Molecular characterization of *Mycoplasma gallisepticum* strains from South African poultry.

by

Lehlohonolo Mathengtheng

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Supervisor: Prof. R.R. Bragg.

This dissertation is dedicated to my parents Mamatsaba and Ohentse, my siblings Lerato and Lebogang, my cousin siblings Diau, Keneilwe, Ntsane and my grandmother Sponkie.

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List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
APS	Ammonium persulfate
bp	base pairs
CO_2	Carbon dioxide
CRD	Chronic Respiratory Disease
CEF	Chick Embryo Fibroblast
^0C	degrees Celcius
DNA	Deoxyribose Nucleic Acid
dNTPs	Deoxyribonucleotide triphosphates
GTS	Gene-targeted sequencing
GI	Growth Inhibition
HI	Haemagglutination Inhibition
IB	Infectious Bronchitis
IP	Immunoperoxidase
IFA	Indirect Fluorescent Antibody
ITS	Intergenic spacer region
kb	Kilobases
kDa	kiloDaltons
Tm	Melting temperature
MI	Metabolism Inhibition
µl	Microliter
ml	Milliliter
mV	Millivolts
min	Minute
M	Molar concentration
ND	Newcastle Disease
NAD	Nicotinamide adenine dinucleotide
oriC	Origin of replication

%	Percentage
PCA	Polyclonal antibody
PCR	Polymerase Chain Reaction
prfA	Polypeptide release factor A
PFGE	Pulse Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
RBC	Red Blood Cells
®	Registered name
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribose Nucleic Acid
rDNA	ribosomal DNA
rRNA	ribosomal RNA
s	Second
SPA	Serum Plate Agglutination
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
tRNA	transfer Ribose Nucleic Acid
UF	Urea-Formamide
UV	Ultra Violet

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The genus *Mycoplasma* has more than a hundred species, with a G+C (Guanine Cytosine) content of 23-40 % (Kleven, 2003). The most important characteristics of the members belonging to this genus is their lack of cell wall, utilization of UGA, a universal bacterial stop codon, to code for tryptophan; as well as the utilization of sterols to strengthen the outermost trilaminar membranes (Hnatow *et al*., 1998). These membranes act as barriers that protect the contents of the *Mycoplasma* cell. Both pathogenic and non-pathogenic species are found in the genus and most species are host-dependent and survive for a limited period outside the host. Boguslavsky *et al.* (2000) reported that a bleb at the tip organelle initiates cytadhesion to host cell membranes in pathogenic species during pathogenesis.

Mycoplasma gallisepticum, an avian pathogen, belongs to this group and is considered to be the most pathogenic of all avian *Mycoplasma* pathogens (Kleven, 2003). Infection with *M. gallisepticum* leads to reduced egg production, growth and hatchability rates thus causing great economic losses (Ferguson *et al*., 2004; Liu *et al*., 2001; Marois *et al*., 2001). Similar clinical signs have been reported in other avian *Mycoplasma* infections such as those of *M. synoviae* and *M. meleagridis* in less severity, though *M. gallisepticum* has a smaller genome size (Levisohn & Kleven, 2000). The size of the genome is 996 422 base pairs (bp)-long with 742 genes as concluded in the R-strain by Papazisi *et al*. (2003). Non-pathogenic species include *M. gallinarum* and *M. gallinaceum*, and should be differentiated from pathogenic species as both are usually isolated together (Hong *et al*., 2005a). This is probably due to similar growth conditions and nutritional requirements.

Differentiation of *Mycoplasma* species based on morphological characteristics is non-effective due to pleomorphic conformation by these species, as well as the lack of ability for Gram staining characterization. Boguslavsky *et al*. (2000) have, however reported the conformation of *M. gallisepticum* and human *Mycoplasma* species to a flask-like shape with attachments at the tip. Serological methods on the other hand, have been shown to be more effective, though with certain limitations. The most effectiveness was achieved with utilization of molecular methods in both the identification and differentiation of *M. gallisepticum* strains.

