemiology and spread of MG and MS (Armour and Ferguson-Noel, 2015; Raviv and Ley, 2013). Contaminated feeders and drinkers with infected fomites have been also reported to transmit MG bacteria (Dhondt et al., 2007).

The natural hosts for MG and MS are the gallinaceous birds, especially chickens and turkeys. For a long time, *Mycoplasmas* were considered as host-specific pathogens (Razin et al., 1998), but in the meantime many reports describe the same *Mycoplasma* species to infect different hosts (Bradbury, 2005).

MG has been isolated from natural infections in ducks (Bencina et al., 1988a), geese (Buntz et al., 1986), song birds (Morishita et al., 1999) as well as from chukar partridges, pheasants, and peafowl (Cookson and Shivaprasad, 1994). Also MS has been isolated from natural infection cases in birds other than chicken and turkey like pigeon (Bencina et al., 1987; Reece et al., 1986), ducks (Bencina et al., 1988a), and geese (Bencina et al., 1988b).

Moreover, an emergence of a novel MG strain in American house finches (*Carpodacus mexicanus*) in the 1990s has been described (Ley et al., 1997; Ley et al., 1996; Luttrell et al., 2001). The new emerging disease resulted in epidemics of severe conjunctivitis in American house finches with estimated hundreds of millions of population losses in few years after the first wave of disease (Delaney et al., 2012; Dhondt et al., 2005; Ley et al., 1996). House sparrows and budgerigars have been experimentally infected with MG and MS and the respective pathogens were reisolated (Bozeman et al., 1984; Gharaibeh and Hailat, 2011; Kleven and Fletcher, 1983), however infected birds seemed less susceptible to infection as they did not show disease manifestations.

Also, some studies investigating the incidence, susceptibility and role of *Mycoplasma* in different bird categories have been described (Farmer et al., 2005; Hartup et al., 2001; Lierz

et al., 2007; Lierz et al., 2008a; Lierz et al., 2008b; Lierz et al., 2000; Poveda et al., 1990), where different Mycoplasma species were detected in new hosts. However, none of these studies investigated or compared infecting *Mycoplasma* pathogens from new hosts down to the sub species level.

On the other hand, reports describing threats of emerging diseases caused by free-ranging birds, like avian influenza A virus, West Nile virus infection and other infections, are increasing. So that, more attention has to be directed towards investigation of the role played by such birds in disease epidemiology (Staley and Bonneaud, 2015).

3. Materials and Methods

3.1. Samples

3.1.1. Samples investigated during the first part of study

Five hundred fifteen Swabs (tracheal, pharyngeal, choanal, lung, synovial and phallus) were collected from 104 different bird flocks (Table 1). These flocks represented cases showing clinical signs of *Mycoplasma* infection (respiratory, locomotor disorders or fertility troubles). Some flocks originated from the same farm but from different occasions.

Table 1: List of samples investigated through first part of study including their identity number, sample type, actual number of birds sampled and some notes concerning case history when available.

	Entry number (Flocks)	animal species	Sample type	No. of swabs
1	632/11/TK	Turkey breeders	Tracheal swab	10
2	774/11/CK	Chicken	Tracheal swab	10
3	824/11/TK	Turkey breeders	Tracheal swab	5
4	884/11/PH	Pheasant	Synovial fluid	1
5	898/11/Tit	Blue and grey tits	Choanal swab	82
6	920/11/CK	Chicken	Tracheal swab	1
7	940/11/CK	Chicken	Tracheal swab	1
8	982/11/GS	Goose	Phallus swab	3
9	985/11/TK	Turkey breeders	Tracheal swab	2
10	998/11/GS	Goose	Tracheal swab	1
11	1001/11/CK	Chicken	Tracheal swab	3
12	1074/11/CK	Chicken	Tracheal swab	10
13	1105/11/TK	Turkey breeders	Tracheal swab	3
14	1233/11/CK	Chicken	Tracheal swab	2
15	1255/11CK	Chicken	Tracheal swab	1
16	1321/11/CK	Chicken	Tracheal swab	5
17	1470/11/CK	Chicken	Tracheal swab	2
18	1478/11/TK	Turkey	tracheal swab	1
19	1482/11/CK	Chicken	tracheal swab	3
20	1571/11/CK	Chicken	tracheal swab	1

21	1601/11/TK	Turkey	Tracheal swabs & synovial fluid	1
22	1608/1/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
23	1608/2/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
24	1608/3/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
25	1608/4/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
26	1608/5/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
27	1608/6/11/TK	Turkey breeders	*Mycoplasma* colonies on agar plates	1
28	1608/7/11/TK	Fattening turkey	*Mycoplasma* colonies on agar plates	1
29	1608/8/11/TK	Fattening turkey	*Mycoplasma* colonies on agar plates	1
30	1732/11/CK	Chicken	Tracheal swab	1
31	1750/11/CK	Chicken	Tracheal swab	1
32	1808/11/CK	Chicken	Tracheal swab	1
33	1861/11/CK	Chicken	Tracheal swab	1
34	1887/11/TK	Turkey	Tracheal swab	3
35	1909/11/CK	Chicken	Tracheal swab	1
36	1931/11/CK	Chicken	Tracheal swab	1
37	1965/11/CK	Chicken	Tracheal swab	2
38	2020/11/PH	Pheasant	Tracheal swab	1
39	2038/11/CK	Chicken	Tracheal swab	1
40	2042/11/TK	Turkey	synovial fluid	4
41	2072/11/CK	Chicken	Tracheal swab	3
42	2081/11/CK	Chicken	Tracheal swab	1
43	2141/11/TK	Turkey	Tracheal swab	3
44	2192/11/CK	Chicken	Tracheal swab	1
45	2464/11/CK	Chicken	Tracheal swab	1
46	2539/11/CK	Chicken	Tracheal swab	1
47	2636/11/CK	Chicken	Tracheal swab	1
48	2730/11/TK	Turkey breeders	Tracheal swab	12
49	24/2012/TK	Turkey breeders	Tracheal swab	24
50	187/12/TK	Turkey breeders	Tracheal swab	25
51	279/12/PG	Turkey breeders	Tracheal swab	10
52	417/12/TK	Turkey	Tracheal swab in Mycoplasma medium	6
53	499/12/TK	Turkey	Tracheal swab in Mycoplasma medium	1
54	561/12/TK	Turkey breeders	Tracheal swab	6
55	720/12/ZF	Zebra finches	Choanal & pharyngeal swabs	19
56	797/12/CK	Chicken	Tracheal and synovial swabs	7
57	904/12/TK	Turkey breeders	Tracheal swab	2

58	1567/12/CK	Chicken	Tracheal swab	4
59	1786/12/CK	Chicken	Tracheal swab	22
60	1858/12/CK	Chicken	Tracheal swab	10
61	1885/12/CK	Chicken	Tracheal swab	11
62	1905/12/SW	Swan	Tracheal swab	1
63	1906/12/CK	Chicken	Tracheal swab	1
64	1907/12/CK	Chicken	Tracheal swab	2
65	1936/12/CK	Chicken	Tracheal swab	1
66	2122/12/CK	Chicken	Tracheal swab	2
67	2124/12/CK	Chicken	Tracheal swab	1
68	2187/12/CK	Chicken	Tracheal swab	1
69	2207/12/CK	Chicken	Tracheal swab	2
70	2208/12/CK	Chicken	Tracheal swab	4
71	2286/12/CK	Chicken	Tracheal swab	9
72	2312/12/PH	Pheasant	Tracheal swab	1
73	2453/12/CK	Chicken	Tracheal swab	2
74	2348/12/TK	Turkey	Synovial fluid	10
75	19/13/CK	Chicken	Tracheal swab	2
76	154/13/CK	Chicken	Tracheal swab	1
77	209/13/CK	Chicken	Tracheal swab	1
78	342/13/CK	Chicken	Tracheal swab	1
79	364/13/CK	Chicken	Tracheal swab	6
80	370/13/CK	Chicken	Tracheal swab	1
81	516/13/PG	Pigeon	Trachea & Air sacs swabs	1
82	612/13/CK	Chicken	Tracheal swab	2
83	649/13/TK	Turkey	Tracheal swab	2
84	833/13/CK	Chicken	Tracheal swab	13
85	925/13/CK	Chicken	Tracheal swab	4
86	1088/13/TK	Turkey	Tracheal swab	2
87	1233/13/CK	Chicken	Tracheal swab	10
88	1372/13/CK	Chicken	Tracheal swab	2
89	1554/13/CK	Chicken	Tracheal swab	1
90	1561/13/CK	Chicken	Tracheal swab	1
91	1847/13/CK	Chicken	Tracheal swab	2
92	1911/13/CK	Chicken	Tracheal swab	2
93	2054/13/CK	Chicken	Tracheal swab	2
94	2087/13/CK	Chicken	Tracheal swab	1

95	2251/13/CK	Chicken	Tracheal swab	10
96	2554/13/CK	Chicken	Tracheal swab	1
97	2591/13/CK	Chicken	Tracheal swab	2
98	77/14/CK	Layer chickens	Tracheal swab	20
99	96/14/CK	Chicken	Tracheal swab	10
100	171/14/CK	Chicken	Tracheal swab	1
101	331/14/CK	chicken	Tracheal swab	20
102	374/14/PH	Pheasant	Tracheal and Lung swabs	11
103	600/14/CK	Chicken	Tracheal swab	1
104	1073/14/CK	Chicken	Tracheal and Lung swabs	2

Sample description: entry number/year of sampling/bird species.
For bird species: CK = chicken; GS= Goose; PH=Pheasants; SW= Swan; Tit= Blue and grey tits; TK = turkey and ZF=Zebra finches
*samples 1608-1/11/TK and 1608-2/11/TK were isolated in 2006 and 2007, respectively, but were investigated during this work.

3.1.2. Samples investigated during the second part of study

During the second part of the study, chickens and contacting free-ranging birds from areas of two organic chicken farms (farm I and farm II). Chickens and contacting free-ranging birds were simultaneously sampled over 3 days.

In farm I, Lohmann brown layers were reared, however, in farm II, Lohmann brown layer breeders were kept. Contacting free-ranging birds were trapped using mist nets built in the free-ranging areas in both farms. The permission No. 39.80.02.40-2013/01 39.3 B/rl, RP Giessen, Giessen federal office authority was obtained before trapping free-ranging birds. Tracheal swabs were collected from chickens, while oro-pharyngeal swabs were collected from free-ranging birds. Captured free-ranging birds we released after sampling and trimming a small part from tail feathers to avoid resampling.

Samples collected from farm I are;

a- 20 tracheal swabs from chicken.

b- 70 pharyngeal swabs from house sparrows (*Passer domesticus*).

c- 3 pharyngeal swabs from blue tits (*Parus caeruleus*).

d- 1 pharyngeal swab from black bird (*Turdus merula*).

Samples collected from farm II are;

a. 20 tracheal swabs from chicken.
b. 43 pharyngeal swabs from house sparrows (*Passer domesticus*).
c. 1 pharyngeal swab from black bird (*Turdus merula*).

3.2. Devices for *Mycoplasma* cultivation

Sanyo CO_2 incubator	Sanyo, München
Light microscope	Carl Zeiss, Oberkochen
pH meter	Mettler-Toledo, Schwerzenbach
Fine Balance	Denver Instrument, Göttingen
Filter membrane	Sartorius, Göttingen
0.22 and 0.45 µm syringe filter membrane	Millex®HA, Millipore, Carrigtwohill, Ireland
Sterile petri dishes	Sartorius, Göttingen
Sterile plastic tubes	Sartorius, Göttingen
Lamina Flow Class II	Nunc, Wiesbaden

3.3. Chemicals for *Mycoplasma* cultivation medium (SP4 broth and SP4 agar medium)

1) PPLO Broth: BD Biosciences, Heidelberg
2) Bacto agar: BD Biosciences, Heidelberg
3) Swine serum: PAA Laboratories, Pasching, Austria

   Heat inactivated at 56°C for 45 minutes in water bath, aliquoted into 50 ml, and stored at -20°C till use.
4) Fresh yeast preparation: Commercial yeast powder

   250 g commercial dried yeast were dissolved in distilled water till 1000 ml total volume. This suspension was boiled in a boiling water bath for 30 min. It was then cooled and centrifuged at 3000 g for 30 min. The supernatant was taken, sterilized by filtration through 0.45 µm filter, aliquoted into 50 ml, and stored at -20°C till use.
5) Nicotinamide Adenine dinucleotide (NAD): Sigma-Aldrich, Munich

   1% NAD solution was prepared by dissolving 0,5 g NAD in 50 ml distilled water. The solution was sterilized by filtration through a 0.45 µm filter (Millex®HA, Millipore, Carrigtwohill, Ireland), aliquoted into 5 ml, and stored at -20°C till use.

6) Glucose: Merck, Darmstadt

   10% Glucose solution was prepared by dissolving 10 g glucose in distilled water till 100 ml total volume. The solution was sterilized by filtration through 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

7) Arginine: Merck, Darmstadt

   10% Arginine solution was prepared by dissolving 10 g arginine in distilled water till 100 ml total volume. The solution was sterilized by filtration through 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

8) Ampicillin: Merck, Darmstadt

   1% Ampicillin solution was prepared by dissolving 1 g ampicillin in distilled water till 100 ml total volume. The solution was filtrated through a 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

9) Penicillin G sodium: Roth, Karlsruhe

   A solution of Penicillin G sodium 100 000 IU/ml was prepared by dissolving 10 000 000 Unites of Penicillin G sodium in distilled water till 100 ml total volume to give a solution of 100,000 units per ml. The solution was filtered through a 0.45 µm filter, aliquoted into 10 ml, and was stored at -20°C till use.

10) Thallium acetate: Roth, Karlsruhe

    5% Thallium acetate solution was prepared by dissolving 5 g Thallium acetate in distilled water till 100 ml total volume. The solution was filtrated through a 0.45 µm filter, aliquoted into 10 ml, and stored at -20°C till use.

11) Phenol red: Roth, Karlsruhe

    0.1% Phenol red solution was prepared by dissolving 0.1 g of phenol red powder in 2,8 ml of 0.1 M NaOH. This mixture was made up to 100 ml with double distilled water to make 0.1% solution and autoclaved at 121°C for 20 minutes, aliquoted into 10 ml, and then stored at -20°C.

3.3.1. SP4 broth medium preparation (1000 ml) (Bradbury, 1998)

Part A

PPLO broth	14.7 g
Distilled water	700 ml

Part B

Swine serum (heat inactivated)	150 ml
Fresh yeast extracts (25% solution)	100 ml
Nicotinamide adenine dinucleotide (NAD) (1% solution)	10 ml
10% Glucose solution (10% solution)	10 ml
10% Arginine solution (10% solution)	10 ml
Penicillin (100 000 U/ml)	10 ml
Ampicillin (1% solution)	10 ml
Thallium acetate (5% solution)	10 ml
Phenol red (0.1 % solution)	20 ml

- Part A is autoclaved at 121 °C for 20 minutes and then kept at 50°C in water bath.
- Components of part B were aseptically mixed together and added to part A.
- PH is then adjusted to 7.4 with 1 M NaOH. Prepared medium is distributed in 1,8 ml aliquots in sterile 6 ml plastic tubes and kept at 4°C for up to 6 months.

3.3.2. SP4 agar medium preparation (1000 ml) (Bradbury, 1998)

Part A

Difco PPLO broth	14.7 g
Bacto agar	10 g
Distilled water	700 ml

Part B

Swine serum (heat inactivated)	150 ml
Fresh yeast extracts (25% solution)	100 ml
Nicotinamide adenine dinucleotide (NAD) (1% solution)	10 ml
10% Glucose solution (10% solution)	10 ml

10% Arginine solution (10% solution)	10 ml
Penicillin (100 000 U/ml)	10 ml
Ampicillin (1% solution)	10 ml
Thallium acetate (5% solution)	10 ml

- Part A is autoclaved at 121 °C for 20 minutes and kept at 50°C in water bath.
- Components of part B were aseptically mixed together and warmed to 50°C in water bath and was then added to part A.
- Prepared agar medium is distributed in sterile 4 cm petri dishes and kept at 4°C for up to 6 months.
- Each newly prepared SP4 broth and SP4 agar was tested for sterility and growth efficiency for MG and MS strains.

3.4. *Mycoplasma* cultivation procedures (Bradbury, 1998)

Mycoplasma cultivation procedures were carried out using SP4 broth and SP4 agar as described (Bradbury, 1998). Tracheal (or pharyngeal) swabs were taken into fresh SP4 broth, vortexing, and swabs were taken out from broth and kept at - 80°C.

Inoculated broth was 10 fold serially diluted till 10^{-3} in fresh SP4 broth. Original and diluted broths were inoculated on SP4 agar plates. Inoculated broth and agar were incubated at 37*°C* and 5 % CO_2 with high humidity. Color changes of broth and appearance of *Mycoplasma* colonies on agar plates (detected under light microscope) were daily evaluated for the first 5 days and then every 2 days for up to 4 weeks. Incubated broth was subcultured onto fresh broth and agar plates directly (in case of broth color change) or every week (in case no broth color change was observed).

In case of *Mycoplasma* colonies' appearance, 3 single colonies (almost morphologically different) were immediately picked and taken onto fresh SP4 broth. Purification of colonies was done by filtration through a 0.45 µm filter membrane and passages onto fresh SP4 broth and agar. This process was repeated for 3 times for complete isolate purification. Pure clones were kept at -80 °C till further processing.

3.5. Chemicals and devices for DNA extraction

DNeasy Blood & Tissue Kit	QIAGEN, Hilden
QIAcube automatic machine for DNA extraction	QIAGEN, Hilden
Nano Drop spectrophotometer	Thermo Scientific, Braunschweig
Thermo mixer Comfort	Eppendorf, Hamburg
Absolute alcohol	Roth, Karlsruhe
Dulbecco's PBS	PAA Laboratories, Pasching, Austria

3.6. DNA extraction

DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) was used for DNA isolation according to the manufacturer's instructions with little modifications according to sample type.

3.6.1. DNA extraction from Mycoplasma cultured broth

In case of successful *Mycoplasma* cultivation onto SP4 media, DNA was extracted from cultured broth. One ml of cultured broth was centrifuged at 21.000 g for 10 minutes. Pellets were twice washed with sterile PBS before suspension in 100 µl PBS. Twenty µl of proteinase K were added to resuspended pellet. Two hundred µl of Buffer AL (lysis buffer) were added, thoroughly mixed by vortexing, followed by incubation at 56°C for 10 minutes. Two hundred µl of absolute alcohol were added and thoroughly mixed by vortexing. The whole mixture was pipetted onto DNeasy Mini spin column placed in a 2 ml collection tube.

The column was centrifuged at 6000 g for 1 min and the flow-through was discarded. The column was placed in a new 2 ml collection tube. Five hundred µl of Buffer AW1 (washing buffer 1) were added to the column followed by centrifugation for 1 min at 6000 g, and the flow-through was discarded. Five hundred µl of Buffer AW2 (washing buffer 2) were added to the column followed by centrifugation for 3 min at 20.000 g, and the flow-through was discarded. The DNeasy Mini spin column was then placed in a clean 1.5 ml eppendorf tube. One hundred µl Buffer AE (elution buffer) were pipetted onto the column membrane, incubated at room temperature for 1 min, and then centrifuged for 1 min at 6000 g. Steps from addition of proteinase K till elution were manually done or automatically by QIAcube automatic machine.

DNA concentration was measured by Nano Drop spectrophotometer (Thermo Scientific, Braunschweig, Germany) and DNA was frozen at -20 °C till examination.

3.6.2. DNA extraction from collected swab samples

In case of unsuccessful *Mycoplasma* cultivation or cultivation of *Mycoplasma* other than MG or MS, DNA was extracted directly from collected swab samples. Cotton tip of swabs were aseptically cut and taken into a sterile 1.5 ml eppendorf tube.

Three hundred fifty µl of sterile PBS were added to the cotton tip of swab followed by thorough pipetting or vortexing. One hundred µl of the PBS suspension were taken into fresh 1.5 ml eppendorf tube.

The following steps from addition of proteinase K, Buffer AL, absolute alcohol, washing, elution and freezing of eluted DNA were done as above mentioned with DNA extraction from *Mycoplasma* culture.