Besides playing a role as pathogens, *Mycoplasma* species have been nicknamed the "crabgrass" of tissue cultures. This is due to the very high levels of contamination in tissue cultures. In a review by Razin (1996), the infection is said to be persistent, frequently difficult to detect and diagnose, and difficult to cure. The origin of contaminating *Mycoplasma* is in components of the culture medium, particularly serum, or in the microbiota of the technician's mouth, spread by droplet infection.

1.2 Classification of *M. gallisepticum*

M. gallisepticum belongs to the division Firmicutes, class Mollicutes, order Mycoplasmatales and the family Mycoplasmataceae (Ley, 2003). Despite their lack of cell wall, *Mycoplasma* are classified into the phylum Firmicutes which consists of low G+C Gram-positive bacteria such as *Clostridium*, *Lactobacillus* and *Streptococcus* based on the 16S rRNA gene analysis. This is the reason some literature refers to *Mycoplasma* as Gram-positive bacteria. Mycoplasmatales is one of the four cultured orders of the class Mollicutes and contains a single family Mycoplasmataceae with two genera: *Mycoplasma* and *Ureaplasma*.

The first classification and differentiation of *M. gallisepticum* from other avian *Mycoplasma* species was done by serotyping and designated serotype A. The

species designation, *M. gallisepticum*, was made in 1960 by Edward and Kanarek (1960). Bradbury *et al*. (1993) worked with *Mycoplasma* species that had phenotypic and antigenic similarities to *M. gallisepticum*, differentiated them by molecular techniques and designated them *M. imitans*.

1.3 Avian mycoplasmosis

1.3.1 Diagnosis

M. gallisepticum is the primary causative agent of Chronic Respiratory Disease (CRD) (Papazisi *et al*., 2002), and can induce avian mycoplasmosis especially under conditions of management stresses and/or other respiratory pathogens. Ley *et al*. (2003) and Hong *et al*. (2005b) reported that infection with *M. gallisepticum* induces further opportunistic infections by *Escherichia coli* and/or viruses but most notably, *M. synoviae* can also cause infections when *M. gallisepticum* infection becomes systemic. Flocks may have serological evidence of the infection with no obvious clinical signs, especially if they encountered the infection at a younger age and have partially recovered. Male birds are said to have more pronounced signs, and the disease is more severe during winter (Ley, 2003).

Because of the expansion of poultry production and construction of large multiage production complexes in a restricted geographic area, it is becoming more and more difficult to maintain *M. gallisepticum*- and *M. synoviae*-negative flocks and therefore the application of vaccination and medication is needed to reduce the production losses and to prevent the transmission of the infection (Hong *et al*., 2005b). In recent years, a reemergence of *Mycoplasma* infection has been observed in poultry, possibly due to these practices of placing large poultry populations in small geographic areas under poor biosecurity (Liu *et al*., 2001).

1.3.2 Transmission and Infection

M. gallisepticum-CRD is transmitted vertically through infected eggs and horizontally by inhalation of contaminated dust, airborne droplets and feathers resulting in rapid disease transmission throughout the flock by subsequent close contact (Papazisi *et al*. 2002; Talha *et al.*, 2003). Vertical transmission has been successfully induced following experimental infection of susceptible chickens (Talha *et al*., 2003). Due to the inability of *M. gallisepticum* to survive long periods outside the host, carrier birds are very essential to its epidemiology. The organism has been reported to have survived in the human nasal passage for 24 hours; on straw, cotton and rubber for 2 days, on human hair for 3 days and on feathers for 2-4 days as cited by Ley (2003).

In a study by McMartin and co-workers (1987), the horizontal transmission of *M. gallisepticum* was described in four phases: phase 1, a latent phase (12-21 days) before antibodies were detected in inoculated birds; phase 2, a period (1-21 days) in which infection gradually appeared in 5-10 % of the population; phase 3 a period (7-32 days) in which 90-95 % of the remaining population developed antibodies; and the fourth phase, a terminal phase (3-19 days) in which the remainder of the population became positive. Increasing the population density increased the rate at which horizontal spread occurred. Horizontal spread has also been found to be much more effective than vertical transmission. When the organism has been successfully transmitted, infection can then proceed.

Before infection can occur, the *Mycoplasma* cell should bind to the host cell membranes. This attachment is mediated by specific interactions between *Mycoplasma* cytadhesins and their corresponding host-cell receptors. The ability of *Mycoplasma* to firmly adhere to cells initiates the process that results in host cell alterations and pathogenesis. Though pathogenic *Mycoplasma* are said to be typically non-invasive organisms that merely colonize epithelial surfaces of the

host tissues, pathogenic human Mycoplasma: *M. penetrans*, *M. pneumoniae*, *M. genitalium* and *M. fermentas* are known to be invasive.

More recently, Winner and co-workers (2000) reported that *M. gallisepticum* is the only avian *Mycoplasma* species that is invasive. Using chick embryo fibroblast (CEF) cells, Boettger and Dohms (2006) observed the same tendency, while *M. synoviae* and non-pathogenic *Mycoplasma* were reportedly non-invasive. Due to the invasiveness of *M. gallisepticum*, the mechanism of host-cell oxidative damage might be similar to that proposed for *M. pnemoniae*, as shown in Figure 1.1 below:

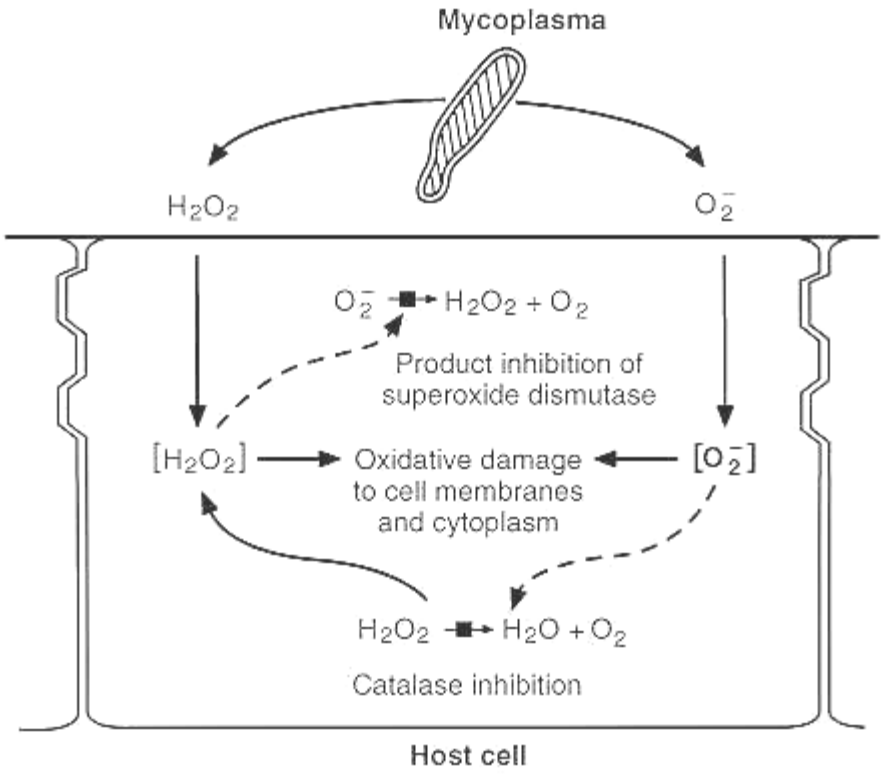

Figure 1.1: Proposed mechanism of oxidative damage to host cells by adhering *M. pneumoniae* (Razin, 1996)

Infection in chickens may superficially resemble respiratory disease caused by other pathogens such as mild strains of Newcastle disease and Avian Infectious Bronchitis, and these diseases may also be present in combination with *M. gallisepticum*. The World Organization for Animal Health (OIE) reported that *M. gallisepticum* in turkeys may be confused with Avian pneumovirus infections and the presence of sinusitis may also suggest infection with *Pasteurella multocida*, *Chlamydia* spp. or *M. synoviae*.