3.7. Chemicals and devices for polymerase chain reaction (PCR)

DreamTaq DNA polymerase master mix (10 X Dream Taq Buffer contains KCL and $(NH_4)_2SO_4$ and 20 mM $MgCl_2$)	Thermo Scientific, Braunschweig
HotStarTaq Master Mix (10 X HotStar Taq Buffer contains Tris·Cl, KCl, $(NH_4)_2SO_4$, 15 mM $MgCl_2$)	QIAGEN, Hilden
dNTPS Mix (2 mM each)	PEQLAB Biotechnologie, Erlangen
Water, nuclease free	QIAGEN, Hilden
TPersonal 48 thermocycler	Biometra, Göttingen
Oligonucleotide primers	Biomers, Ulm

3.8. Genus *Mycoplasma* PCR (Lierz et al., 2007) (modified from (van Kuppeveld et al., 1992)

Generally, the PCRs of the present study were performed using ready mixed Master Mixes; DreamTaq DNA polymerase master mix or HotStarTaq Master Mix, according to the manufacturer's instructions. PCR reaction master mix contained 2.5 µl 10 X Dream Taq Buffer, dNTP Mix, forward and reverse primers, Taq polymerase and template DNA. PCR reaction master mix was completed to 25 µl with nuclease free water. Thermal programs were carried out in the TPersonal 48 thermocycler according to exact thermal conditions for each PCR reaction. PCR products were electrophoresed into pre-stained agarose gel and were detected under ultraviolet transillumination.

Primers for Genus *Mycoplasma* PCR;

Forward primer (GPF) 5'- GCT GGC TGT GTG CCT AAT ACA -3'

Reverse primer (MGSO) 5'- TGC ACC ATC TGT CAC TCT GTT AAC CTC -3'

Master Mix composition:

Component	**Quantity (µl)**
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer GPF (10 pmol/µl)	0.4
Reverse primer MGSO (10 pmol/µl)	0.4
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	18.2
Total volume	25

Thermal program:

Step	**Temperature, °C**	**Time**	**Number of cycles**
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	56	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	1

3.9. Polymerase chain reaction (PCR) for MG diagnosis and genotyping

3.9.1. *Mycoplasma gallisepticum* (MG) species-specific PCR (Lierz et al., 2008b)

Forward primer (MGF) 5'-CCA AGG CGA TGA CGT GTA GTT-3'

Reverse primer (MGR) 5'-CTG CAG CAC CGA AGT ATT CGC TC-3'

Master Mix composition:

Component	**Quantity (µl)**
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer MGF (10 pmol/µl)	0.4
Reverse primer MGR (10 pmol/µl)	0.4
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	18.2
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	60	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	1

3.9.2. *Mycoplasma gallisepticum* cytadhesin 2 (*Mgc2*) PCR (Garcia et al., 2005)

Forward primer (*Mgc2*-F) 5'-CGC AAT TTG GTC CTA ATC CCC AAC A-3'

Reverse primer (*Mgc2*-R) 5'-TAA ACC CAC CTC CAG CTT TAT TTC C-3'

Master Mix composition:

Component	Quantity (µl)
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer *Mgc2*-F (10 pmol/µl)	1.0
Reverse primer *Mgc2*-R (10 pmol/µl)	1.0
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	16.9
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	35
Annealing	58	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.9.3. MG 16S–23S rRNA IGSR (MG IGSR) PCR (Raviv et al., 2007a)

Forward primer (MG-IGSR-F) 5'-GTA GGG CCG GTG ATT GGA GTT A-3'

Reverse primer (MG-IGSR-R) 5'-CCC GTA GCA TTT CGC AGG TTT G-3'

Master Mix composition:

Component	Quantity (µl)
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer MG-IGSR-F (10 pmol/µl)	1.0
Reverse primer MG-IGSR-R (10 pmol/µl)	1.0
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	16.9
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	3 min	1
Denaturation	94	20 sec	30
Annealing	55	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.9.5. Semi-nested MG 16S-23S IGSR PCR

We designed this semi-nested MG 16S-23S IGSR PCR through the present study to improve sensitivity of MG 16S-23S IGSR PCR. The new PCR design included two PCRs. Briefly, the first PCR targeted a longer segment of the MG 16S-23S IGSR due to inclusion of a new forward primer which is located upstream of the former forward primer, while the second PCR was the same used in the conventional MG 16S-23S IGSR PCR (Figure 1).

3.9.5.1. MG 16S-23S IGSR PCR-1

For this PCR, a genus-specific *Mycoplasma* primer (16S-F-MYC) located in the 16S rRNA gene (Volokhov et al., 2006) was utilized as a forward primer. The 16S-F-MYC primer was found to be located upstream of the forward primer of the MG 16S-23S IGSR PCR (Figure 1). The 16S-F-MYC primer was paired with the MG reverse primer (MG IGSR R) located in the 23S rRNA (Raviv et al., 2007a) (Figure 1). This PCR included 20 cycles of denaturation, annealing and extension, in addition to initial denaturation and final extension.

Forward primer (16S-F-MYC) 5'- GGT GAA TAC GTT CTC GGG TCT TGT ACA CAC -3' (Volokhov et al., 2006)

Reverse primer (MG IGSR R) 5'-CCC GTA GCA TTT CGC AGG TTT G-3' (Raviv et al., 2007a)

Master Mix composition:

Component	**Quantity (µl)**
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer (16S-F-MYC) (10pmol/ul)	1.0
Reverse primer (MG IGSR R) (10pmol/ul)	1.0
Dream Taq DNA polymerase (5 Units/ul)	0.1
Template DNA	2.5
Water, nuclease-free	16.9
Total volume	25

Thermal program:

Step	**Temperature, °C**	**Time**	**Number of cycles**
Initial denaturation	94	3 min	1
Denaturation	94	20 sec	20
Annealing	55	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.9.5.2. MG 16S-23S IGSR PCR-2 (Raviv et al., 2007a)

Procedures of this PCR were done as above mentioned for the MG 16S–23S rRNA IGSR (MG IGSR) PCR (Raviv et al., 2007a). This PCR was done using MG specific primers (MG IGSR F and MG IGSR R) with inclusion of 1 µl amplification product of the first MG 16S-23S IGSR PCR as a template DNA (Figure 1).

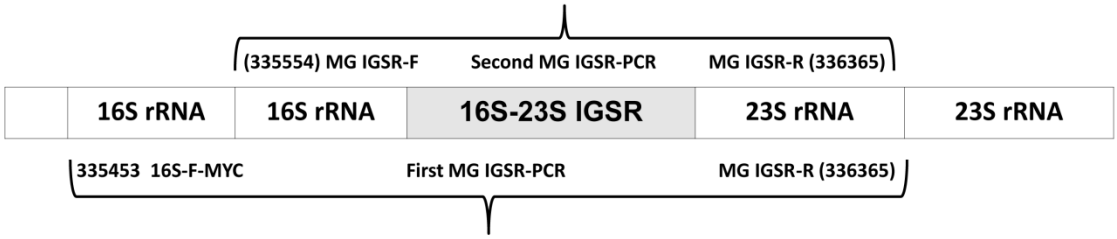

Figure 1: Schematic diagram of semi-nested MG 16S-23S IGSR-PCR indicating primers and their start point (for Forward primers) and end point (for reverse primer) in MG-R-high-strain (NC_017502.1). For the first PCR, 16S-F-MYC and MG IGSR-R primers were used, while for the second PCR, MG IGSR-F and MG IGSR-R primers were used.

3.9.6. MG phase-variable putative adhesin (*pvpA*) PCR (Liu et al., 2001)

Forward primer (*PvpA*-F) 5'-GCC AMT CCA ACT CAA CAA GCT GA -3'

Reverse primer (*PvpA*-R) 5'-GGA CGT SGT CCT GGC TGG TTA GC-3'

Master Mix composition:

Component	**Quantity (µl)**
10X HotStar Taq Buffer	2.5
dNTP Mix, 2 mM each	2.5
Forward primer *PvpA*-F (10pmol/µl)	1.0
Reverse primer *PvpA*-R (10pmol/µl)	1.0
HotStar Taq DNA polymerase (5 Units/µl)	0.10
Template DNA	2.5
Water, nuclease-free	15.35
Total volume	25

Thermal program:

Step	**Temperature, °C**	**Time**	**Number of cycles**
Initial denaturation	94	15 min	1
Denaturation	94	30 sec	40
Annealing	55	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.9.7. Semi-nested MG *PvpA*-PCR (Liu et al., 2001)

This semi-nested MG *pvpA*-PCR was used to improve sensitivity of the conventional MG *pvpA*-PCR. The new PCR design included two PCRs. Briefly, the first PCR is the same like the

conventional MG *pvpA*-PCR , while the second PCR targeted a shorter segment of the MG *pvpA* due to inclusion of a new forward primer which is located downstream of forward primer (Figure 2).

3.9.7.1. MG *pvpA* PCR-1 (Liu et al., 2001)

For this PCR, *PvpA*-F and *PvpA*-R primers used in MG *pvpA* PCR were utilized (Figure 2). Procedures of the first MG *pvpA* PCR are the same as above mentioned in the MG putative cytadhesin protein A (*PvpA*) PCR (Liu et al., 2001) but for only 20 cycles (denaturation, annealing and extension).

3.9.7.2. MG *pvpA* PCR-2 (Liu et al., 2001)

For this PCR, a new primer (MG *pvpA*-F2) located downstream of forward primer (MG *pvpA*-F), was used as a forward primer. The new forward primer (MG *pvpA*-F2) was used together with the MG *pvpA*-R (Liu et al., 2001) (Figure 2). This PCR was used to improve sensitivity although it targeted a smaller genome segment than conventional MG *pvpA* PCR. The second MG *pvpA* PCR was performed by including 1 µl of first MG *pvpA* PCR as a template DNA (Figure 2).

Forward primer (*PvpA*-F2) 5'-GGT AGT CCT AAG TTA TTA GGT C -3'

Reverse primer (*PvpA*-R) 5'-GGA CGT SGT CCT GGC TGG TTA GC-3'

Master Mix composition:

Component	**Quantity (µl)**
10X Hotstar Taq Buffer	2.5
dNTP Mix, 2 mM each	2.5
Forward primer (*PvpA*-F2) (10pmol/ul)	0.5
Reverse primer (*PvpA*-R) (10pmol/ul)	0.5
Hotstar Taq DNA polymerase (5 Units/ul)	0.15
Template DNA (from first PCR product)	1.0
Water, nuclease-free	17.85
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	15 min	1
Denaturation	94	30 sec	35
Annealing	55	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

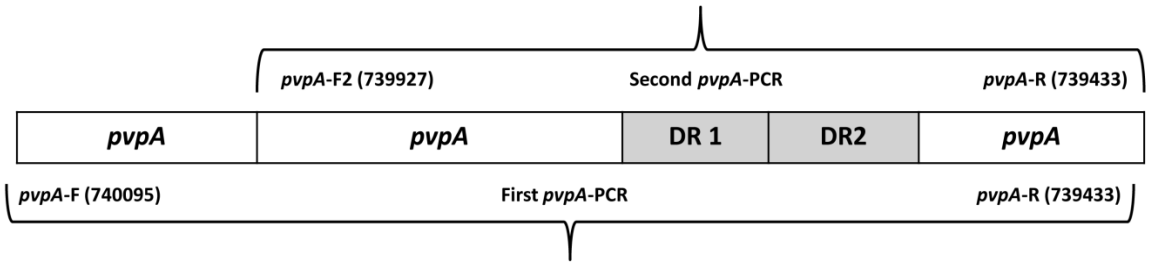

Figure 2: Schematic diagram of semi-nested MG *PvpA*-PCR indicating primers and their start point (for Forward primers) and end point (for reverse primer) in MG-R-high-strain (NC_017502.1). For the first PCR, MG-*PvpA*-1F and MG-*PvpA*-2R primers were utilized, while for the second PCR, MG-*PvpA*-3F and MG-*PvpA*-2R primers were used (Modified from (Liu et al., 2001)).

3.10. Polymerase chain reaction (PCR) for MS diagnosis and genotyping

3.10.1. *Mycoplasma synoviae* (MS) species-specific PCR (Marois et al., 2000)

Forward primer (MSF) 5'-GAG AAG CAA AAT AGT GAT ATC A-3'

Reverse primer (MSR) 5'-CAG TCG TCT CCG AAG TTA ACA A-3'

Master Mix composition:

Component	Quantity (µl)
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer MSF (10pmol/µl)	0.4
Reverse primer MSR (10pmol/µl)	0.4
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	18.2
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	56	30 sec	
Extension	72	30 sec	
Final extension	72	10 min	1

3.10.2. MS variable lipoprotein and haemagglutinin (*vlhA*) PCR (Jeffery et al., 2007)

Forward primer (*vlhA*-F) 5'- TAC TAT TAG CAG CTA GTG C-3'

Reverse primer (*vlhA*-R) 5'- AGT AAC CGA TCC GCT TAA T-3'

Master Mix composition:

Component	Quantity (µl)
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer MS-cons-F (10pmol/µl)	0.4
Reverse primer MS-cons-R (10pmol/µl)	0.4
Dream Taq DNA polymerase (5 Units/µl)	0.1
Template DNA	2.5
Water, nuclease-free	18.1
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	35
Annealing	54	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.10.3. Revised MS variable lipoprotein and haemagglutinin (*vlhA*) PCR (Wetzel et al., 2010)

For this MS-*vlhA*-PCR, degenerating primers were used to improve sensitivity efficiency.

Forward primer (Rev-*vlhA*-F) 5'- CCA TTG CTC CTG CTG TTA T -3'

Reverse primer (Rev-*vlhA*-R) 5'- KMT KCT GTT GTA GTT GCT TCA A -3'

Master Mix composition:

Component	Quantity
10X Dream Taq Buffer	2.5
dNTP Mix, 2 mM each	1.0
Forward primer Rev-*vlhA*-F (10pmol/µl)	1.0
Reverse primer Rev-*vlhA*-R (10pmol/µl)	1.0
Dream Taq DNA polymerase (5 Units/µl)	0.15
Template DNA	2.5
Water, nuclease-free	16.85
Total volume	25

Thermal program:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation	94	4 min	1
Denaturation	94	30 sec	40
Annealing	55	30 sec	
Extension	72	60 sec	
Final extension	72	10 min	1

3.10.4. Nested MS *vlhA*-PCR

This nested PCR was designed through the present study to improve sensitivity of conventional MS *vlhA*-PCR assays. Briefly, it was observed that MS-cons-F and MS-cons-R primers of Jeffery design (Jeffery et al., 2007) are located upstream of Rev-*vlhA*-F and Rev-*vlhA*-R primers of Wetzel design (Wetzel et al., 2010) in the conserved segment of the MS *vlhA* gene. The new nested MS *vlhA*-PCR design combined both Jeffery design and Wetzel design in a nested PCR design with minor modification (Figure 3).

The first MS *vlhA*-PCR was conducted using MS-cons-F and MS-cons-R primers with thermal conditions as described (Jeffery et al., 2007). It included 20 cycles of denaturation, annealing and extension, in addition to initial denaturation and final extension.

One µl of the first PCR product was used as template DNA in the second MS-*vlhA*-PCR. The second MS *vlhA*-PCR was done using *vlhA*-rev-F and *vlhA*-rev-R primers with thermal conditions as described (Wetzel et al., 2010).

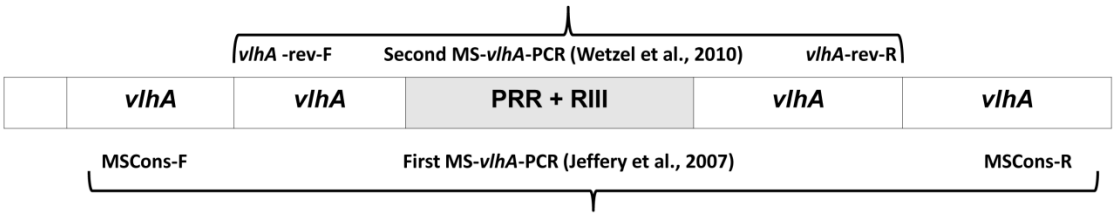

Figure 3: Schematic diagram of nested MS *vlhA*-PCR indicating primers and their start point (for Forward primers) and their end point (for reverse primer) in MS 53 strain (AE017245.1).

3.11. Chemicals and devices for agarose gel electrophoresis

Agarose NEEO ultra-Qualität	Roth, Karlsruhe
GeneRuler 100 bp DNA ladder and 6X Loading Dye	Thermo Scientific, Braunschweig
GelRed stain (10,000 X)	Biotium, Hayward, USA
UV-transilluminator (Biometra TI 5)	Biometra, Göttingen
Gel image Documentation system (BioDocAnalyze digital)	Biometra, Göttingen

Tris-Borat-EDTA-Buffer (TBE)-Buffer (10X concentration) (1000 ml solution)

108 g	Tris	Roth, Karlsruhe
55 g	Boric acid	Merck, Darmstadt
40 ml	0,5 M EDTA (Roth) pH 8,0	Merck, Darmstadt

PH is adjusted to 8,0 with 4 M NaOH and complete up to 1000 ml with distilled water.

For agarose gel preparation and electrophoresis buffer, 1X TBE solution was used.

3.12. Agarose gel electrophoresis

A range of 1-2% agarose concentrations in 1X TBE was prepared according to the expected molecular weight of the PCR products. GelRed stain (at 1:10,000 working concentration) was added to agarose solution. The solution was heated to boiling in a microwave for 3 minutes and left to warm before pouring in Gel casting chamber. PCR products were mixed with 6X loading dye before loading onto solidified casted gel. DNA ladder or GeneRuler 100 bp DNA ladder were included to confirm the molecular weight of amplicons. Electrophoresis was carried out for 45 minutes at 120 Voltage. Bands were detected under UV-transilluminator and photos were documented using BioDocAnalyze digital.

3.13. Chemicals and devices for PCR product purification

GeneJET PCR Purification Kit	Thermo Scientific, Braunschweig
GeneJET gel Purification Kit	Thermo Scientific, Braunschweig
Thermo mixer Comfort	Eppendorf, Hamburg
UV-transilluminator (Biometra TI 5)	Biometra, Göttingen
Gel image Documentation system (BioDocAnalyze digital)	Biometra, Göttingen
3 M sodium acetate, pH 5.2 solution	Roth, Karlsruhe

3.14. PCR product purification

PCR product was purified from PCR solution (in case of appearance of only specific band on agarose electrophoresis) or from agarose (in case of appearance of non-specific bands beside the specific band on agarose electrophoresis).

3.14.1. PCR product purification from PCR solution

For PCR fluid purification, GeneJET PCR Purification Kit (Thermo Scientific, Braunschweig) was used according to manufacturer's recommendations. Briefly, PCR fluid was adjusted to 100 µl with sterile PBS followed by thorough mixing. Hundred µl of Binding Buffer (yellow colour) were added followed by thorough mixing. In case the color of the solution is orange or violet, 10 µl of 3 M sodium acetate solution were added to the mixture. The mixture was brought onto the GeneJET purification spin column.

The spin column was centrifuged at 6000 g for 1 minute and the flow-through was discarded. The spin column was washed with 700 µl Wash Buffer and the flow-through was discarded. Empty spin columns were centrifuged for additional 1 minute for complete removal of wash buffer residues. Elution of purified DNA was done in 30 µl elution buffer.

3.14.2. PCR product purification from agarose

For PCR purification from agarose, GeneJET gel Purification Kit (Thermo Scientific, Braunschweig) was used according to the manufacturer's recommendations. Specific bands were cut from agarose using a clean scalpel and put into a 2 ml eppendorf tube. Binding buffer was added to the excised band with 1:1 volume. The agarose mixture was melted by incubation at 50-60 °C for 10 minutes on a thermo mixer till homogenization. In case the color of the solution was orange or violet, 10 µl of 3 M sodium acetate solution were added to the mixture. The mixture was brought onto the GeneJET purification spin column.

The following steps were the same as mentioned above for the PCR product purification from PCR fluid.

3.15. Program used for sequence analysis and alignment

Sequencing company	LGC Genomics GmbH, Berlin
Lasergene; DNASTAR, Inc. , Madison, USA (for sequencing editing, alignment and phylogenetic tree construction)	GATC Biotech, Konstanz

3.16. PCR product sequencing, sequence analysis and processing of amplicons

Sequencing of PCR products was performed at LGC Genomics GmbH, Berlin, Germany. Resulting sequences were blasted against GenBank data (http://www.ncbi.nlm.nih.gov/). Sequence editing, alignments and phylogenetic tree construction were done using Lasergene; DNASTAR, Inc., Madison, USA (GATC Biotech, Konstanz). Resulting sequences and respective sequences obtained from GenBank were edited to have the same start and end nucleotide sequence. Afterwards, sequences were aligned by the Clustal W method. Phylogenetic trees were constructed and the relationship between sequences was estimated by MegAlign program (Lasergene; DNASTAR, Inc., Madison, USA).