1.3.3 Clinical signs and Isolation

M. gallisepticum infection has a wide variety of clinical manifestations, of which CRD is the most significant in chickens and turkeys. The pathology associated with this disease is characterized by severe air sac infection where *M. gallisepticum* is the primary pathogen (Hong *et al*., 2005b; Liu *et al*.,2001). Marois *et al*., (2001) reported *M. gallisepticum* causing upper respiratory tract infections in patridges and pheasants.

M. synoviae on the other hand, following *M. gallisepticum* infection, can result in respiratory disease in chickens and turkeys, while *M. iowae* infection leads to reduced hatchability and embryo mortality in turkeys (Hong *et al*., 2005b). The infections can be asymptomatic but are mostly associated with coryza, conjunctivitis, coughing and sneezing.

Other symptoms reported are nasal exudates, tracheal rales and breathing through a partially open beak with unilateral and bilateral sinusitis occurring particularly in turkeys and game birds. In these poults, infraorbital sinuses can become so swollen that the eyelids are closed as illustrated in Figure 1.2.

Two cases of *M. gallisepticum* infection in different avian species in backyard gamebird operations investigated by Bencina *et al*. (2003) in Slovenia resulted in 20% mortality in pheasants due to severe respiratory disease but very low

pathogenesis of the organism was reported for chickens and turkeys reared at the same site. Infected finches may reveal ocular and nasal discharge and swollen eyelids in addition to conjunctivitis, while in chickens and turkeys *M. gallisepticum* can cause acute respiratory disease especially in young birds, with the turkeys showing more susceptibility. A more chronic form of the disease may occur and cause reduced egg production in breeders and layers. Kleven *et al*. (2004) also reported fowl cholera in Nebraskian turkey flocks from which *M. gallisepticum* strains K4029 and K4043 were first isolated in 1995.

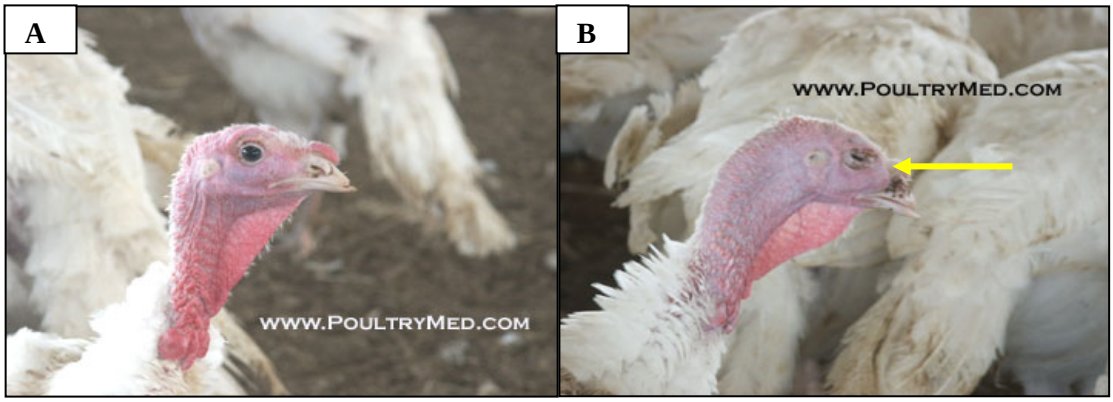

Figure 1.2: A, Healthy turkey before infection; B, Clinical signs of *Mycoplasma gallisepticum*. The arrow shows the swollen sinuses that led to closure of the eye.(www.poultrymed.com)

Samples are taken from live birds, fresh carcasses or carcasses of birds that have been frozen when fresh and should be maintained under conditions that will preserve them. Samples like tracheal and lung washes are usually maintained on ice for about 3-4 hours then stored at -20^{0}C for subsequent analysis (Papazisi *et al*., 2002).