3.17. Chemicals and devices for restriction fragment length polymorphism of MG putative cytadhesin (*PvpA*-RFLP)

Digestion Buffer G (10X)	Thermo Scientific, Braunschweig
GeneRuler Low Range DNA ladder (from 25 bp to 700 bp)	Thermo Scientific, Braunschweig
Water bath	Köttermann Labortechnik, Uetze-Hänigsen

Restriction endonuclease enzymes for *PvpA*-RFLP

Enzyme	**Recognition site**	**Concentration**	**Company**
PvuII	5'...C A G↓C T G...3' 3'...G T C↑G A C...5'	10 U/µl	Thermo Scientific, Braunschweig
XmiI (AccI)	5'...G T↓M K A C...3' 3'...C A K M↑T G...5'	10 U/µl	Thermo Scientific, Braunschweig
Bme (ScrFI)	5'...C C↓N G G...3' 3'...G G N↑C C...5'	10 U/µl	Thermo Scientific, Braunschweig

3.18. *PvpA*- restriction fragment length polymorphism (*PvpA*-RFLP) (Pillai et al., 2003)

Procedures for *pvpA*-PCR were performed as mentioned above for the MG *pvpA*-PCR and as described (Liu et al., 2001). Afterwards, the *pvpA*-PCR product was digested by each of *PvuII, AccI* and *ScrFI* restriction enzymes separately. The digestion mixture contained 18 µl of the PCR product, 3 µl 10X Buffer G and 2 µl restriction enzyme. The digestion mixture was completed to 30 µl total volume with distilled water. Digestion mixtures were incubated in water bath at 37°C for 8 hours and was then separated on 3% agarose gel pre-stained with GelRed (1:10,000

working concentration) for 3-4 hours at 85 Voltage. GeneRuler Low Range DNA ladder was included to confirm the molecular weight of amplicons. Bands were detected under UV-transilluminator and documented using BioDocAnalyze digital.

3.19. Chemicals and devices for amplified fragment length polymorphism (AFLP)

DNA automatic sequencer (DNA-Analyzer 4200)	LI-COR Inc., USA
GenelmaglR Release 4.05	Scanalytics Inc., USA
Endonuclease restriction enzymes	Thermo Scientific, Braunschweig
Digestion Buffer G (10X)	Thermo Scientific, Braunschweig
T4 DNA ligase enzyme	Thermo Scientific, Braunschweig
T4 ligase Buffer	Thermo Scientific, Braunschweig
DNA adapters and oligonucleotides primers	Biomers, Ulm

Chemicals for denaturing polyacrylamide gel

Acrylamide solution (LongRanger Gel solution)	Biozym, Oldendorf
Urea	Roth, Karlsruhe
APS (Ammonium persulphate)	Roth, Karlsruhe
Boric acid	Roth, Karlsruhe
EDTA	Roth, Karlsruhe
TEMED	Roth, Karlsruhe
Formamid (deionized)	Roth, Karlsruhe
Fuchsin	AppliChem, Darmstadt

Restriction endonuclease enzymes used for AFLP

Enzyme	**Recognition site**	**Concentration**	**Company**
BglII	5'...A↓G A T C T...3' 3'...T C T A G↑A...5'	10 U/µL	Thermo Scientific, Braunschweig
MunI (MfeI)	5'...C↓A A T T G...3' 3'...G T T A A↑C...5'	10 U/µL	Thermo Scientific, Braunschweig

DNA adapters and primers sequences used for AFLP

Oligonucleotide	Sequence (5'---3')	Company
BGL adapter	CGG ACT AGA GTA CAC TGT	Biomers, Ulm
	GAT CGA CAG TGT ACT CTA GTC	Biomers, Ulm
MFE adapter	AAT TCC AAG AGC TCT CCA GTA	Biomers, Ulm
	TAG TAC TGG AGA GCT CTT GG	Biomers, Ulm
Mfe1 (labeled with DY781)	GAG AGC TCT TGG AAT TGA	Biomers, Ulm
Bgl2F (labeled with DY781)	GAG TAC ACT GTC GAT CT	Biomers, Ulm

3.20. Amplified fragment length polymorphism (AFLP) (Hong et al., 2005b)

Approximately 400 ng of MG or MS genomic DNA were double digested with 10 units of *BglII* and 10 units of *MfeI* enzymes in a total volume of 20 ul. The digestion mixture was incubated in a water path for 3 hours at 37°C. Two pmol of the BGL adaptor and 20 pmol of the MFE adaptor were ligated to 5 µl (100 ng) of digested DNA, in the presence of 10 U of T4 DNA ligase enzyme in a final volume of 20 µl ligation mixture. Ligation was done overnight at room temperature. Amplification of ligated-restricts was performed in a volume of 25 µl PCR reaction mix. PCR reaction mix included 5 µl of 10-fold diluted ligation mixture, 2.5 µl of 10X Dream Taq Buffer, 5 µl dNTPS (2 mM each), 5 pmol of Bgl2F and Mfe1 primers and 1.5 units of Dream Taq DNA polymerase. The Thermal program consisted of initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 60 sec, annealing at 54°C for 60 sec and extension at 72°C for 90 sec, and a final extension at 72°C for 10 min. PCR product was 1:5 diluted with Licor Buffer (Formamide, EDTA and Fuchsin) followed by denaturation at 93°C for 3 min and quickly chilled on ice. The PCR product was separated on an 8% denaturing polyacrylamide gel. PCR fragments between 71 and 431 bp were detected on a DNA automatic sequencer (DNA-Analyzer 4200, LI-COR Inc., USA). Fragment sizes and pattern variations were analyzed by GenelmaglR Release 4.05, (Scanalytics Inc., USA). Background subtraction, data normalization, and cluster analysis was performed using the Pearson correlation and un-weighted pair group methods with average linkages.

3.21. Index of discrimination (D) (Hunter and Gaston, 1988)

The discriminatory power of each typing method has been compared through the discrimination (D) index (Hunter and Gaston, 1988). D-index refers to the ability of a typing method to distinguish between different strains and measures the probability that two unrelated strains will be placed into different groups. D-index is calculated using Simpson diversity or discrimination (D) index, which takes into account the number of types defined by the method and the relative frequencies of these types.

A D index of > 0.90 is considered adequate, and a D-index > 0.95 is considered as a good typing method. This index can be calculated by the following equation;

$$D=1-\frac{1}{N(N-1)}\sum_{n=1}^{S} nj(nj-1)$$

N is the total number of strains in the sample population, S is the total number of types described, and nj is the number of strains belonging to the jth type.

3.22. Reference and vaccine strains

- MG strains

  MG 6/85 strain (vaccine strain) (Mycovac-L, Intervet)

  MG F-strain (vaccine strain) (F Vax-MG, Schering-Plough Animal Health)

- MS reference strain

  MS WUV 1853 strain (reference strain)

4. Results

The present study included two parts or stages. Through the first part of the study, MG and MS diagnostic and genotyping methods were investigated and compared. Through this part, birds with suspension of *Mycoplasma* infection were sampled. Collected samples were tested for *Mycoplasma* spp. by cultivation and Genus Mycoplasma PCR and positive samples for *Mycoplasma* spp. were further tested by MG/MS species-specific PCR and. Positive MG or MS samples were subjected to genotyping. The discriminatory powers of different genotyping methods were compared.

Through the second part of present study, samples from naturally infected chicken and the contacting non-poultry birds were collected and examined for MG and MS infection. Genetic profiles of MG and MS from both hosts were compared by established genotyping method.

4.1. MG and MS incidence and genotyping

In this part of the study, 515 swabs (tracheal and choanal) from 104 poultry and backyard flocks were examined (Table 1). These cases represented suspected cases for *Mycoplasma* infection (cases with respiratory or locomotor disorders or fertility troubles). In this part of the study, swabs (tracheal, pharyngeal, choanal, lung, synovial and phallus) were collected from different flocks (Table 1).

Some of investigated flocks originated from the same farm but from different occasions. This case was met with 3 farms;

1- Turkey farm located in East Germany from which 8 MG isolates originating from 8 different flocks were received and included in the present study. The eight flocks were divided as following;
   i. Two historical turkey breeder flocks (identity No.: 1608-1/11/TK and 1608-2/11/TK) from 2006 and 2007 MG outbreaks, respectively.
   ii. Four turkey breeder flocks (identity no.: 1608- 3, 4, 5, 6/11/TK) from MG outbreaks in 2011.
   iii. Two fattening turkey flocks (identity No.: 1608-7/11/TK and 1608-8/11/TK) from 2011 MG outbreaks.

2- Turkey breeder farm in Thüringen, Germany, where 4 flocks (identity No.: 632/11/TK, 2730/11/TK, 24/12/TK and 187/12/TK) were examined through 2011 and 2012.
3- Organic chicken breeder farm located in Wiesbaden, Germany, from which 5 flocks (identity No.: 2122/12/CK, 833/13/CK, 1233/13/CK, 1372/13/CK and 331/14/CK) were examined through 2012, 2013 and 2014.

4.1.1. MG and MS identification by PCR and culture

Out of 104 examined flocks, 76 tested positive in a *Mycoplasma* genus-specific PCR. From these positive flocks, 26 and 37 tested positive for MG- and MS- species specific PCR, respectively (Table 2). These PCR results were confirmed by amplicon sequencing. Double MG/MS infection was revealed in thirteen of the positive flocks (Table 2). On the other hand, cultivation was successful only with samples from 8 flocks (for MG) and 6 flocks (for MS), respectively (Table 2). MG isolates originated from one turkey farm (1608/11/TK) located in East Germany but from different occasions and different years (Table 2). However 3 of the MS isolates (2730/11/TK, 24/12/TK and 187/12/TK) originated from one turkey breeder farm but from 3 different MS outbreaks that appeared at the winter of 2011 and 2012. Each of the other 3 MS isolates (2072/11/CK, 2081/11/CK and 2141/11/CK) originated from different farms (Table 2).

Table 2: List of samples that tested positive for MG and MS isolation and species-specific PCR

	Entry number	Sampling date	MG Isolation	MG PCR	MS Isolation	MS PCR	Notes
1	632/11/TK	04.2011	-	-	-	+	Turkey breeders in Thüringen
2	1321/11/CK	06.2011	-	+	-	-	Chicken with resp. signs
3	1608/1/11/TK	2006	+	+	-	-	Turkey breeders in East Germany
4	1608/2/11/TK	2007	+	+	-	-	Turkey breeders in East Germany
5	1608/3/11/TK	07.2011	+	+	-	-	Turkey breeders in East Germany
6	1608/4/11/TK	07.2011	+	+	-	-	Turkey breeders in East Germany
7	1608/5/11/TK	07.2011	+	+	-	-	Turkey breeders in East Germany
8	1608/6/11/TK	07.2011	+	+	-	-	Turkey breeders in East Germany
9	1608/7/11/TK	07.2011	+	+	-	-	Fattening turkey in East Germany – MG 6/85 strain vaccinated
10	1608/8/11/TK	07.2011	+	+	-	-	Fattening turkey in East Germany – MG 6/85 strain vaccinated
11	2038/11/CK	08.2011	-	+	-	-	NA
12	2072/11/CK	09.2011	-	-	+	+	Chicken with resp. signs
13	2081/11/CK	09.2011	-	+	+	+	Chicken with resp. Signs
14	2141/11/TK	10.2011	-	-	+	+	NA
15	2730/11/TK	12.2011	-	-	+	+	Turkey breeders in Thüringen
16	24/2012/TK	01.2012	-	-	+	+	Turkey breeders in Thüringen
17	187/12/TK	01.2012	-	-	+	+	Turkey breeders in Thüringen
18	1567/12/CK	07.2012	-	-	-	+	Chicken with resp. Signs
19	1786/12/CK	08.2012	-	+	-	+	Chicken with resp. Signs
20	1858/12/CK	09.2012	-	+	-	+	Chicken with resp. Signs Chicken
21	1907/12/CK	09.2012	-	+	-	+	NA
22	1936/12/CK	09.2012	-	+	-	+	NA
23	2122/12/CK	10.2012	-	+	-	+	Organic Chicken breeders in Wiesbaden, with infertility problems
24	2187/12/CK	10.2012	-	+	-	-	NA
25	2207/12/CK	10.2012	-	-	-	+	Chicken with resp. Signs & infertility problems
26	2286/12/CK	10.2012	-	-	-	+	Chicken with resp. Signs
27	2453/12/CK	11.2012	-	+	-	+	Chicken with resp. Signs

	Entry number	Sampling date	MG Isolation	MG PCR	MS Isolation	MS PCR	Notes
28	19/13/CK	01.2013	-	-	-	+	NA
29	154/13/CK	01.2013	-	-	-	+	NA
30	209/13/CK	01.2013	-	-	-	+	NA
31	342/13/CK	02.2013	-	-	-	+	NA
32	364/13/CK	03.2013	-	+	-	+	Chicken with resp. Signs
33	370/13/CK	02.2013	-	-	-	+	Chicken with resp. Signs
34	612/13/CK	03.2013	-	+	-	+	Chicken with resp. Signs
35	833/13/CK	04.2013	-	-	-	+	Organic Chicken breeders in Wiesbaden, with infertility problems
36	925/13/CK	04.2013	-	+	-	+	NA
37	1233/13/CK	06.2013	-	-	-	+	Organic Chicken breeders in Wiesbaden, with infertility problems
38	1372/13/CK	06.2013	-	-	-	+	Organic Chicken breeders in Wiesbaden, with infertility problems
39	1554/13/CK	07.2013	-	-	-	+	NA
40	1561/13/CK	07.2013	-	+	-	+	Chicken with swollen sinuses & resp. & infertility problems
41	1847/13/CK	08.2013	-	+	-	-	NA
42	1911/13/CK	08.2013	-	-	-	+	NA
43	2054/13/CK	09.2013	-	-	-	+	NA
44	2087/13/CK	09.2013	-	-	-	+	Organic chicken with resp. Signs
45	2251/13/CK	09.2013	-	-	-	+	Chicken with resp. Signs & eggshell misshape
46	2591/13/CK	11.2013	-	+	-	-	NA
47	77/14/CK	02.2014	-	+	-	+	Organic layer chicken with resp. Signs
48	331/14/CK	02.2014	-	+	-	+	Organic Chicken breeders in Wiesbaden, with infertility problems
49	600/14/CK	03.2014	-	-	-	+	Chicken with resp. Signs
50	1073/14/CK	07.2014	-	-	-	+	NA

This table showing 26 MG samples by species-specific PCR and 8 from them is positive with MG cultivation. Also 37 samples were positive with MS species-specific PCR and 6 from them had cultivable MS. A total of 13 samples are positive for MG/MS co-infection.
Sample description: entry number/year of sampling/bird species.
For bird species: CK = chicken; GS= Goose; PH=Pheasants; SW= Swan; Tit= Blue and grey tits; TK = turkey and ZF=Zebra finches
*samples 1608-1/11/TK and 1608-2/11/TK were isolated in 2006 and 2007, respectively, but were investigated during this work.
(+) =positive, (-) = negative and (NA) = Not available.

4.1.2. MG typing by *mgc2* sequencing

PCR and sequencing of *mgc2* differentiated 3 different sequence types within MG positive samples (n = 26) that belonged to two main clusters. The first cluster included 8 samples that produced an approximately 230 bp amplicon. Six samples from them belonged to one sequence type (M-I) (identical to *mgc2* of MG 6/85 vaccine strain), while the other two samples belonged to another sequence type (M-II). Cluster II contained 18 samples that showed amplicons of approximately 300 bp and belonged to sequence type M-III, identical to *mgc2* of MG ts-11 vaccine strain (Figure 4). Concerning the turkey MG isolates, *mgc2* sequence alignment differentiated 8 turkey MG isolates (1608-11/TK) into two sequence types (M-I and M-III); the two fattening turkey isolates from 2011 belonged to sequence type M-I (identical to MG 6/85 vaccine strain) and the other 6 breeder isolates from 2006, 2007 and 2011 belonged to sequence type M-III (Table 3). The D-index of *mgc2* sequencing was 0.52.

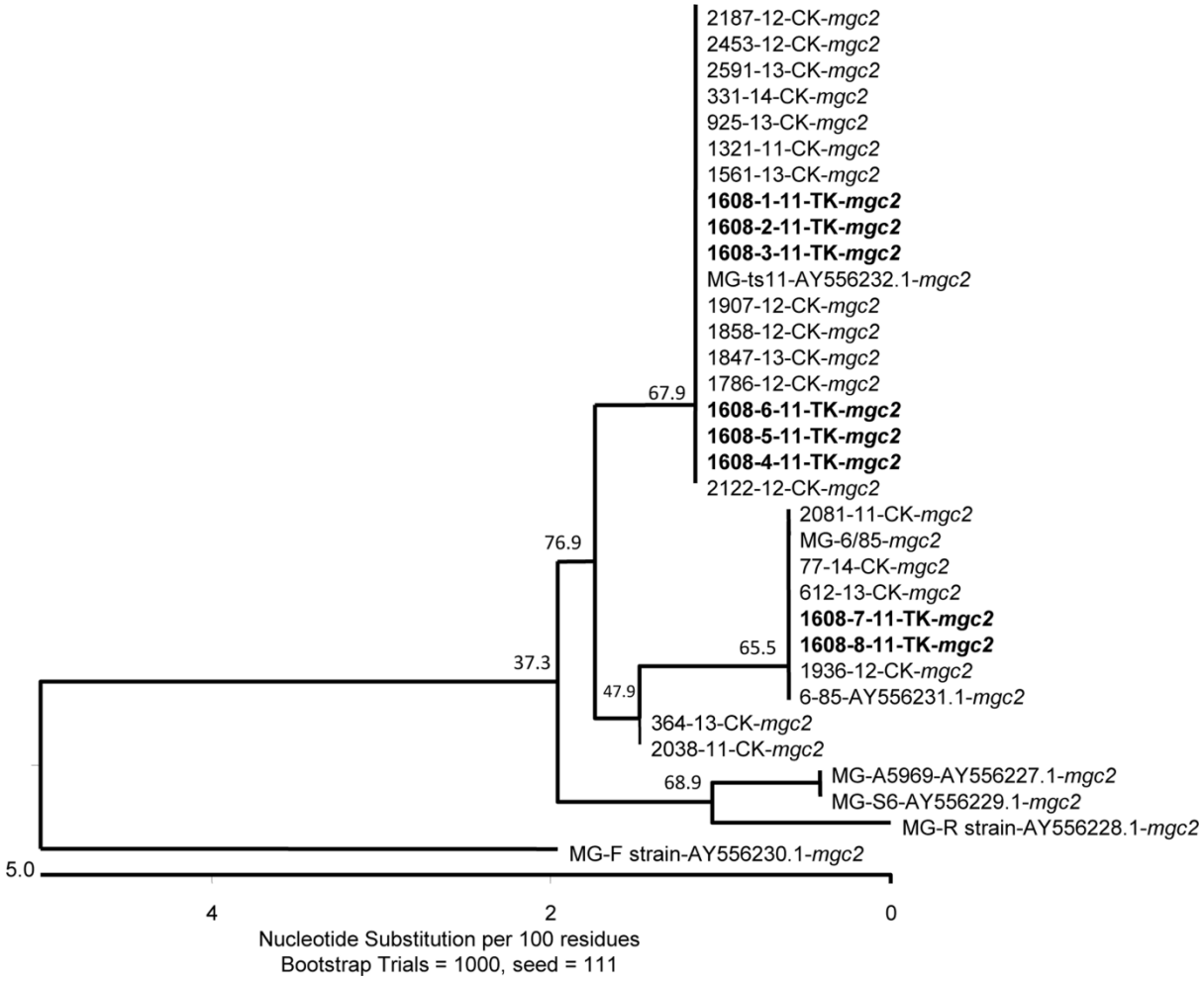

Figure 4: Phylogenetic tree of the *mgc2* sequences of MG samples and reference strains. Sequences of MG samples and compared MG reference strains were trimmed and edited to have the same starting and ending sequences. MG samples demonstrated 3 different *mgc2* sequence types. The turkey MG isolates (identity no. 1608-11/TK and shown in bold font) demonstrated two *mgc2* sequence types. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

Sample description: entry number-year of sampling-bird species (CK = chicken and TK = turkey)-genome segment

MG reference strain description: MG-Reference strain Identity-Accession number-genome segment

4.1.3. MG typing by MG 16S-23S IGSR sequencing

With MG-IGSR PCR, amplicons of approximately 800 bp were produced with MG samples (n=26) with no visible size differences between samples on agarose electrophoresis. A range of 1 to 6 nucleotides differences between samples was shown by MG-IGSR sequence alignment. MG-IGSR sequence alignment differentiated 10 sequence types (from I-I to I-X) that fell into 4 main clusters; I, II, III and IV within samples (Figure 5 and Table 3). Cluster I included 6 samples that were closely related to the 6/85 vaccine strain. Cluster II and cluster III included 9 samples each, with no clear relationship to known reference strains. Samples 2038-11-CK and 925-13-CK composed cluster IV. Concerning the turkey MG isolates, MG-IGSR sequence alignment differentiated them into three sequence types (I-A3, I-B4 and I-C1); fattening turkey isolates from 2011 belonged to sequence type I-A3 (identical to MG 6/85 vaccine strain), breeder isolates from 2006 and 2011 seemed identical and belonged to sequence type I-C1, and breeder isolate from 2007 demonstrated different MG-IGSR sequence type I-B4 (Table 3). MG-IGSR sequencing showed a D-index of 0.86.

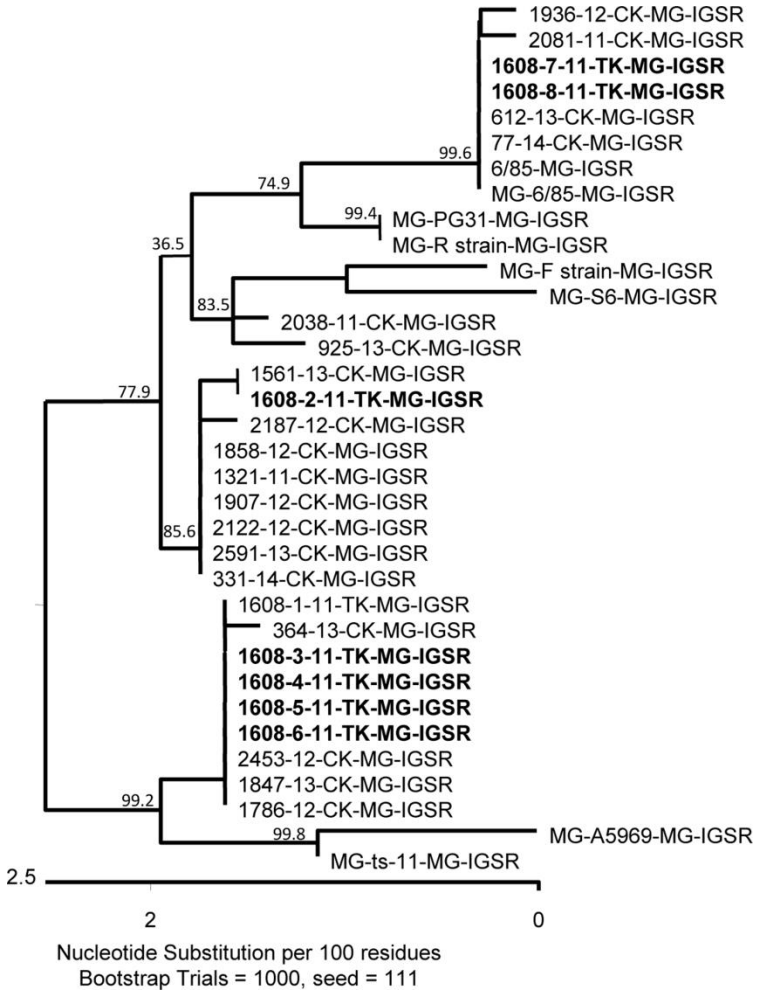

Figure 5: Phylogenetic tree of MG 16S-23S IGSR sequences for MG samples and MG reference strains. Sequences of MG samples and compared MG reference strains were trimmed and edited to have the same starting and ending sequences. MG samples demonstrated 10 16S-23S IGSR sequence types. Turkey MG isolates (shown in bold font) belonged to three different 16S-23S IGSR sequence types. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

Sample description: entry number-year of sampling-bird species (CK = chicken and TK = turkey)-genome segment

MG reference strain description: MG-Reference strain Identity-Accession number-genome segment

4.1.4. MG typing by *pvpA* sequencing

With *pvpA*-PCR, MG samples showed PCR amplicons with three different molecular weights; approximately 600 (14 samples), 660 (10 samples) and 770 bp (1 sample). Sample 2187/12/CK was not tested by *pvpA*-PCR due to shortage of DNA. In total, *pvpA* sequencing differentiated 14 *pvpA* sequence types that belonged to 6 clusters within samples (Figure 6). Cluster I included 5 samples from which four samples showed a *pvpA* sequence identical to that of 6/85 vaccine strain. Cluster II included also 5 samples (4 MG turkey breeder isolates from 2011 together with historical MG turkey breeder isolates from 2006) that showed relationship to *pvpA* of MG ts-11 vaccine and MG-R reference strains. Cluster III and IV included 5 samples each. Cluster V included turkey breeder MG isolate from 2007 and chicken MG isolate 1321-11/CK. Clusters VI included 3 MG isolates with no clear relationship to known MG reference strain (Figure 6). *PvpA* sequences of all samples showed two copies of direct repeats (DR), while chicken sample 1561/13/CK demonstrated three DRs with approximately 770 bp *pvpA*-PCR product (Table 3). D-index of *pvpA* sequencing of 0.94 was shown (Table 3).

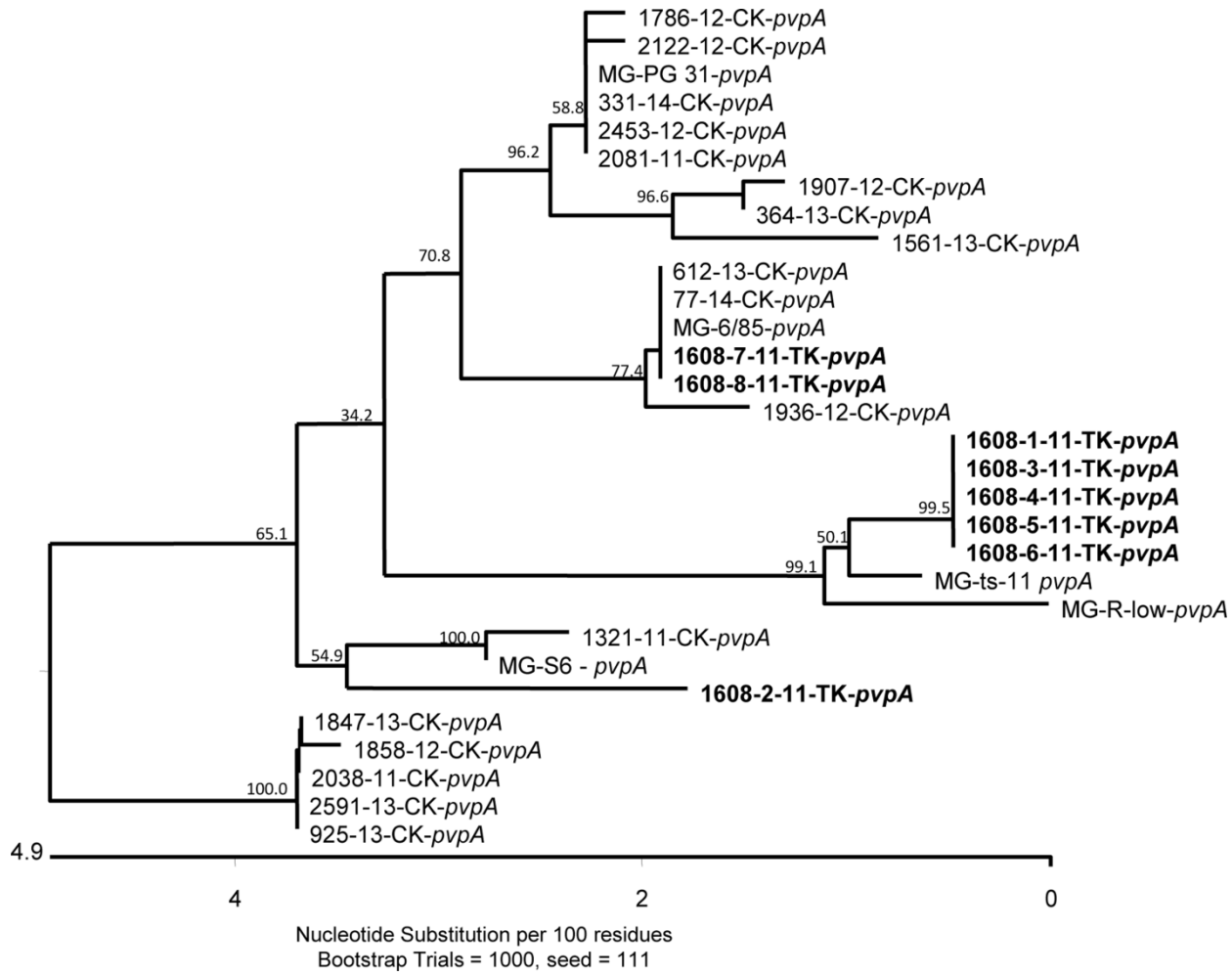

Figure 6: Phylogenetic tree for *pvpA* sequences of MG samples and MG reference strains. Sequences of MG samples and compared MG reference strains were trimmed and edited to have the same starting and ending sequences. Samples demonstrating 14 *pvpA* sequence types. Eight turkey MG isolates (shown in bold font) showed three different sequence types. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

4.1.5. Multiple gene-targeted sequencing (GTS) of *mgc2/pvpA*/MG-IGSR sequencing

Collective analysis of *mgc2*/*pvpA*/MG-IGSR sequencing discriminated 18 sequence types (from I to XVIII) over all samples (Table 3). Multiple GTS analysis of *mgc2*/*pvpA*/MG-IGSR differentiated turkey MG isolates into 3 sequence types (I, II and IV); 4 breeder isolates from 2011 and one breeder isolate from 2006 were identical and belonged to GTS type I. Fattening turkey isolates belonged to GTS type II and were 100% identical to that of MG 6/86 vaccine strain. Breeder isolate from 2007 was different and demonstrated GTS type IV (Table 3). The D-index of multiple GTS was 0.95.

Table 3: MG typing results by sequencing of the *mgc2, pvpA* and MG-IGSR, multiple GTS

	MG strain	***mgc2* bp**	***mgc2* seq.**	**16-23 IGSR (bp)**	**16-23 IGSR seq.**	***pvpA* (bp)**	***pvpA* seq.**	**Multiple GTS (*mgc2*/ IGSR/*pvpA*)**
1	1321/11/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-E	III
2	1608-1/11/TK*	A (~ 300 bp)	M-I	813	I-C1	660	P-D	I
3	1608-2/11/TK*	A (~ 300 bp)	M-I	813	I-B4	600	P-F	IV
4	1608-3/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	I
5	1608-4/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	I
6	1608-5/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	I
7	1608-6/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	I
8	1786/12/CK	A (~ 300 bp)	M-I	813	I-C1	600	P-C2	V
9	1858/12/CK	A (~ 300 bp)	M-I	814	I-B2	660	P-A2	I
10	1907/12/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-G	VI
11	2122/12/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-C3	VII
12	2187/12/CK	A (~ 300 bp)	M-I	813	I-B3	-	-	XVIII
13	2453/12/CK	A (~ 300 bp)	M-I	813	I-C1	600	P-C1	VIII
14	925/13/CK	A (~ 300 bp)	M-I	809	I-E	660	P-A1	IX
15	1561/13/CK	A (~ 300 bp)	M-I	813	I-B4	770	P-I	X
16	1847/13/CK	A (~ 300 bp)	M-I	813	I-C1	660	P-A1	XI
17	2591/13/CK	A (~ 300 bp)	M-I	813	I-B1	660	P-A1	XII
18	331/14/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-C1	XIII
19	1608-7/11/TK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	II
20	1608-8/11/TK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	II

21	364/13/CK	B (~ 230 bp)	M-II	813	I-C2	600	P-H	XIV
22	612/13/CK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	II
23	1936/12/CK	B (~ 230 bp)	M-II	815	I-A1	600	P-B2	XV
24	77/14/CK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	II
25	2038/11/CK	B (~ 230 bp)	M-III	814	I-D	660	P-A1	XVI
26	2081/11/CK	B (~ 230 bp)	M-III	815	I-A2	600	P-C1	XVII

Descriptive codes: Identity number /year/species). CK = chicken; TK = turkey; seq. = sequencing; ~ = approximately

*samples 1608-1/11/TK and 1608-2/11/TK were isolated in 2006 and 2007, respectively, but were investigated during present study.

4.1.6. Accession numbers for MG isolates

Resulting sequences of MG-IGSR and *pvpA* of this study were submitted to GenBank under accession numbers KP710059 to KP710084 (for MG-IGSR) and KP881244 to KP881268 (for *pvpA*).

4.1.7. Global comparison of German MG

Sequences of *mgc2, pvpA* and MG IGSR from MG strains found in other countries were downloaded from GenBank. Sequences were edited to have the same start and end nucleotide sequences and were then aligned with respective sequences of the present study. With the global comparison of *mgc2, pvpA and* MG-IGSR sequences, German MG strains seemed to form a separate cluster with particular genetic profile. Some MG isolates from other geographic localities were genetically similar in one or more genome segments. With comparison of *mgc2* gene sequences, some Russian and Italian MG strains showed relationship to German strains (Figure 7). With MG-IGSR sequences, German MG strains demonstrated characteristic profile and only some Russian MG strains showed similarity with German 6/85 like strain cluster (Figure 8). Through *pvpA* gene comparison, speciality of German MG strains as well as the relationship between some Russian and German isolates in 6/85 like cluster were shown (Figure 9).

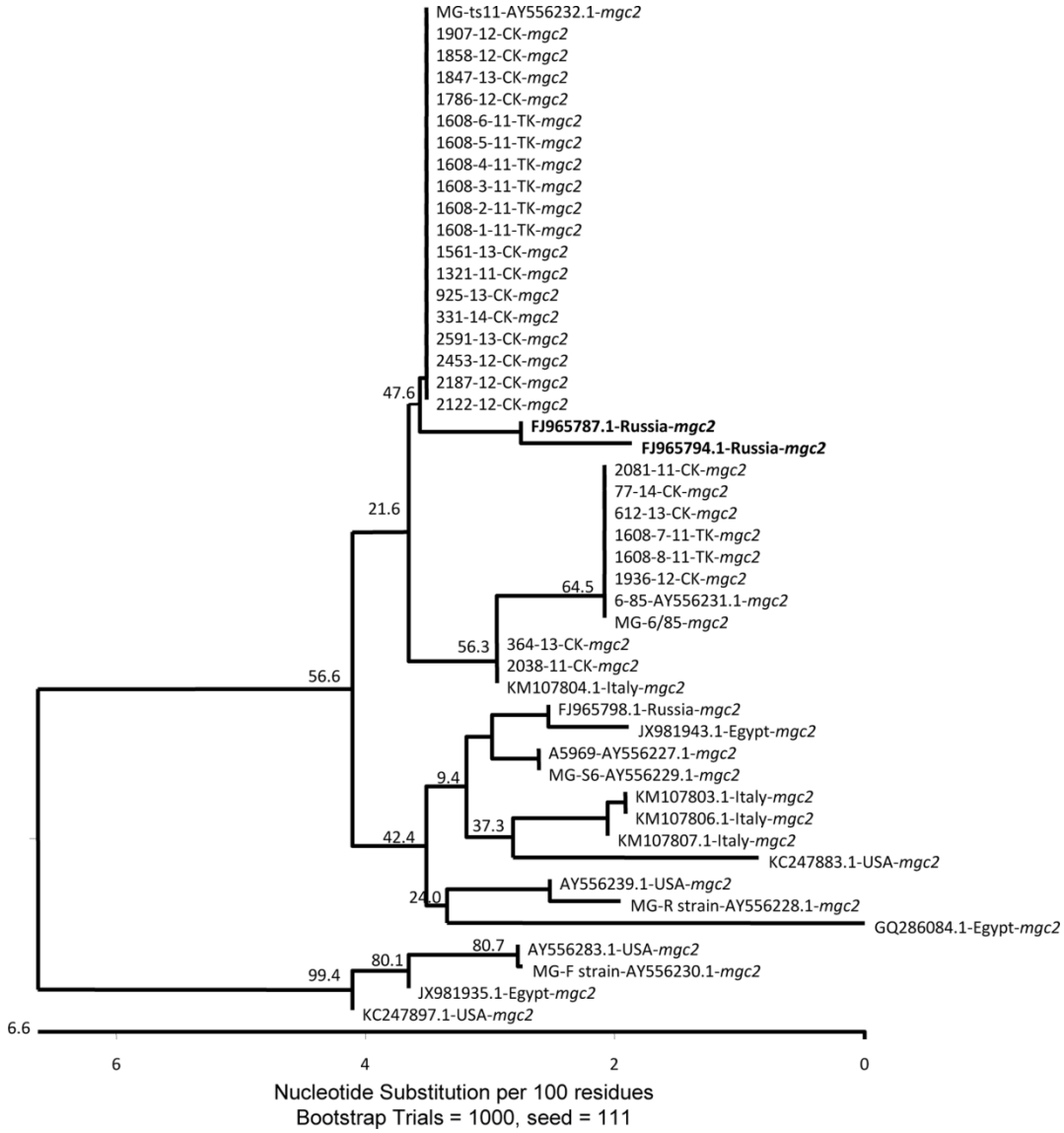

Figure 7: Phylogenetic tree for *mgc2* sequences of MG samples, MG reference strains and MG strains in USA, Russia, Egypt and Italy. Sequences were trimmed and edited to have the same starting and ending sequences. Some Russian MG strains (shown in bold font) shared relationship to German MG. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

Sample description: entry number-year of sampling-bird species (CK = chicken and TK = turkey)-genome segment

MG reference strain description: MG-Reference strain Identity-Accession number-genome segment

Description of MG strains from other countries: Accession number-Country-genome segment

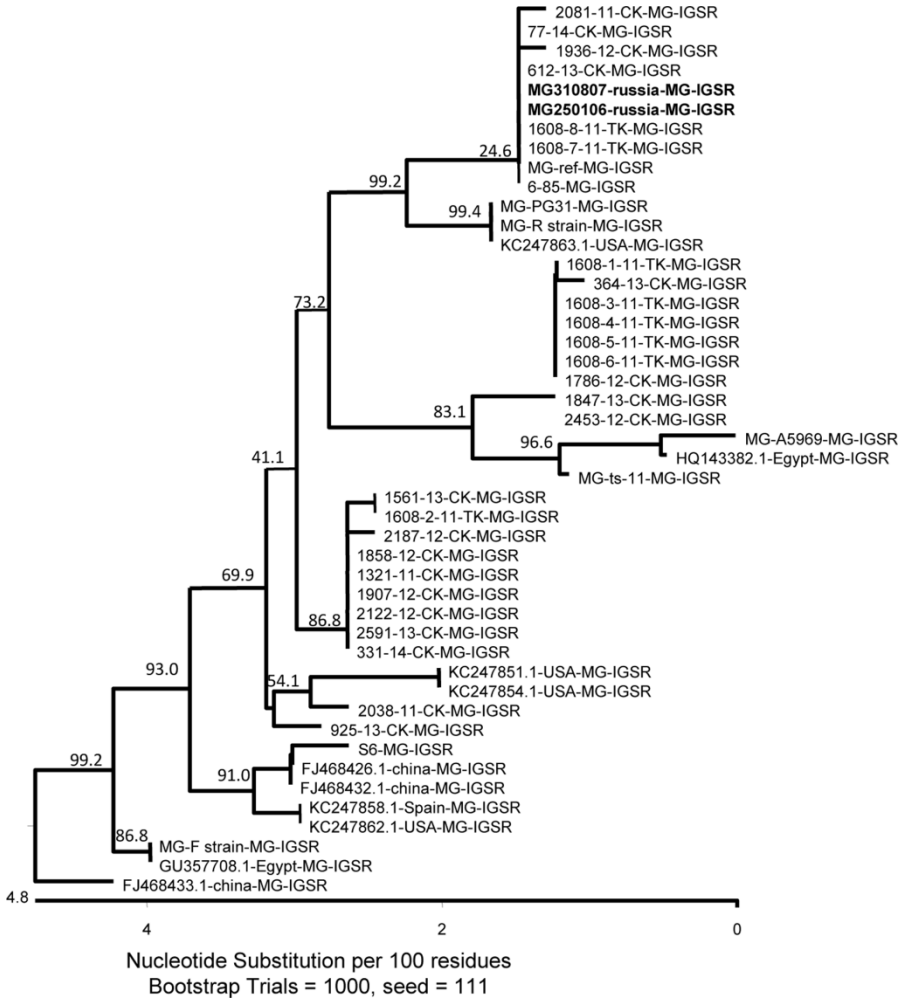

Figure 8: Phylogenetic tree of 16S-23S IGSR sequences for MG samples and MG strains found in USA, Russia, China, Egypt and Spain, together with reference strains. Sequences were trimmed and edited to have the same starting and ending sequences. Some Russian MG strains (shown in bold font) shared relationship to German MGs of the 6/85 strain cluster. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