Small pieces of tissue should be placed in *Mycoplasma* broth and swabs should be vigorously agitated in 1-2 ml of *Mycoplasma* broth and then discarded if transportation is needed. In such cases, a means of chilling should be included and dilution done because the presence of specific antibodies or antibiotics or other inhibitory substances in tissues may inhibit *Mycoplasma* growth unless they are diluted out.

1.3.3.1 Isolation from live birds

From live birds, isolates may be obtained from the sinus exudates (Ferguson *et al*., 2005) as well as conjuctival sinuses from clinically ill birds (Ley *et al*., 1997a). Sera and nasal washes are also performed prior to procedures such as vaccination (Papazisi *et al*., 2002). Bencina *et al*. (2003) reported on an *M. gallisepticum* strain that was highly pathogenic for chicken embryos isolated from the sinus of a pheasant.

When taking swab samples, isolation can be done from the choanal cleft, oropharynx, oesophagus, trachea, cloaca and phallus. Blood collection is another option for *M. gallisepticum* isolation and has been previously collected from the wing vein of chickens. Nasal washes are performed with the head of the bird held at a slightly downward angle and wash samples are collected by gravity into sterile plastic beakers (Papazisi *et al*., 2002). *M. gallisepticum* may also be present from oviducts and semen of a rooster (Ley, 2003).

1.3.3.2 Isolation from dead birds

In the case of dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea or air sacs while exudates can be aspirated from the infraorbital sinuses and joint cavities (OIE). In a study conducted by Bencina *et al*. (2003) *M. gallisepticum* strain ULB 992 that synthesizes a small amount of *M. gallisepticum* C3, a truncated form of *M. gallisepticum* C1 also lacking PvpA, was isolated from the infraorbital sinus of a dead peafowl.

1.3.3.3 Isolation from eggs

Samples are also taken from embryonated eggs, dead-in-shell embryos, chickens or poults that have broken the shell but failed to hatch. When using

embryonated eggs, samples may be taken from the inner surface of the vitelline membrane and from the oropharynx and airsacs (OIE).

1.3.4 Growth of *M. gallisepticum*

The members of the genus *Mycoplasma* are known to require very complex media for adequate growth. This is attributed to their replication, the high interdependence between the organisms and their hosts as well as the sterol requirement for the trilaminar membranes. In addition to these properties, *M. gallisepticum* is fastidious *in vitro* (Levisohn & Kleven, 2000) and tends to grow very slowly on a protein-rich medium containing 10 to 15% added animal serum and is rather resistant to certain antimicrobial agents which are frequently added in the medium to retard growth of contaminant bacteria and fungi (Hong *et al*., 2005a). Several suitable culture media have been formulated such as *Mycoplasma* agar (Avian *Mycoplasma* solid medium from *Mycoplasma* experience, Reigate, U.K.) or *Mycoplasma* broth (Avian *Mycoplasma* liquid medium from *Mycoplasma* experience) (Mekkes & Feberwee, 2005).

The medium developed by Fey *et al*. (as cited by Ley, 2003) is widely used for isolation of *M. gallisepticum* and *M. synoviae*. *Mycoplasma* medium generally contains a protein digest and a meat-infusion base supplemented with serum or a serum fraction, yeast fraction, glucose and bacterial inhibitors. *M. gallisepticum* is cultured in *Mycoplasma* broth or on agar at 37^0C in a CO_2 –rich atmosphere until the broth shows colour change or colonies appear on plates (Harasawa *et al*., 2004).