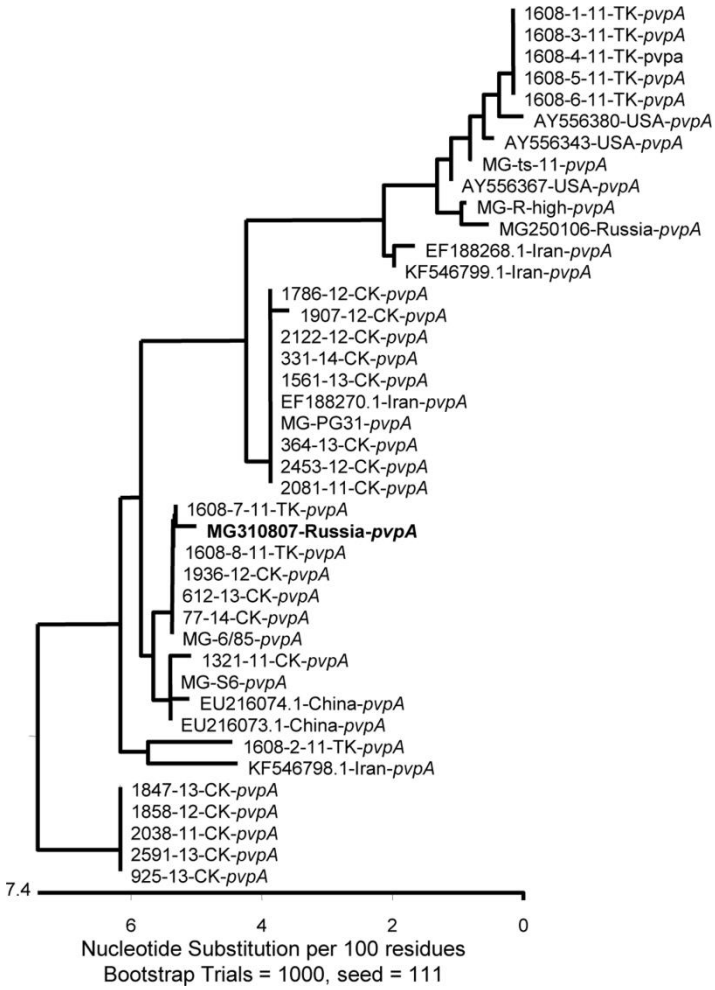

Figure 9: Phylogenetic tree of *pvpA* sequences for MG samples and MG strains found in USA, Russia, China and Iran, together with MG reference strains. Sequences were trimmed and edited to have the same starting and ending sequences. Some Russian MG strains (shown in bold font) shared relationship to German MGs of the 6/85 strain cluster. Iranian MG strain fell into the German cluster with relationship to MG PG31 strain. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

4.1.8. MG typing by *pvpA*-RFLP

Twenty-five samples were examined by *pvpA*-PCR. Chicken sample 2187/12/CK was excluded due to shortage of DNA. MG samples showed *pvpA* amplicons of three sizes, approximately 600 bp (14 samples), 660 bp (10 samples) and 770 bp (1 sample). However, *pvpA*-RFLP differentiated 6 RFLP groups within samples (from I to VI) (Table 4). The D-index of *pvpA*-RFLP was 0.81.

Table 4: Results of *pvpA*-RFLP genotyping for MG samples by using *PvuII*, *AccI*, and *ScrFI* restriction endonuclease enzymes

	MG strain	PCR product length (bp)	*PvuII* fragments (bp)	*PvuII*-RFLP-group	*AccI* fragments (bp)	*AccI*-RFLP-group	*ScrFI* fragments (bp)	*ScrFI*-RFLP-group	RFLP-group
1	1608-1/11/TK	660	380+230+50	A	340+320	A	240+240+180	A	I
2	1608-3/11/TK	660	380+230+50	A	340+320	A	240+230+180	A	I
3	1608-4/11/TK	660	380+230+50	A	340+320	A	240+230+180	A	I
4	1608-5/11/TK	660	380+230+50	A	340+320	A	240+240+180	A	I
5	1608-6/11/TK	660	380+230+50	A	340+320	A	240+240+180	A	I
6	1608-7/11/TK	600	380+120+50+50	B	Not cut	B	360+240	B	II
7	1608-8/11/TK	600	380+120+50+50	B	Not cut	B	360+240	B	II
8	2081/11/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
9	1786/12/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
10	1907/12/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
11	1936/12/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
12	2122/12/CK	600	380+120+50+50	B	Not cut	B	360-240	B	II
13	2453/12/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
14	364/13/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
15	612/13/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
16	77/14/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
17	331/14/CK	600	380+120+50+50	B	Not cut	B	360+240	B	II
18	2038/11/CK	660	Not cut	D	Not cut	C	275+240+145	D	III

19	1858/12/CK	660	Not cut	D	Not cut	C	275+240+145	D	III
20	925/13/CK	660	Not cut	D	Not cut	C	275+240+145	D	III
21	1847/13/CK	660	Not cut	D	Not cut	C	275+240+145	D	III
22	2591/13/CK	660	Not cut	D	Not cut	C	275+240+145	D	III
23	1608-2/11/TK	600	380+170+50	C	Not cut	B	375+225	C	IV
24	1321/11/CK	600	380+120+50+50	B	Not cut	B	240+225+135	E	V
25	1561/13/CK	770	380, 120, 120, 50, 50, 50	E	Not cut	D	530+230	F	VI

4.1.9. MG typing by Amplified Fragment Length Polymorphism (AFLP)

AFLP applied to turkey MG isolates (n = 8) revealed 4 AFLP electrophoretic profiles (Figure 10 and Table 5). Breeder isolate from 2006 showed electrophoretic profile (profile A1) that was similar (but not identical) to that of 4 breeder isolates from 2011 (profile A2). The two fattening turkey isolates were identical and showed electrophoretic profile (profile B) that was similar (but not identical) to the 6/85 vaccine strain. Unique AFLP profile was demonstrated with breeder isolate from 2007 (profile C) (Figure 10 and Table 5).

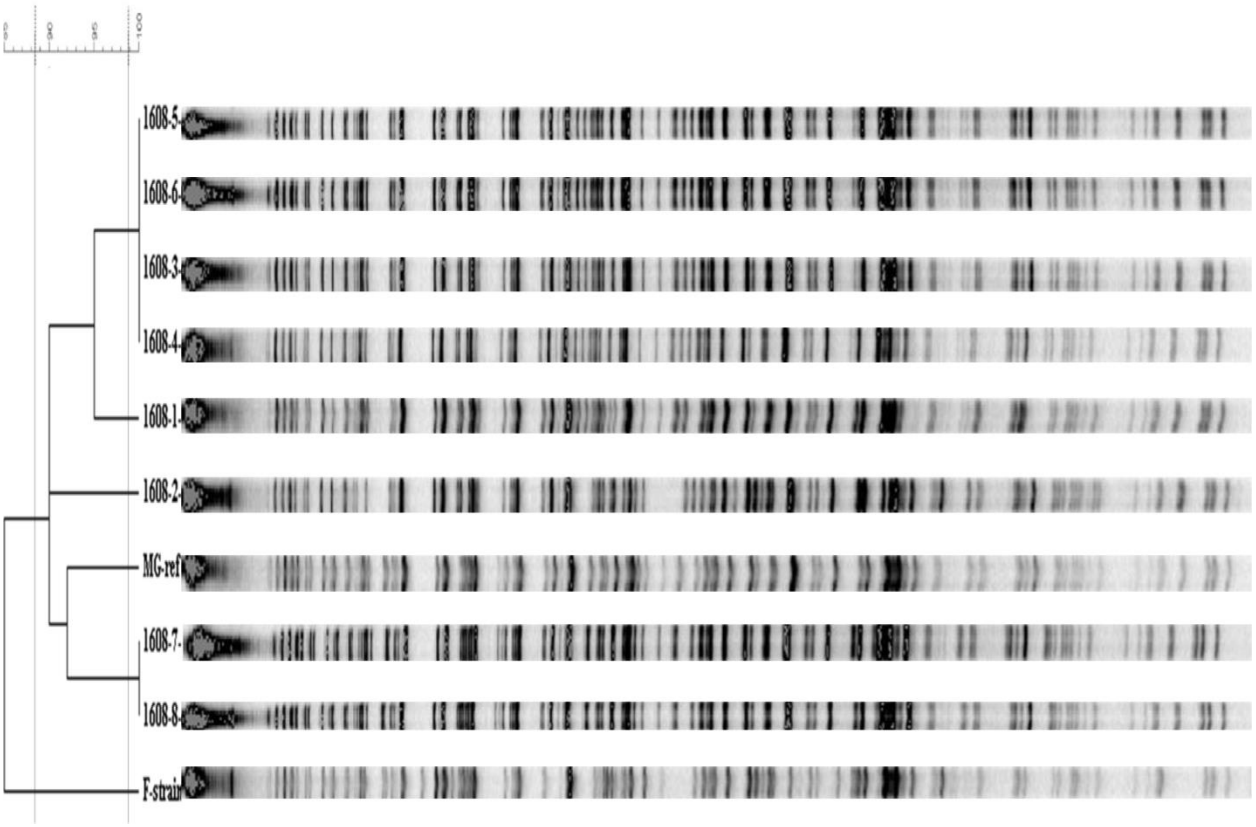

Figure 10: AFLP analysis of 8 turkey MG isolates, MG 6/85 strain and MG F strain. Four turkey breeder isolates from 2011 (1608-3, 4, 5 and 6/11/TK) have identical patterns. They are similar (but not identical) to breeder MG isolate from 2006 (1608-1/11/TK). The two fattening turkey isolates (1608-7 and 8/11/TK) have identical patterns and they are similar to MG 6/85 strain. Turkey breeder MG isolate from 2007 (1608-2/11/TK) showed unique AFLP profile. Clustering of samples (dendrogram) was performed through LI-COR e-seq Release 2,0 program (LI-COR Inc, Lincolin, USA) using the un-weighted pair-group method with arithmetic averaging.

Table 5: MG typing results of sequencing the *mgc2, pvpA* and MG-IGSR, multiple GTS and the *pvpA*-RFLP and the AFLP

	MG strain	*mgc2* bp	*mgc2* seq.	16-23 IGSR (bp)	16-23 IGSR seq.	*pvpA* (bp)	*pvpA* seq.	*pvpA*-RFLP	Multiple GTS (*mgc2*/ IGSR/*pvpA*)	AFLP
1	1321/11/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-E	R-V	III	
2	1608-1/11/TK*	A (~ 300 bp)	M-I	813	I-C1	660	P-D	R-I	I	A-1
3	1608-2/11/TK*	A (~ 300 bp)	M-I	813	I-B4	600	P-F	R-IV	IV	C
4	1608-3/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	R-I	I	A-2
5	1608-4/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	R-I	I	A-2
6	1608-5/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	R-I	I	A-2
7	1608-6/11/TK	A (~ 300 bp)	M-I	813	I-C1	660	P-D	R-I	I	A-2
8	1786/12/CK	A (~ 300 bp)	M-I	813	I-C1	600	P-C2	R-II	V	
9	1858/12/CK	A (~ 300 bp)	M-I	814	I-B2	660	P-A2	R-III	I	
10	1907/12/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-G	R-II	VI	
11	2122/12/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-C3	R-II	VII	
12	2187/12/CK	A (~ 300 bp)	M-I	813	I-B3	-	-	-	XVIII	
13	2453/12/CK	A (~ 300 bp)	M-I	813	I-C1	600	P-C1	R-II	VIII	
14	925/13/CK	A (~ 300 bp)	M-I	809	I-E	660	P-A1	R-III	IX	
15	1561/13/CK	A (~ 300 bp)	M-I	813	I-B4	770	P-I	R-VI	X	

16	1847/13/CK	A (~ 300 bp)	M-I	813	I-C1	660	P-A1	R-III	XI	
17	2591/13/CK	A (~ 300 bp)	M-I	813	I-B1	660	P-A1	R-III	XII	
18	331/14/CK	A (~ 300 bp)	M-I	813	I-B1	600	P-C1	R-II	XIII	
19	1608-7/11/TK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	R-II	II	B
20	1608-8/11/TK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	R-II	II	B
21	364/13/CK	B (~ 230 bp)	M-II	813	I-C2	600	P-H	R-II	XIV	
22	612/13/CK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	R-II	II	
23	1936/12/CK	B (~ 230 bp)	M-II	815	I-A1	600	P-B2	R-II	XV	
24	77/14/CK	B (~ 230 bp)	M-II	815	I-A3	600	P-B1	R-II	II	
25	2038/11/CK	B (~ 230 bp)	M-III	814	I-D	660	P-A1	R-III	XVI	
26	2081/11/CK	B (~ 230 bp)	M-III	815	I-A2	600	P-C1	R-II	XVII	

This table shows collective results of multiple GTS analysis, *pvpA*-RFLP and AFLP within MG samples. Multiple GTS analysis of *mgc2*/*pvpA*/MG-IGSR differentiated 18 sequence types (from I to XVIII). *PvpA*-RFLP differentiated 6 genotypes (from R-I to R-VI). AFLP could differentiate 4 genotypes within MG isolates.

Sample description: entry number/year of sampling/bird species.

For bird species: CK = chicken; GS= Goose; PH=Pheasants; SW= Swan; Tit= Blue and grey tits; TK = turkey and ZF=Zebra finches

*samples 1608-1/11/TK and 1608-2/11/TK were isolated in 2006 and 2007, respectively, but were investigated during this work.

seq. = sequencing; ~ = approximately

*samples 1608-1/11/TK and 1608-2/11/TK were isolated in 2006 and 2007, respectively, but were investigated during present study.

4.1.10. MS typing by *vlhA* sequencing

The *vlhA* gene of 36 MS samples was amplified and sequenced. Sample 154/13/CK was excluded because of DNA shortage. *VlhA* sequencing discriminated 15 *vlhA* sequence types. Two *vlhA* sequence types (I and II) predominated and included 22 samples (Figure 11). *VlhA* sequence type I included 12 MS samples. From those samples; three MS isolates (2730/11/TK, 24/12/TK and 187/12/TK) that originated from a turkey breeder farm in Thüringen, while the fourth isolate (632-11/TK) from an earlier MS outbreak in the same farm showed different *vlhA* sequence types (Figure 11). *VlhA* sequence type II included 10 MS samples in total. Four from these MS samples (833/13/CK, 1233/13/CK, 1372/13/CK and 331/14/CK) belonged to organic chicken farm located in Wiesbaden; however the fifth sample (2122-12/CK) from the same farm but from MS outbreak in 2012 showed different *vlhA* sequence type. All samples showed one *vlhA* amplicon, while sample 1561/13/CK showed two *vlhA* amplicons with two *vlhA* sequence types (1561-L-13-CK and 1561-S-13-CK) (Figure 11). The D-index of *vlhA* sequencing was 0.83.

4.1.11. Global comparison of German MS

With global comparison of MS-*vlhA,* German MS isolates seemed to have their particular genetic profile. The two German predominating MS strains (*vlhA* sequence types I & II) and Dutch MS strains showed identical *vlhA* gene sequences.. Also, some German MS strains showed similar *vlhA* with Japanese and Iranian MS strains (Figure 12).

4.1.12. Accession numbers for MS isolates

Resulting *vlhA* sequences of this part of study have been submitted to GenBank under accession numbers KP893761 to KP893801.

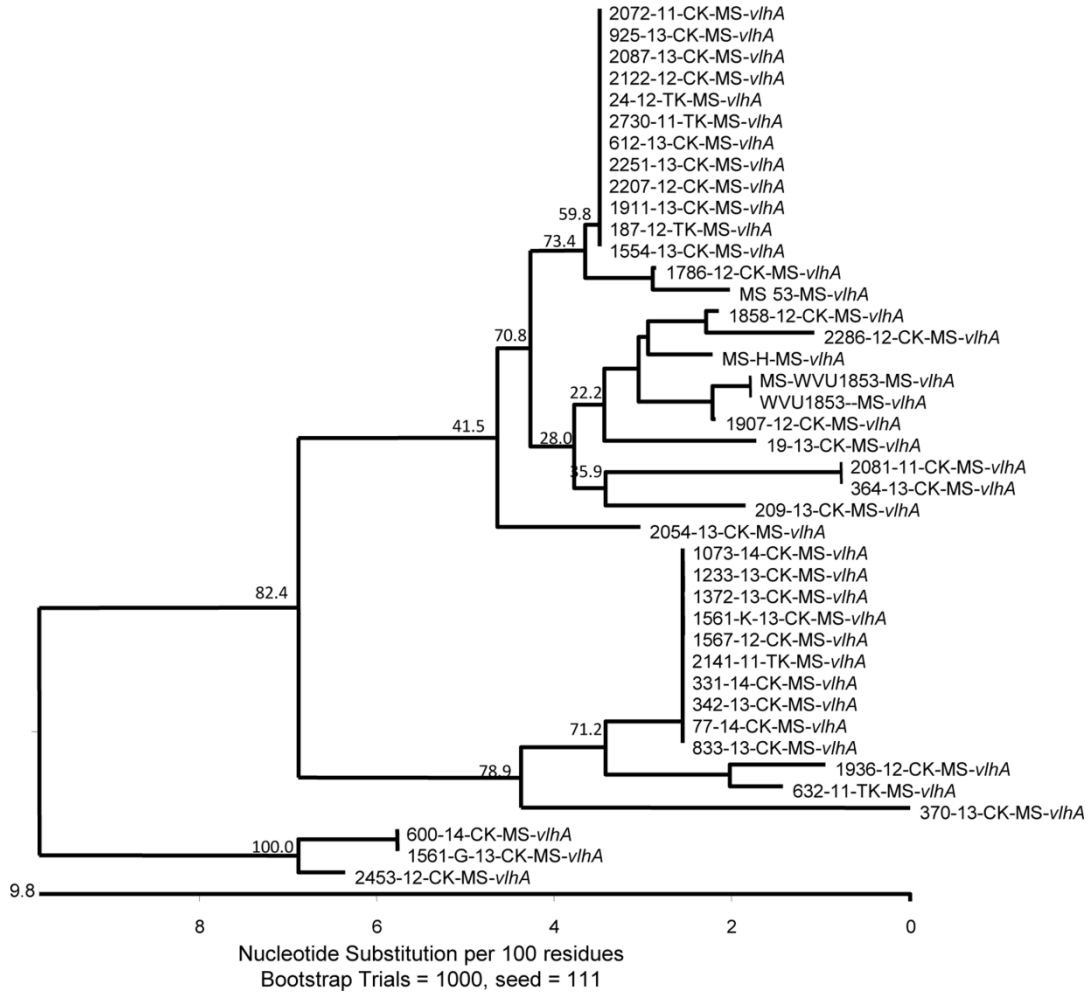

Figure 11: Phylogenetic tree for *vlhA* sequences of MS samples and MS reference strains. Sequences were trimmed and edited to have the same starting and ending sequences. MS samples demonstrated 15 *vlhA* sequence types. Two *vlhA* sequence types were more frequent than other types and were found in 22 MS samples. Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

Sample description: entry number-year of sampling-bird species (CK = chicken and TK = turkey)-genome segment

MS reference strain description: MS-Reference strain Identity-genome segment (*vlhA*)

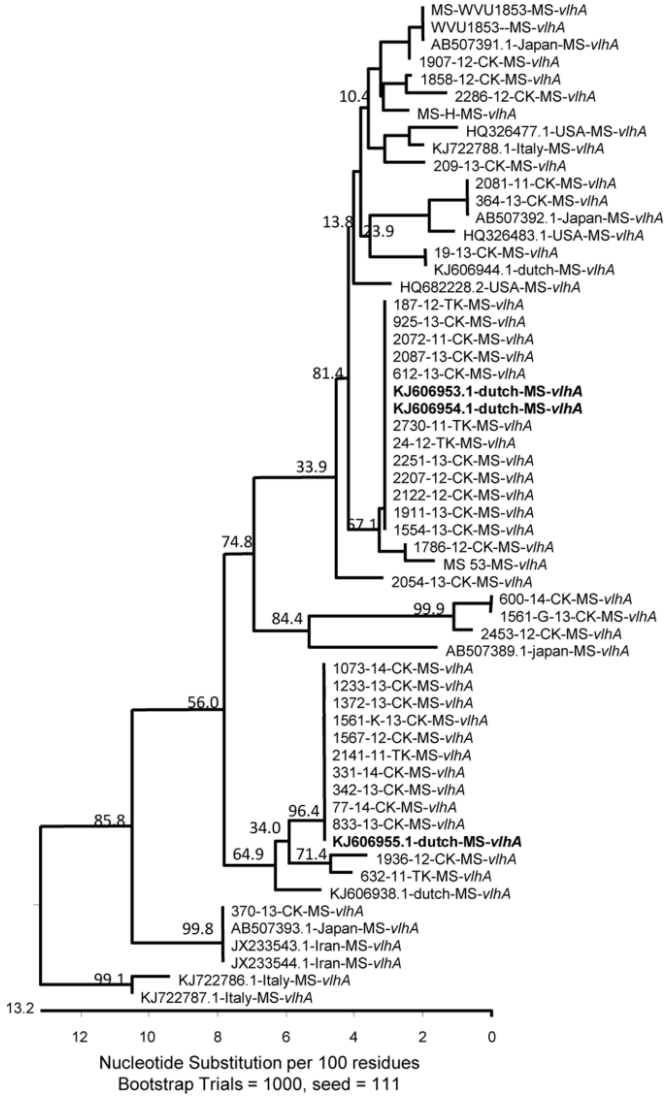

Figure 12: Phylogenetic tree of *vlhA* sequences of MS samples and MS found in USA, Dutch, Japan, Italy and Iran, together with MS reference strains. Sequences were trimmed and edited to have the same starting and ending sequences. Some Dutch MS strains (shown in bold font) shared identical *vlhA* sequence with the two main German MS strains. Identical Phylogenetic tree produced with the use of Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI).

Sample description: entry number-year of sampling-bird species (CK = chicken and TK = turkey)-genome segment

MS reference strain description: MS-Reference strain Identity-Accession number-genome segment

Description of MS strains from other countries: Accession number-Country-genome segment (*vlhA*)

4.1.13. MS typing by Amplified Fragment Length Polymorphism (AFLP)

MS isolates (n = 6) were differentiated into 3 electrophoretic profiles by AFLP. MS isolates (2730/11/TK, 24/12/TK and 187/12/TK) originating from turkey farm in Thüringen showed nearly identical AFLP profile. Turkey MS isolate 2141/11/TK and chicken MS isolate 2072/11/CK were genetically related and compromised another AFLP profile. The third AFLP profile was produced with chicken MS isolate 2081/11/CK (Figure 13).

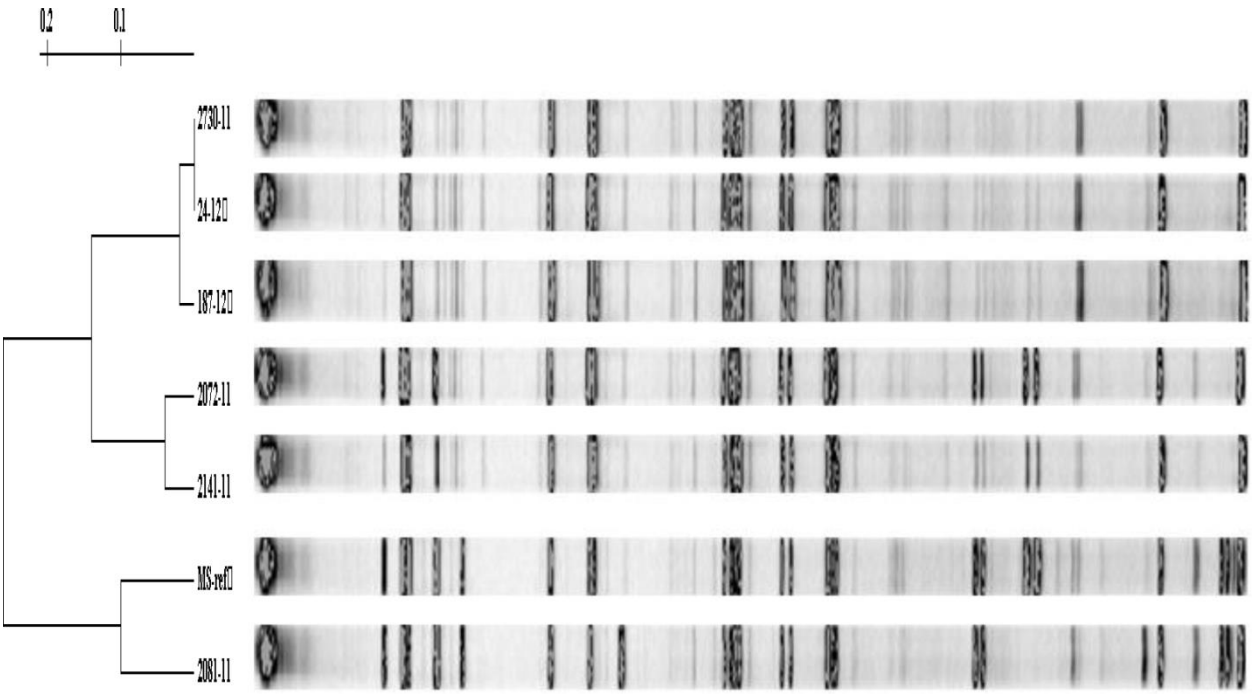

Figure 13: AFLP analysis of 6 MS isolates and MS WVU 1853 reference strain. Turkey breeder MS isolates 2730/11/TK, 24/12/TK and 187/12/TK originating from one turkey breeder farm are showed identical AFLP profile. Turkey MS isolate 2141/11/TK and chicken MS isolate 2072/11/CK are similar. Chicken MS isolate 2081/11/CK are showing a third AFLP profile. Clustering of samples (dendrogram) was performed through LI-COR e-seq Release 2,0 program (LI-COR Inc, Lincolin, USA) using the un-weighted pair-group method with arithmetic averaging.

4.2. MG and MS in poultry and free-ranging birds

Through our investigation on MG and MS in poultry farms in the first part of the study, two organic chicken farms tested positive with MG/MS species-specific PCR. Large numbers of free-ranging birds, especially house sparrows (*Passer domesticus*), were observed to come and share drinkers and feeders with reared birds during the daily open-air free rearing periods. The two farms were selected to study the role of free-ranging birds in MG/MS transmission under complete natural conditions. In farm I (Identity No.: 77/2014), Lohmann brown layers with mild respiratory manifestations, production problems and egg deformities, were reared. In farm II (Identity No.: 331/2014), Lohmann brown layer breeders suffering from fertility and hatchability problems, were kept. Farms were about 25 km from each other.

. Samples from chicken and contacting free-ranging birds were simultaneously collected over 3 days. Samples were investigated for MG and MS by culturing and species-specific PCR. MG strains were compared and typed through multiple GTS analysis of *mgc2* gene, *pvpA* gene and the MG 16S-23S IGSR; the *vlhA* gene was sequenced and compared for MS typing.

Samples of free-ranging birds tested negative with conventional MG IGSR-PCR, *pvpA*-PCR and *vlhA*-PCR. Semi-nested MG IGSR-PCR, semi-nested *pvpA*-PCR and nested *vlhA*-PCR were designed through the present study to improve sensitivity of respective PCRs.

4.2.1. MG and MS detection in free-ranging birds

From the area of farm I, 28 out of 74 samples (38%) from free-ranging birds tested positive for the *Mycoplasma* genus-specific PCR. Out of these *Mycoplasma* positive samples, 11 (15%) and 4 (5.4%) tested positive with MG- and MS- specific PCR, respectively. In farm II, 13 out of 44 samples (30%) from free-ranging birds were tested positive for *Mycoplasma* genus-specific PCR. From these positive samples, 5 tested positive for MG- (11%) and MS- (11%) species specific PCR, respectively. In total, from both farms' areas (n=118 free-ranging birds' samples), nucleic acids of genus *Mycoplasma*, MG and MS were detected in 41(35%), 16 (14%) and 9 (8%) birds, respectively. None of samples from free-ranging birds showed *Mycoplasma* growth on culture. However, chicken samples showed *Mycoplasma* colonies but, none of the colonies tested positive for MG or MS.

4.2.2. Sensitivity of newly designed PCRs

Samples from free-ranging birds tested positive with MG species-specific PCR while they were negative with the conventional MG IGSR PCR and MG *pvpA*-PCR. Also samples from free-ranging birds tested positive with MS species-specific PCR while they were negative with both conventional MS *vlhA*-PCRs (Jeffery et al., 2007; Wetzel et al., 2010). So we designed and used the semi-nested MG-16S-23S IGSR, semi-nested *pvpA* and nested *vlhA*-PCR assays to overcome this problem with free-ranging birds investigated through the second part of the study.

Newly designed semi-nested MG-16S-23S IGSR, semi-nested *pvpA* and nested *vlhA*-PCR assays showed better sensitivity than respective conventional PCR assays. Two samples from free-ranging birds from each farm tested positive with semi-nested MG-16S-23S IGSR PCR, however they were negative with conventional MG-16S-23S IGSR PCR. This was also true for the semi-nested *pvpA* PCR, where 4 and 3 additional samples from free-ranging birds from farm I and farm II, respectively, tested positive. For the nested *vlhA* PCR, 4 and 5 additional samples from farm I and farm II, respectively, tested positive, while they were negative with both conventional MS-*vlhA*-PCR assays.

4.2.3. MG typing by *mgc2* sequencing

Free-ranging birds from farm I and II as well as chickens from farm I produced amplicons of approximately 230 bp with the *mgc2*-PCR. In chicken samples from farm II, a product of approximately 300 bp was amplified. *Mgc2* sequence alignment showed that free-ranging birds from both farms had the same nucleotide sequence identical to that of chickens from farm I. This sequence showed 100% identity with *mgc2* sequences of the MG 6/85 vaccine strain. *Mgc2* sequences of chickens from farm II were identical to that of the MG ts-11 strain. A gap of 63 nucleotides differentiating the two *mgc2* sequences was shown (Figure 14).

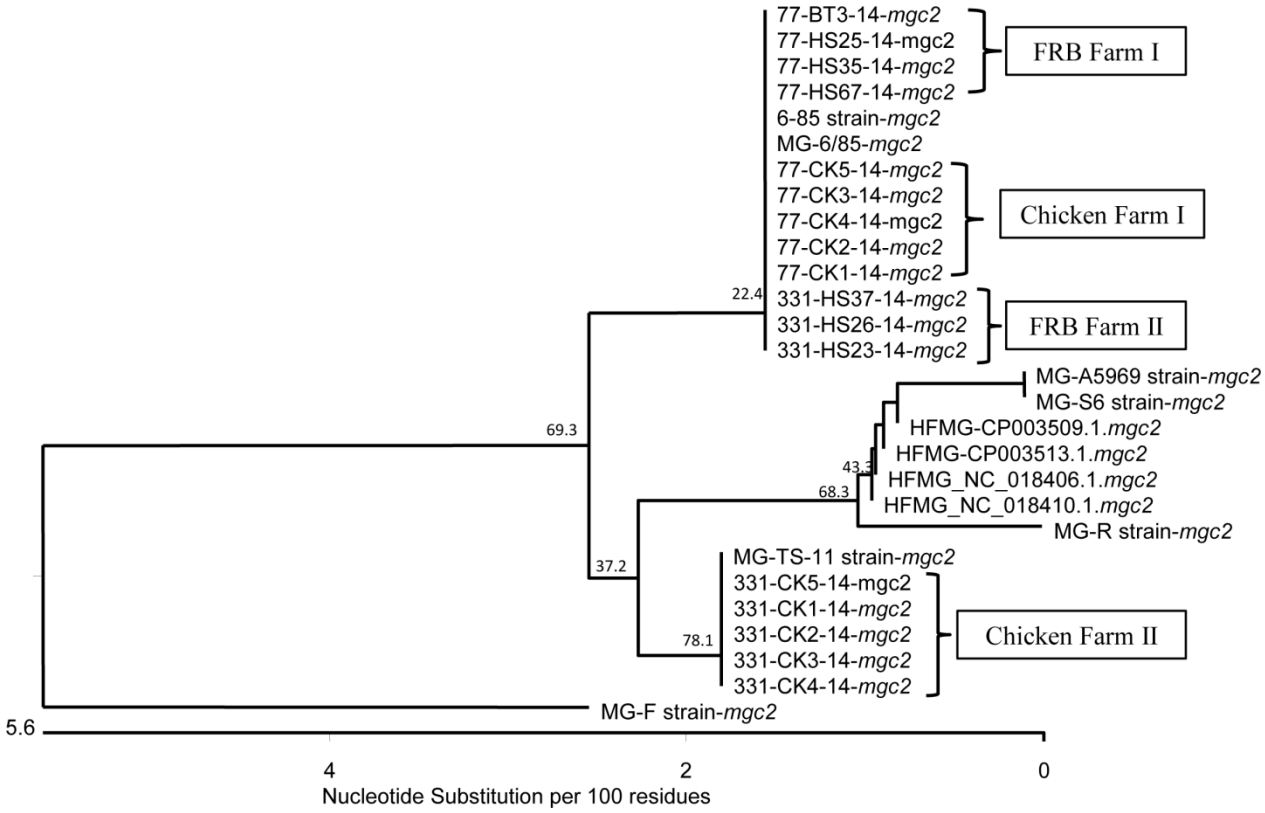

Figure 14: Phylogenetic tree for *mgc2* sequences for MG samples from chicken and contacting free-ranging birds in relation to MG reference strains using the Clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI). Free-ranging birds from farm I and II and chickens from farm I demonstrated identical *mgc2* sequence that was 100% homologous to that of the MG 6/85 strain. Chickens from farm II showed different *mgc2* sequence that was identical to that of the MG ts-11 strain. Both *mgc2* sequences were different from that of house finches MG (HFMG) sequences.

Sample description: sample entry number (77 for farm I and 331 for farm II)-year of sampling-bird species (CK = chicken, HS = House sparrow; BT= Blue tits)-genome segment

MG reference strain description: MS-Reference strain Identity-genome segment (*mgc2*)

Description of MG from house finches (HFMG): HFMG-Accession number- segment (*mgc2*)

FRB= Free-ranging birds

4.2.4. MG typing by MG 16S-23S IGSR sequencing

With conventional MG 16S-23S IGSR PCR, none of the free-ranging bird samples showed an amplicon; however with the newly designed semi-nested MG 16S-23S IGSR PCR, 4 samples from free-ranging birds (two from each farm) produced amplicons of about 800 bp. Samples did not show size difference on agarose gel electrophoresis. Sequencing of amplified products revealed two IGSR sequence types within samples. Similar to the results of *mgc2*, free-ranging birds from both farms together with chickens of farm I showed the same sequence type that was identical to the 16S-23S IGSR of MG 6/85 vaccine strain. Chickens from farm II showed different sequence type that revealed 98% homology to 16S-23S IGSR of MG-PG31 and MG-R strains (Figure 15).

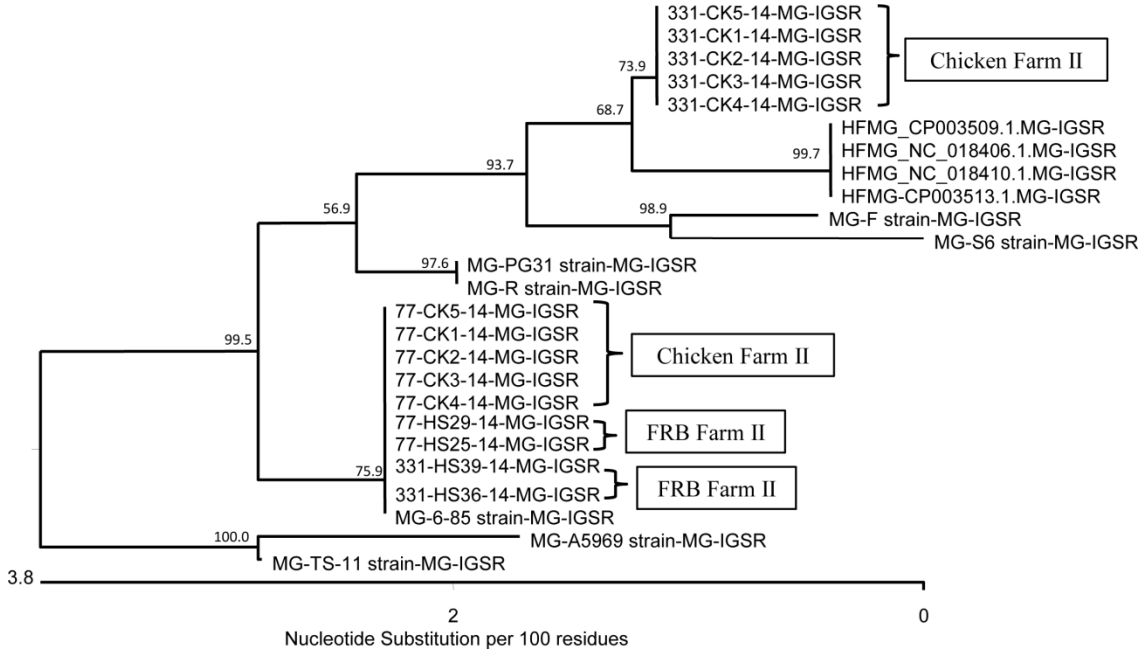

Figure 15: Phylogenetic tree of MG 16S-23S IGSR sequences for MG samples from chicken and contacting free-ranging birds and their relation to MG reference strains using the clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI). Free-ranging birds from farm I and II and chickens from farm I showed identical MG IGSR sequence type that was identical also to the MG 6/85 strain. Chickens from farm II showed different MG IGSR sequence type. Both clusters seemed different from HFMG sequences.

Sample description: sample entry number (77 for farm I and 331 for farm II)-year of sampling-bird species (CK = chicken, HS = House sparrow; BT= Blue tits)-genome segment

MG reference strain description: MS-Reference strain Identity-genome segment (IGSR)

Description of MG from house finches (HFMG): HFMG-Accession number- segment (IGSR)

FRB= Free-ranging birds

4.2.5. MG typing by *pvpA* sequencing

With conventional MG *pvpA*-PCR, no amplicon was produced with samples from free-ranging birds; however with semi-nested *pvpA*-PCR, 4 and 3 samples of free-ranging birds from farm I and II, respectively, produced amplicons and were sequenced. With *pvpA* sequence comparison and alignment, two *pvpA* sequence types were shown within samples. Similar to results of *mgc2* and MG-IGSR, *pvpA* sequences of free-ranging birds originating from both farms as well as of chickens from farm I were identical and corresponded to that of the MG 6/85 strain and the MG strain TLS-2 (GenBank accession no. JN113336.1). House sparrow samples 331/HS23/14 and 331/HS37/14 from the farm II area showed one nucleotide polymorphism in the *pvpA* sequence from other free-ranging birds; however they were still in the same cluster (Figure 16).

PvpA sequence of chicken samples from farm II showed a different *pvpA* sequence type that was identical to that of MG PG31 strain (Figure 16).