Increased humidity and tension in the atmosphere have been reported to enhance growth. Such conditions can be achieved by the inclusion of damp paper or cotton wool, and by flushing the container with 5-10% CO_2 in nitrogen, by placing a lighted candle in the container or by using a CO_2 incubator or suitable gas-generating system. The glucose in the medium is fermented and this

allows for the pH to be lowered, causing the phenol red indicator to change from red to orange/yellow thus making it possible to visually detect growth in broth tubes (Ley, 2003).

1.3.5 Serology

Serological procedures are useful for flock monitoring in *M. gallisepticum* control programs and to aid in diagnosis when infection is suspected. A positive serological test, together with history and signs typical of the *M. gallisepticum* disease, allows a presumptive diagnosis pending isolation and/or identification of the organisms (Ley, 2003). Immunological and DNA detection methods can be used to identify mycoplasmal isolates. These include the Indirect Fluorescent Antibody (IFA) and Immunoperoxidase (IP) tests, both of which are simple, sensitive, specific and rapid to perform. Growth inhibition (GI) and metabolism inhibition (MI) tests can also be done in addition to IFA and IP (OIE).

Ferraz and Danelli (2003) studied hemagglutination inhibition (HI) tests and immunoblot assays to detect antigenic differences among vaccine and wild *M. gallisepticum* strains. Immunoblot assay resulted in a Polyclonal antibody (PCA) reactivity pattern that did not vary much from that of HI. For their preparation of cellular antigen, an *M. gallisepticum* strain was firstly stored in glycerol, with lyophilized vaccine strain being maintained at 4^0C, while the other vaccine was maintained at -20^0C. All strains are then cultivated in Hayflick modified medium with 10% equine serum (Ferraz & Danelli, 2003). Western blot followed these preparations and the nitrocellulose sheet that had been used to observe the peptide bands was incubated with PCA.

Direct immunofluorescence can also be used to identify *M. gallisepticum* (Ferguson *et al*., 2004). For GI and MI tests, the OIE states that purified (cloned) cultures are required but not for the IFA and IP tests. IFA and IP are very good tools as they can detect the presence of more than one species of *Mycoplasma*,

as the colonies specific for the antiserum will react while others will not. Kleven and co-workers (2004) tested sera for antibody against *M. gallisepticum* using serum plate agglutination (SPA) and HI. Agglutination reactions were graded from 0 (negative) to 4 (strong, rapid reaction) and agglutination scores ≥1 and HI titers of ≥1:40 were considered positive.

Immunofluorescence and IP procedures for diagnosis are generally applied to suspected laboratory isolates rather than directly to infected exudates. This is because the organisms are too small to be recognized conclusively under the light microscope and because the corresponding negative and positive control exudates/tissue is unlikely to be readily available.

In the GI test, the growth of *Mycoplasma* is inhibited by specific antiserum, enabling species to be identified. It is relatively insensitive and sera must be at a high titer, monospecific and prepared in mammalian hosts as poultry sera do not inhibit *Mycoplasma* growth efficiently (OIE). Ferraz and Danelli (2003) made use of HI tests because *M. gallisepticum* is capable of haemagglutinating avian red blood cells (RBCs) and specific antibodies in sera cause inhibition.

Furthermore, a strain that grows well and haemagglutinates reliably should be selected. The HI test requires a satisfactory haemagglutinating *M. gallisepticum* antigen, washed chicken or turkey RBCs and the test sera. The antigen can be either a fresh broth culture or a concentrated washed suspension of *M. gallisepticum* in phosphate buffered saline.

In addition to the haemagglutinating antigens, other antigens of great interest are the cytadhesins. Some of these cytadhesins and heamagglutinins have been described as immunodominant and these are proteins or lipoproteins with molecular weights of 60-75 kDa (Ley, 2003).

1.4 The complete genome of *M. gallisepticum*

Due to their degenerative evolution, it is not surprising that all *Mycoplasma* species have minimal genetic information. One of the first species whose genome was completely sequenced is *M. genitalium* that is 580 kb long. More species have been sequenced since then, including *M. gallisepticum*.