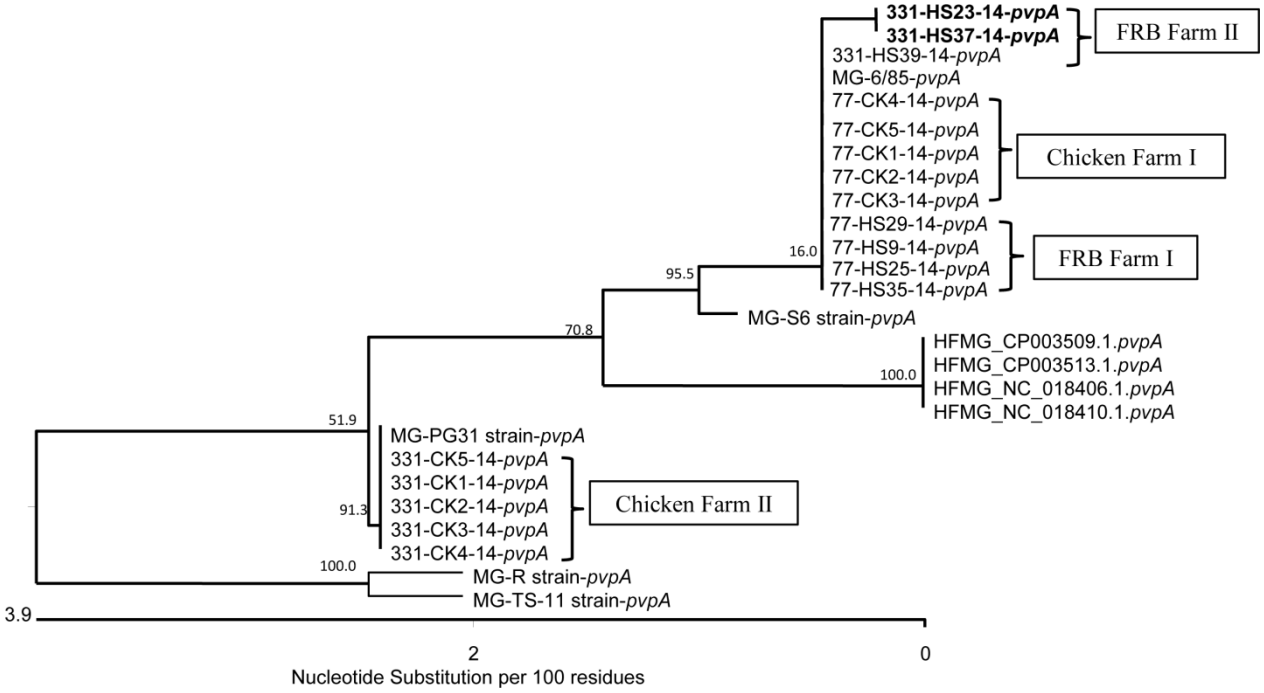

Figure 16: Phylogenetic tree for *pvpA* sequences of MG samples and MG reference strains using the clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI). Free-ranging birds from farm I and II and chickens from farm I demonstrated identical *pvpA* sequence that was 100% homologous to the MG 6/85 strain. Chickens from farm II demonstrated different *pvpA* sequence identical to the MG PG-31 strain. Both *pvpA* sequence types appeared different from HFMG sequences. Two house sparrows (331-HS23-14 and 331-HS37-14) showed one nucleotide variation from other house sparrows; however they still appear in the sample cluster.

Sample description: sample entry number (77 for farm I and 331 for farm II)-year of sampling-bird species (CK = chicken, HS = House sparrow; BT= Blue tits)-genome segment

MG reference strain description: MS-Reference strain Identity-genome segment (*pvpA*)

Description of MG from house finches (HFMG): HFMG-Accession number- segment (*pvpA*)

FRB= Free-ranging birds

4.2.6. Multiple GTS of *mgc2*/*pvpA*/MG-IGSR sequencing

By collecting the sequencing data of three MG genome fragments (*mgc2*/*pvpA*/MG-IGSR) through multiple GTS analysis, free-ranging birds from both farms areas showed an identical circulating MG strain. This strain demonstrated the same *mgc2*, *pvpA*, and MG-IGSR sequences data like the MG 6/85 strain. Multiple GTS analysis of the three genome segments proved also identity between MG of free-ranging birds from both farms and that of chicken from farm I. However chicken from farm II had different MG strains that did not show relationship to specific MG reference strain. MG strains from chicken in farm II had *mgc2* sequences similar to MG ts-11 vaccine strain, MG IGSR similar to MG PG31 and MG R strain, and *pvpA* sequences identical to MG PG31 strain.

4.2.7. MS genotyping by *vlhA* sequencing

Nine samples from free-ranging birds (4 from farm I and 5 from farm II) tested positive with the newly designed nested *vlhA*-PCR, however they tested negative with both Jeffery or Wetzel conventional *vlhA* PCRs. Sequencing of amplicons proved two *vlhA* sequence types within most samples from free-ranging birds in both farms; however one sample (331/HS6/14) from farm II showed different *vlhA* sequence type. Chickens from farm I showed two sequence types identical to those of the free-ranging birds; whereas chicken from farm II showed only one *vlhA* sequence type in common with the two of free-ranging birds. Each of two house sparrows from farm I (77/HS35/14 and 77/HS46/14) produced two *vlhA* amplicons with two different molecular weights on gel electrophoresis. Each amplicon belonged to one of the two *vlhA* sequence types (Figure 17).

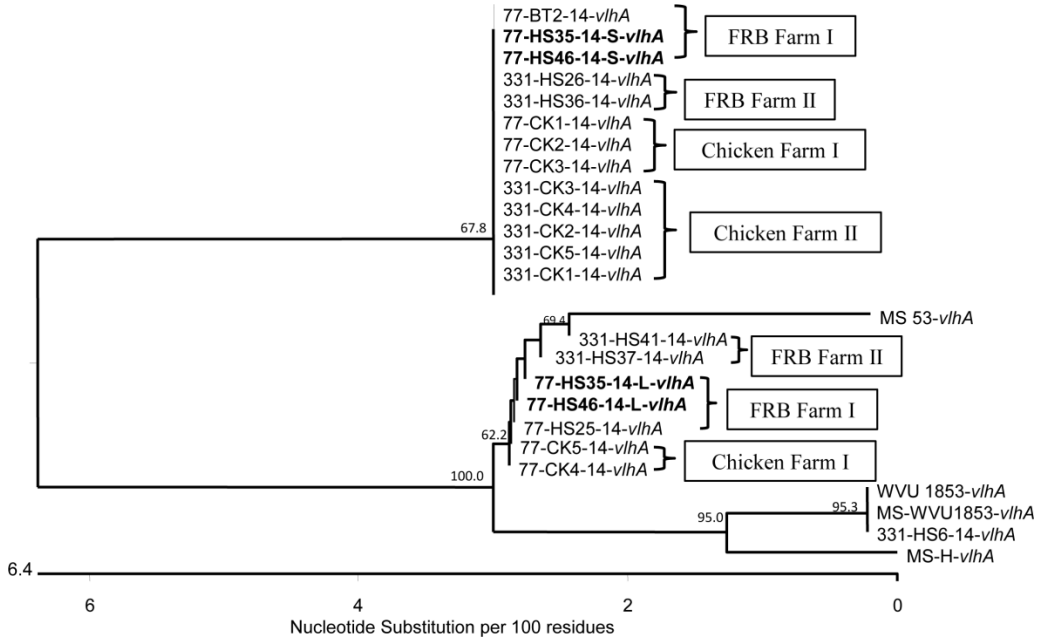

Figure 17: Phylogenetic tree for *vlhA* sequences of MS samples and MS reference strains using the clustal-W alignment of MegAlign program (DNASTAR Lasergene, Madison, WI). Free-ranging birds from both farms demonstrated two *vlhA* sequences. Chickens from farm I showed the same two *vlhA* sequences like free-ranging birds; however chickens from farm demonstrated only one *vlhA* sequence identical to the free-ranging birds. Each of house sparrows 77-SP35-14 and 77-SP46-14 (shown in bold font) from farm I showed two *vlhA* sequences.

Sample description: sample entry number (77 for farm I and 331 for farm II)-year of sampling-bird species (CK = chicken, HS = House sparrow; BT= Blue tits)-genome segment

MG reference strain description: MS-Reference strain identity-genome segment (*vlhA*)

FRB= Free-ranging birds

5. Discussion

5.1. MG and MS incidence and genotyping

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are the most relevant *Mycoplasma* species known to infect avian hosts (Kleven, 2008b; OIE, 2008). Massive economic losses due to MG/MS infection, expressed in carcass downgrading and condemnation at processing, low feed conversion and medication cost, make it as one of the costliest diseases confronting poultry industry worldwide (Raviv and Ley, 2013). Definite MG and MS diagnosis with pathogen identification down to the subspecies level is essential for tracing the infecting pathogen and subsequent evaluation of implemented control strategies.

Through the first part of the present study, MG infection was confirmed in 26 flocks via species-specific PCR. However, MG culturing was successful only in 8 from the investigated flocks. MS infection was also confirmed in 37 flocks that tested positive with MS species-specific PCR, while only 6 MS isolates from them were cultivable.

Similar results with MG and MS diagnosis have been described in previous studies, where MG-PCR was more sensitive than culture and tested positive with 65%, while culture was positive with only 33% from investigated samples (Kahya et al., 2010). MS-PCR also showed higher sensitivity (61% from investigated samples) than culture (25% from investigated samples) in natural clinical samples (Marois et al., 2000). However, some studies described comparable sensitivity between culture and PCR in experimentally infected chicken (Feberwee et al., 2005b). Higher sensitivity results of PCR than culture seems logical as PCR can detect nucleic acid of live and dead microorganisms, while culture is only possible with live microorganisms. Furthermore, some organisms or isolates are fastidious (like MG and MS) and their cultivation is not often successful.

The nature of the pathogen examined in each case might explain the reason behind this difference in sensitivity results between PCR and culture described in these studies. Whereas comparable sensitivity between PCR and culture was described with experimental infection study in which laboratory cultivable isolates were used (Feberwee et al., 2005b), PCR proved

higher sensitivity than culture in the other two studies (Kahya et al., 2010; Marois et al., 2000) where natural clinical samples including cultivable and uncultivable isolates were investigated.

Our results demonstrated also higher incidence of MS (n = 37) than MG (n = 26) in 104 flocks investigated. These results conform to recent reports describing an increase in incidence and clinical significance of MS in other European countries like Belgium and the Netherlands (Haesendonck et al., 2014; Landman, 2014; Michiels et al., 2016). Our results agree partially with reports from Germany describing 75% MS incidence by PCR in commercial layers flocks while none of investigated flocks tested positive with MG PCR (Kohn et al., 2009).

The current situation and the increase in MS incidence might be due to two reasons; the subclinical form known in most MS infection cases (Kleven et al., 1975; Lockaby et al., 1999) as well as the implemented control strategies. Many national control programs focus on MG (as more clinically relevant avian *Mycoplasma*) with less strict strategies towards MS control. MS control by eradication of infected poultry flocks in many countries, including Germany, is applied only to infected breeder and grandparent flocks and does not include layers or broilers (Landman, 2014). In some countries where the MS incidence and clinical impact have increased, new MS control and eradication strategies including layers have been launched (Landman, 2014). So that, implementing more strict MS control strategies in Germany seems critical and should be considered.

On the other hand, genotyping methods that offer adequate intra-species discriminatory capability are critical for diagnosticians and epidemiologists for tracing infecting pathogens. Moreover, expanding usage of live vaccines for control programs in many countries necessitate the presence of robust typing methods able to differentiate between vaccine and field strains.

Concerning MG typing by sequencing through the first part of present study, our results showed D-indexes of 0.52, 0.86, 0.94 and 0.95 with the *mgc2,* MG IGSR, *pvpA* and the multiple GTS (*mgc2/pvpA/* MG IGSR) sequencing, respectively. The discriminatory results of single gene segments (*mgc2,* MG IGSR and *pvpA*) agreed with and supported each other. Starting with *mgc2* sequencing, it was able to differentiate 3 sequence types in MG samples in total (Figure 4). MG-IGSR showed a better discriminatory power than *mgc2* sequencing and could differentiate 10 sequence types that belonged to 4 main clusters within samples (Figure 5). *PvpA* sequencing agreed with results of *mgc2* and MG-IGSR sequencing with an even higher discriminatory power

differentiating 14 sequence types into 6 main clusters within samples (Figure 6). Moreover, combination of *mgc2*, MG-IGSR and *pvpA* sequencing results through a multiple GTS analysis proved highest discriminatory power with a good D-index (0.95) and differentiated 18 sequence types within MG samples (Table 3).

Higher D-index than our results has been reported with *mgc2,* where *mgc2* sequencing showed a D-index of 0.91 through genotyping of 67 MG isolates from different countries in addition to 10 MG reference strains (Ferguson et al., 2005). Also MG IGSR sequencing of 38 unrelated MG isolates and reference strains should a D-index of 0.95 (Raviv et al., 2007a). However, comparable results with *pvpA* ranged from 0.92 (Ferguson et al., 2005) to 0.97 (Sprygin et al., 2010a) as well as with multiple GTS (0.97) (Ferguson et al., 2005) have been described.

Variation in *mgc2* discriminatory power between our results and previous reports might be explained by the segment of the *mgc2* gene investigated. Whereas a segment with a range of approximate 230 bp to 300 bp was investigated in the present study, the other study (Ferguson et al., 2005) investigated a segment of about 820 bp. Also, the genetic relationship and similarity between some of our MG strains (originating from particular geographic locality or even from the same farm) which is not the case in the other study (Ferguson et al., 2005) is another possible factor for such a difference. This genetic relationship between some of our MG strains might also explain our relatively MG IGSR lower discriminatory power (D- index 0.86) compared to other reports describing higher D-index of MG IGSR (0.95) (Raviv et al., 2007a) where unrelated MG strains were investigated.

On the other hand, the high D-index of *pvpA* proved through our study (0.94) and previous studies (ranging from 0.92 to 0.97) (Ferguson et al., 2005; Sprygin et al., 2010a) confirm its benefit as a single locus sequence target for MG genotyping. Moreover, combination of sequencing results from three genome segments (*pvpA*, MG IGSR, *mgc2)* showed the highest discriminatory power and proved to be a robust typing method capable of intra-species differentiation.

Also through the present study, the *pvpA*-RFLP discriminated only 6 different genotypes within MG samples and showed a D-index of 0.81. Similar *pvpA*-RFLP results have been also described where it could differentiate 7 RFLP groups within 66 MG strains originating from house finch, chicken and turkey were examined (Pillai et al., 2003). Results of the *pvpA*-RFLP were

completely in accordance with *pvpA* sequencing results with superiority of sequencing that showed a D-index of 0.94 (Table 4). But with considering the relatively low discriminatory power of *pvpA*-RFLP (0.81) in addition to its laboriousness, time consumption and costs compared to sequencing (D-index 0.94), *pvpA* sequencing (either as a single locus sequencing (SLS) or as part of multiple GTS analysis) seems to be more reliable for MG typing and *pvpA*-RFLP might be more suitable where sequencing is not available.

On the other hand and in order to compare the multiple GTS analysis with the whole genome finger printing techniques for MG typing, this was possible with only 8 turkey MG isolates where both multiple GTS analysis and the amplified fragment length polymorphism (AFLP) were applied. With genotyping of the MG isolates (n = 8) examined by both multiple GTS and AFLP, the discriminatory power of multiple GTS (3 GTS types) was slightly lower than that of AFLP (4 AFLP electrophoretic patterns) (Figure 10 and table 5). Multiple GTS showed identity between turkey breeder MG isolate from 2006 (1608-1/11/TK) and the four turkey breeder isolates from 2011 (1608-3, 4, 5 and 6/11/TK), however AFLP proved the close relationship (not identity) between the historical and recent MG isolates. The high discriminatory capability of AFLP (as one of the whole genome finger printing techniques) for *Mycoplasma* typing has been described (Feberwee et al., 2005a; Hong et al., 2005a; Hong et al., 2005b).

The difference between the discriminatory power of multiple GTS and AFLP might be explained by the informational content gained by both methods. Whereas AFLP uses information of the complete genome, multiple GTS accesses only information of a few genome segments. Moreover, the multiple GTS does not depend on cultivated bacteria, unlike the AFLP, and could be performed directly from clinical samples. Thus 18 non-cultivable MG pathogens could be typed by multiple GTS, while they were not typable by AFLP. Considering this in addition to the laboriousness and inability of inter-laboratory data exchange of AFLP compared to the GTS (Armour et al., 2013), GTS seems more feasible in practice.

On the other hand, MG strains investigated through the present study by multiple GTS analysis could be differentiated into 4 main clusters; a MG 6/85 like strain cluster, a turkey breeder cluster, in addition to two characteristic clusters with no relationship to known MG reference strains.

The 6/85 like strain cluster included 5 MG isolates. From those, four MG isolates (1608-7/11/TK, 1608-8/11/TK, 612/13/CK and 77/14/CK) showed identical GTS data like the MG 6/85 vaccine strain, while the fifth isolate (1936/12/CK) shared similar (but not identical) sequence data to the vaccine strain. These five MG isolates represented nearly 20% of the MG isolates (26 MG isolates) found. MG infections and outbreaks with live MG vaccines like strains have been reported with MG 6/85, MG ts-11 and MG F like strains (El Gazzar et al., 2011; Gharaibeh et al., 2011; Kleven et al., 2004; Throne Steinlage et al., 2003). In these studies, the origin of those vaccines like MG strains was not always clear whether they originate from vaccine strains or from naturally field isolates. This is because some of the infected poultry flocks with vaccines like strains had no history with live MG vaccination.

MG 6/85 like isolates of the present study represented an example for this ambiguity. Whereas the MG isolates 1608-7/11/TK and 1608-8/11/TK might originate from MG 6/85 vaccine strain because the respective flocks had a history of 6/85 vaccination. This is not the case for isolates 612/13/CK and 77/14/CK. Such cases of ambiguity constitute a real challenge accompanying usage of live MG vaccines and should be considered along with the implementation of vaccination strategies. The second cluster (turkey breeder cluster) included one breeder MG isolate from 2006 (1608-1/11/TK) and 4 isolates from 2011 (1608-3, 4, 5 and 6/11/TK) originating from one turkey farm. This genetic relationship was demonstrated by both the multiple GTS analysis and AFLP (Table 4). This close relationship highlights the importance of the implementation of strict eradication and control measurements after MG outbreaks. Additionally, it illustrates the epidemiological significance of periodical surveillance with genotype identification for tracing the origin of infecting pathogens.

Interesting was also the sample 1561/13/CK that showed a characteristic MG- *pvpA* sequence of an about 770 bp amplicon in size with inclusion of three direct repeat sequences (DRs) in the *pvpA* gene. The MG genome has been documented to contain two copies of DRs in the C-terminus of the *pvpA* gene, with the exception of MG F vaccine strain that contains only one copy of the DRs (Boguslavsky et al., 2000; Ferguson et al., 2005; Jiang et al., 2009; Liu et al., 2001; Szczepanek et al., 2010). Also, molecular characterization of the proline-rich C-terminal segment of the *pvpA* gene for some MG strains and isolates proved two direct repeat sequences (DR-1 and DR-2) (Boguslavsky et al., 2000). For our knowledge, this is the first report describing

three copies of DRs in an MG genome. Further studies on the mechanism of antigenic variability of MG are recommended.

Global comparison of *mgc2*, MG IGSR and *pvpA* sequences of MGs of the present study with respective gene segments of MGs from other countries demonstrated particularity of German MG strains. This comparison illustrated as well the genetic relationship between some Russian MG isolates and the German MG 6/85 like isolates (Figures 7, 8 and 9).

Infection with MG 6/85 vaccine like strains have already been reported in other countries (Kleven et al., 2004) where it was not possible to determine exactly if the infecting strain was of a vaccine origin or represented a field isolate, which is comparable to our situation in the present study. These findings illustrate the epidemiological importance of data exchange offered by sequencing and draw our attention to the challenges accompanying usage of live MG vaccines for control programs. The establishment of a global data base for MG is in progress (Armour et al., 2013) and improvements and international cooperation are highly required. However complete sequences of some MG strains have been published (Papazisi et al., 2003; Szczepanek et al., 2010), more investigations and comparisons between other MG strains are expected due to immediate advances in sequencing technologies and bioinformatics.

Moving to MS that showed higher incidence than MG. Genotyping of MS strains (n=37) by *vlhA* sequencing revealed circulation of 15 MS strains in German poultry farms with predomination of two main MS strains. *VlhA* sequencing showed a D-index of 0.83 (Figure 11).

Similar discriminatory power of *vlhA* gene sequencing have been shown through previous studies, where it could differentiate 10 sequence types within 35 MS field isolates in Australia (Jeffery et al., 2007), 8 sequence types within 21 Iranian MS isolates (Bayatzadeh et al., 2014), 9 sequence types within 27 Dutch MS isolates (Dijkman et al., 2014) and 9 sequence types within 19 Japanese MS isolates (Ogino et al., 2011).

Concerning the cultivable MS isolates (n = 6) examined by both *vlhA* sequencing and AFLP, high degree of agreement between results of both methods was shown with slightly higher discriminatory power of AFLP. Both methods discriminated 3 genogroups within isolates. *VlhA* grouped turkey MS strains (2730/11/TK, 24/12/TK and 187/12/TK) and chicken strain 2072/11/CK into one sequence type, turkey MS isolate (2141/11/TK) into another type, while chicken MS isolate 2081/11/CK represented the third *vlhA* sequence type (Figure 11). However,

genotyping of the MS isolates by AFLP showed also 3 groups (I, II and III) according to their electrophoretic pattern, grouping was relatively different from that of the *vlhA* sequencing. Whereas group I included the turkey MS isolates 2730/11/TK, 24/12/TK and 187/12/TK, group II included the chicken (2072/11/CK) and turkey (2141/11/TK) MS isolates, and group III in which the chicken MS isolate 2081/11/CK was included (Figure 13).

Comparable results between *vlhA* sequencing and AFLP have been also described (Dijkman et al., 2014), however the slightly higher power of AFLP could be explained by the different informational content investigated by both methods. But with considering the non-cultivable MS isolates investigated only with *vlhA* sequencing (n = 31) and the easy data exchange of *vlhA* compared with AFLP and the laboriousness of AFLP, sequencing of the *vlhA* seems to be more applicable. Furthermore, due to the relatively inadequate D-index of *vlhA* gene (0.83), the inclusion of other genome segments seems necessary for better MS typing. Other MS gene portions like the 16S-23S intergenic spacer region might allow for better MS strain differentiation.

VlhA sequencing was also helpful and proved 100% homology between MS strains 2730/11/TK, 24/12/TK and 187/12/TK that originated from three successive outbreaks in a turkey breeder farm in the winter of 2011/2012. These strains were different from the MS strain 632/11/TK, the causative agent of the preceding outbreak in March 2011. Similarly, *vlhA* sequencing proved the genetic relatedness between the 4 MS strains 833/13/CK, 1233/13/CK, 1372/13/CK and 331/14/CK that represented 4 successive MS outbreaks appeared in one organic chicken farm. Those 4 MS strains, that showed identical *vlhA* sequence, represented MS outbreaks from middle 2013 till the beginning of 2014. The *vlhA* sequence of these 4 MS strains was different from that of the MS strain of previous outbreak (2122/12/CK) occurred in the middle of 2012 in the same farm. However the homology of *vlhA* gene sequence from MS strains originating from successive outbreaks seems logic, heterogeneity from preceding strains might be due to infection with different MS strains or the same MS strain but with variant *vlhA* nucleotide sequence that has been reported to undergo high antigenic variability (May and Brown, 2011; Noormohammadi, 2007; Slavec et al., 2011).

The global comparison of resulting *vlhA* sequences demonstrated the genetic relatedness between Dutch MS strains and the two predominant MS strains in Germany (Figure 12). This

possibility for strain comparison by sequencing represents another advantage of sequencing as a typing method offering relatively easier inter-laboratory data exchange. This convergence between German and Dutch MS strains might be explained by the geographical proximity, but it illustrates as well the epidemiological significance of joint international control strategies.

Again with the sample 1561/13/CK that generated two *vlhA* amplicons. These amplicons showed different molecular weights in size and nucleotide composition and consequently two different sequence types (Figure 11). Obviously this sample originates from a mixed infection with two different MS strains. Moreover, this sample represented a MG/MS co-infection. The sample showed also a characteristic MG- *pvpA* sequence (770 bp amplicon) with three DRs in the *pvpA* gene. Although, similarity of some genome fragments caused by horizontal gene transfer (HGT) events between MG and MS have been demonstrated through genome comparison (Szczepanek et al., 2010; Vasconcelos et al., 2005), no sequence similarity between the MG *pvpA* and MS *vlhA* gene sequences was reported. So that, inclusion of the characteristic MG- *pvpA* sequence together with the two MS-*vlhA* amplicons in this sample (1561/13/CK) could not be definitely clarified and further investigations should be considered.

Collectively and based on the results of the first part of the study, we conclude that *pvpA* gene sequencing followed by IGSR showed the best discriminatory power as a single locus sequence typing (SLST) method and their combination in the multiple GTS (*mgc2/pvpA/*MG-IGSR) provides good discriminatory power for MG typing. Multiple GTS and *vlhA* sequencing for MG and MS, respectively, allow for intra-species identification as well as global strain comparison and consequently for a better understanding of the epidemiology and genetic evolution of circulating strains. Furthermore, the increasing MS incidence might require updating of implemented control strategies with more strict measures. A broader periodical surveillance and stronger international cooperation and coordination for MG/MS control programs should be considered.

5.2. MG and MS in poultry and free-ranging birds

For long time, MG and MS were thought to be host specific, however reports describing natural infections with MG and MS pathogens in new hosts are increasing (Bencina et al., 1987; Bozeman et al., 1984; Delaney et al., 2012; Dhondt et al., 1998; Lierz et al., 2000; Morishita et

al., 1997). This means that the hypothesis of MG and MS host specificity is difficult to be accepted. Also, the actual knowledge concerning the role for non-poultry birds in *Mycoplasma gallisepticum* (MG) and *Mycoplasma synoviae* (MS) epidemiology is limited and requires more investigations.

So we aimed to investigate the intra-species genetic relationship of MG and MS strains originating from infected poultry and their contacting free-ranging birds under complete natural conditions. Moreover, based on the discriminatory results of typing methods demonstrated through the first part of study, identification of the infecting pathogens down to the sub species level was done using multiple GTS analysis of *mgc2/pvpA/*MG-IGSR (for MG) and *vlhA* sequencing for MS.

Out of 118 free-ranging birds examined through this part of the study, nucleic acids of MG and MS were detected in 16 and 9 birds with 14% and 8% incidence, respectively; however none of these MG and MS strains were cultivable. All the birds, that were captured over three days what constituted a representative sample for free-ranging birds' population in areas of infected chicken farms, were apparently healthy birds.

Recent survey reports from Belgium have described MG (0.5%) and MS (1.3%) in free-ranging birds, where 5 and 12 out of 890 free-ranging birds tested positive for MG and MS, respectively (Michiels et al., 2016). Also through a surveillance study in the USA, 3 out of 358 (0.8%) free-ranging birds tested positive with MG-PCR and none of investigated birds showed clinical manifestations (Farmer et al., 2005). Also the two different studies (Gharaibeh and Hailat, 2011; Kleven and Fletcher, 1983), no clinical signs were seen on house sparrows experimentally infected with MG.

The higher incidence for MG and MS in free-ranging birds found in the present study in comparison to the Belgian (Michiels et al., 2016) and north American (Farmer et al., 2005) studies might be explained by the nature of the samples investigated. Whereas our samples were obtained from only two locations with infected chickens with avoidance of double sampling the same bird (by marking the samples bird before release), the Belgian and American samples were collected from different geographic localities.

Failure of MG and MS isolation from free-ranging birds might be due to many reasons; the nature of sample (oropharyngeal not tracheal), and the fastidious nature of MG and MS, the low

susceptibility of free-ranging birds to MG and MS (Dhondt et al., 2008; Kleven and Fletcher, 1983). Supposing that the free-ranging birds are just mechanical carriers for MG and MS might also be a reason for isolation failure. Although pathogen isolation is considered a standard for infection diagnosis, the detection of nucleic acids of MG and MS in free-ranging birds suggests that these birds are at risk and could play a role for pathogen transmission.

On the other hand and due to the apparently low MG and MS DNA load in oropharyngeal swabs collected from free-ranging birds, we tried to improve the sensitivity of such PCRs by application of semi-nested (MG 16S-23S IGSR-PCR and MG *pvpA*-PCR) and nested (MS *vlhA*-PCR) PCR designs. Semi-nested *pvpA*-PCR was reported to be 10-fold more sensitive than conventional *pvpA*-PCR (Liu et al., 2001). Although the exact sensitivity degree of the newly designed semi-nested MG 16S-23S IGSR-PCR and nested MS *vlhA*-PCR assays was not determined as it was not a main aim through the present study, the new PCR assays showed higher sensitivity compared to the respective conventional PCRs. The newly designed nested and semi-nested PCRs could to a far degree overcome the problem of low detection frequency in samples and 4, 7 and 9 samples from free-ranging birds tested positive with the semi-nested MG-16S-23S IGSR PCR, semi-nested *pvpA* PCR and nested MS *vlhA* PCR, respectively. However, none of these samples tested positive with the respective conventional PCR.

Through MG genotyping by multiple GTS analysis of the *mgc2* gene, MG 16S-23S IGSR and the *pvpA* gene, the sequencing results of the 3 segments agreed and supported each other to a far distance. Intra-species differences and relationship were firstly shown by *mgc2* sequencing. These findings were confirmed by sequencing of MG 16S-23S IGSR and *pvpA* gene and subsequently the multiple GTS analysis. Similar results were shown in previous studies (Ferguson et al., 2005; Gharaibeh et al., 2011), where sequencing of more than one genome segment (multiple GTS analysis) proved to be helpful in explaining the intra-species degree of relatedness between MG strains.

Multiple GTS analysis of MG strains proved the identity of strains originating from free-ranging birds' populations on areas of both farms as well as in chickens of farm I. Surprisingly, this circulating MG strain shared the same *mgc2*, MG 16S-23S IGSR and *pvpA* sequence data like the MG 6/85 vaccine strain. Moreover, this MG strain was found through the first part of the present study to be frequent (in 5 out of 26 MG positive samples) in German commercial

poultry and backyard flocks. Similar cases of infection with live MG vaccine-like strains like the MG 6/85 (Kleven et al., 2004; Throne Steinlage et al., 2003), ts-11 (El Gazzar et al., 2011), and F strain (Gharaibeh et al., 2011) vaccines have also been reported. Although the MG 6/85 vaccine is used for some MG control programs in German poultry farms, the origin of this 6/85 like strain could not be definitely proven because none of both farms had a history of MG 6/85 strain vaccination. The possibility that the origin of this MG 6/85 like strain either from a nearby vaccinated farm or from a field isolate of similar genetic profile to the MG 6/85 vaccine could not be definitely clarified. Deeper sequencing analysis for other genome segments might help for clarification and differentiation between such closely related strains. Concerning different sequence data of MG found in chickens from farm II, it might be due to infection with different MG strains or strain competition known to occur between infecting MG strains for replacing each other (Turner and Kleven, 1998).

Similar to MG findings, MS typing demonstrated predomination of two MS lineages in populations of free-ranging birds from areas of both farms. Identical *vlhA* sequences were shown in samples of chickens from farm I. However, chickens from farm II showed one MS strain identical to the predominating MS strains found in free-ranging birds. Also interesting were the two predominating MS strains in free-ranging birds that shared identical *vlhA* sequence data with the two predominant MS strains found in the first part of the study (Figure 11 and 17). Those two MS strains showed high occurrence in German poultry farms and were found in 22 out of 37 MS strains detected (Figure 11). Moreover, these two strains showed also identical *vlhA* sequence with Dutch MS strains (Figure 12).

On the other hand, each of the samples 77/sp35/14 and 77/sp46/14 showed two different *vlhA* amplicons with two different *vlhA* sequence types (Figure 17). In a previous *in vivo* study, variations in MS-*vlhA* gene sequence and MS-*vlhA* protein have been demonstrated in tracheal cultures from the same chicken taken 8 and 18 days post infection (Slavec et al., 2011). Other reports described high degree of MS diversity and reversible surface antigenic variation (Noormohammadi, 2007; Pflaum et al., 2016). So, explaining the immediate findings by presence of two separate MS strains in each bird or *in vivo* variation of one MS strain could not be definitely confirmed or excluded. Moreover, such *vlhA* variation demonstrated in free-ranging birds, in addition to *pvpA* nucleotide variation in house sparrow samples 331/HS23/14

and 331/HS37/14 (Figure 16), might draw attention that these birds could be more than a mechanical vehicle for pathogen transmission and might act as a vesicle where antigenic changes can occur. Also, sharing of free-ranging birds in both locations for the same MG and MS strains might explain that these birds have their own circulating pathogens, and consequently threats of adaptation of these pathogens to new hosts could not be excluded (Delaney et al., 2012).

Based on immediate finding and the genetic relatedness between MG and MS strains originating from chicken and contacting free-ranging birds, suitable control measures that prevent or minimize this contact should be considered before adaptation of MG, or in particular MS, pathogens to a new avian species.

6. Summary

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are avian pathogens of worldwide significant economic impact. Definite diagnosis with pathogen identification down to sub-species level represents the first bulwark for correct control approach. Furthermore, the role of non-poultry birds in MG and MS epidemiology has not been extensively investigated and outbreaks of MG and MS in poultry farms continue to reappear despite implemented control measurements.

The present study includes two parts. Through the first part, 104 German poultry flocks were investigated for MG and MS infection and the occurred *Mycoplasma* spp. were typed and compared. MG showed lower incidence and was detected in 26 flocks (25%), however MS was identified in 37 flocks (36%).

MG genotyping was carried out through *pvpA*-restriction fragment length polymorphism (*pvpA*-RFLP) analysis, sequencing of the MG cytadhesin 2 (*mgc2*) gene, the MG 16S-23S intergenic spacer region (IGSR) and the phase-variable protein A (*pvpA*) gene that showed discrimination indexes (D-index) of 0.81, 0.52, 0.86 and 0.94, respectively. Multiple gene-targeted sequencing (GTS) analysis of *mgc2*/*pvpA*/IGSR discriminated 18 sequence types (from I to XVIII) within MG strains and showed the highest D-index (0.95). Multiple GTS revealed a characteristic genetic profile of German MG strains that could be differentiated into 4 clusters; a 6/85 like strain, a turkey breeder, in addition to two German characteristic clusters with no relationship to known MG reference strains.

MS genotyping was carried out via sequencing of the MS variable lipoprotein and hemagglutinin (*vlhA*) gene that showed a D-index of 0.83. MS genotyping proved circulation of 15 *vlhA* sequence types with predomination of two sequence types. Global comparison of *vlhA* sequences proved genetic relationship between German and Dutch MS strains.

Results demonstrated also the importance of periodical surveillance for MG and MS with species identification down to the strain level. Multiple GTS analysis (for MG) and *vlhA* sequencing (for MS) proved to be dependable, practical genotyping methods capable of intra-species discrimination that offers also easier inter-laboratory data exchange enabling a better understanding of the genetic evolution of circulating pathogens. Increased MS incidence

requires updating of implemented control strategies. International cooperation and coordination for MG/MS control are epidemiologically very important.

Through the second part of the study, chicken and contacting free-ranging birds from two naturally MG/MS infected chicken farms (I and II) were simultaneously investigated and compared for MG and MS. From 118 captured free-ranging birds, DNA of genus *Mycoplasma* was detected in 41 birds. From those, MG-specific and MS-specific DNA were detected in 16 and 9 samples, respectively. The genetic profile of MG was compared through multiple gene-targeted sequencing (GTS) analysis of *mgc2*, *pvpA* and MG IGSR. Free-ranging birds from both farms' areas as well as chickens of farm I showed the same MG lineage that shared identical GTS profile like MG 6/85 vaccine strain. MG of chickens from farm II demonstrated a different GTS profile. The genetic profile of MS was investigated through sequencing of the MS *vlhA* gene. Free-ranging birds captured on both farms showed two circulating MS lineages which were also detected in chickens of farm I. However, chickens from farm II had only one MS strain in common with free-ranging birds.

In conclusion, results of the present study demonstrate the importance of periodical surveillance for MG and MS. Multiple GTS analysis (for MG) and *vlhA* sequencing (for MS) proved to be robust, practical genotyping methods capable of intra-species discrimination. Sequencing allows also for easier data exchange between laboratories and consequently a more comprehensive understanding of the genetic relationship of internationally circulating strains. Increased MS occurrence necessitates updating of the immediate control strategies. Stronger international cooperation and coordination for MG/MS control are recommended.

Moreover, results highlight also the possibility of an epidemiological role played by free-ranging birds, especially the wide spread house sparrows, in MG and MS spread between poultry farms. Hence, suitable control measures to decrease the contact between poultry and free-ranging birds should be quickly launched.

7. Zusammenfassung

Title: Epidemiologische und genetische Studien zu *Mycoplasma gallisepticum* und *Mycoplasma synoviae* bei Geflügel und frei lebenden Vögeln

Mycoplasma gallisepticum (MG) und *Mycoplasma synoviae* (MS) sind vogelpathogene Bakterien mit weltweit bedeutenden wirtschaftlichen Auswirkungen. Die genaue Diagnose mit der Pathogenidentifizierung bis zum Unterartenniveau ist die Basis für entsprechende Kontrollmaßnahmen. Außerdem ist die Rolle von frei lebenden Vögeln in der MG- und MS-Epidemiologie noch nicht umfassend untersucht worden und Ausbrüche von MG und MS in Geflügelfarmen treten weiterhin auf, trotz durchgeführter Kontrollmaßnahmen.

Die vorliegende Studie umfasst zwei Teile. Im ersten Teil wurden 104 Geflügelherden in Deutschland auf MG und MS untersucht, und die nachgewiesenen Mykoplasmen typisiert und miteinander sowie mit der Literatur verglichen. MS zeigte ein häufigeres Vorkommen und wurde in 37 (36%), MG dagegen nur in 26 (25%) Herden entdeckt.

Es wurde eine MG-Genotypisierung durch *pvpA*-Restriction Fragment Length Polymorphism (*pvpA*-RFLP) Analyse, Sequenzierung des MG Cytadhesin 2 (*mgc2*)-Gens, MG-16-23 Intergenic Spacer Region (IGSR) und MG Phase-Variable Protein A (*pvpA*)-Gens durchgeführt. Diese Untersuchungen ergaben Discrimination Indices (D-Index) von 0,81, 0,52, 0,86 und 0,94. Die multiple gene-targeted sequencing (GTS) Analyse der *mgc2*/*pvpA*/IGSR-Gene ergab 18 Sequenztypen (von I bis XVIII) mit dem höchsten D-Index (0.95). Die multiple GTS zeigte ein charakteristisches genetisches Profil der deutschen MGs, die in 4 Cluster unterschieden werden konnten; ein MG 6/85-ähnliches Cluster, ein Puteneltern- Cluster, und zusätzlich zwei deutsche charakteristische Cluster ohne Beziehung zu bekannten MG-Referenzstämmen.

Die MS-Genotypisierung wurde durch Sequenzanalyse des MS-Variable Lipoprotein und Hemagglutinin (*vlhA*)-Gens untersucht, welches einen D-Index von 0,83 aufwies. Die MS-Genotypisierung zeigte 15 *vlhA*-typen mit einer besonderen Häufigkeit von zwei Typen. Der Vergleich des gesamten *vlhA*- *Gens* mit in der GenBank hinterlegten Sequenzen zeigte genetische Beziehungen zwischen deutschen und holländischen MS- Sequenzen auf.

Im zweiten Teil der Studie wurden Hühner und frei lebende Vögel von zwei natürlichen MG/MS infizierten Hühnerfarmen (I und II) gleichzeitig beprobt, und auf MG und MS untersucht, sowie gefundene Sequenzen genotypisiert. Von 118 gefangenen frei lebenden Vögeln wurde DNA der Klasse *Mycoplasma* in 41 Vögeln entdeckt. Von diesen 41 positiven Tieren wiesen 16 MG-spezifische und 9 MS-spezifische DNA auf. Das genetische Profil von MG wurde durch eine Multiple GTS Analyse der folgenden Gene *mgc2*, *pvpA* und MG IGSR verglichen. Frei lebende Vögel aus beiden Farmen sowie Hühner der Farm I wiesen identische MG-Genotypen auf, die auch identisch mit dem GTS-Profil des MG 6/85 Impfstammes war. Das GTS-Profil von MG der Hühner von Farm II war jedoch unterschiedlich. Das genetische Profil von MS wurde durch eine MS-*vlhA*-Sequenzierung untersucht. Frei lebende Vögel auf beiden Farmen wiesen zwei zirkulierende MS-Typen auf, die auch in Hühnern der Farm I entdeckt wurden. Jedoch hatten Hühner von der Farm II nur einen MS-Typ gemeinsam mit der frei lebenden Vögeln derselben Farm.

Die Ergebnisse der vorliegenden Studie unterstreichen die Wichtigkeit der regelmäßigen Untersuchung von Geflügelbetrieben auf MG und MS sowie die genaue Analyse der Stammvorkommen. Multiple GTS-analyse (für MG) und *vlhA*-sequenzierung (für MS) haben sich als praktische Genotypisierungsmethoden zur Differenzierung innerhalb der jeweiligen Spezies erwiesen. Die Sequenzierung bietet auch die Möglichkeit zum Datenaustausch zwischen Laboren. Das hohere Vorkommen von MS sollte zur Aktualisierung der Kontrollstrategien dieses Erregers in Geflügelbetrieben führen. Verstärkte internationale Zusammenarbeit und Koordination für die Kontrolle von MG/MS werden empfohlen.

Außerdem unterstreichen die Ergebnisse eine epidemiologische Rolle der frei lebenden Vögel, besonders der Haussperlinge, bei der MG- und MS-Übertragung zwischen Geflügelfarmen. Geeignete Kontrollmaßen für solche Vögel sollten in Betracht gezogen werden.

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I dedicate this dissertation to all of you

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Declaration

I declare; this dissertation submitted is a work of my own, written without any illegitimate help by any third party and only with materials indicated in the dissertation. I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me. At any time during the investigations carried out by me and described in the dissertation, I followed the principles of good scientific practice as defined in the “Statutes of the Justus Liebig University Giessen for the Safeguarding of Good Scientific Practice.

Mohamed Ahmed

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