Papazisi *et al*. (2003) completed the genome of *M. gallisepticum* using the R-strain. The authors were able to assign function to 469 of the 742 genes in the genome. A total of 150 genes were found to encode for conserved hypothetical proteins while 123 remain as hypothetical proteins. Due to this large number of putative proteins to which function has not been assigned, the inner functioning of this pathogen is relatively unknown.

Important proteins, the cytadhesins, are also encoded for in the genome and are known to play a fundamental role in *M. gallisepticum* adhering to the host cells as pathogenesis is initiated. There are about five or more identified cytadhesins but only three will be concentrated upon in this review. These cytadhesins show homology in all *M. gallisepticum* strains and homology to the human *Mycoplasma* pathogens has also been reported Hnatow *et al*. (1998).

Furthermore, the genome contains two copies of the rRNA genes and 33 tRNA genes that correspond to all amino acids. One set of the rRNA genes is organized as an operon, with adjacent 16S, 23S and 5S genes; and a second copy of the 16S rRNA gene lies 221 kb upstream of the 23S and 5S rRNA genes.

A single polypeptide release factor (*prfA*) was identified, consistent with the use of only UAA and UGA as stop codons. Similar to most bacteria, the origin of replication (*oriC*) is located in the region of the *dnaA* gene. Comparative analysis of the *oriC* regions of sequenced *Mycoplasma* genomes predicts putative DnaA boxes in the area surrounding the *dnaA* gene Papazisi *et al*. (2003).

1.4.1 Cytadhesion genes

1.4.1.1 Cytadhesion gene *Mgc2*

The *Mgc2* gene is 912 bp-long and encodes the 32.6 kDa cytadhesion protein Mgc2 that is clustered at the tip organelle and was as such functionally implicated in cytadhesion (Boguslavsky *et al*., 2000). Comparison of Mgc2 to the *M. pneumoniae* protein P30 and the P32 sequences of *M. genitalium* showed 40.9% and 31.4% homology, respectively, as determined by Hnatow *et al*. (1998). This is of notable importance as P30 and P32 also function as cytadhesins that aid their respective *Mycoplasma* in adhering to mucosal membranes and hence initiate infection in humans. Mgc2 has the same function in avian *Mycoplasma* species.

Hnatow *et al*. (1998) further reported that there are 30 shared proline residues at amino acid positions 73 in P30, 68 in P32 and 62 in Mgc2. In addition to this, the carboxy end of Mgc2 (aa. 185 to 304), like that of P30 and P32, has the characteristics of cytoskeletal matrix proteins and the carboxy end of the molecule is distinguished by two identical overlapping 24 amino acid sequences. The *Mgc2* gene is fairly well conserved in *M. gallisepticum* and can be used as a basic reference in molecular identification of isolates. Using suitable restriction enzymes on *Mgc2*, *M. gallisepticum* isolates can be differentiated to strain level.

1.4.1.2 Cytadhesion gene *PvpA*

The *PvpA* gene is the most recently identified cytadhesion gene to *Mgc2* and *GapA* and is similar in function to both. The cytadhesion protein encoded for by *PvpA* is designated PvpA and exhibits higher homology to the P30 and P32 proteins than Mgc2, with 54 and 52% homology, respectively (Boguslavsky *et al*., 2000). PvpA has several important features: (i) it is an integral membrane surface protein with free C-terminus, (ii) it possesses an epitope shared by three distinct

variant surface lipoproteins of the bovine pathogen *M. bovis*, (iii) it is subject to spontaneous high-frequency variation in expression, (iv) exhibits size variation among strains and (v) is not a lipoprotein. Liu *et al*. (2001) reported that structurally, PvpA is a non-lipid integral membrane protein with a surface-exposed C-terminal portion.

The surface-exposed C-terminus of PvpA has high proline content (28%) and contains identical direct repeat sequences of 52 amino acids each designated DR-1 and DR-2. The presence of proline-rich regions in the surface-exposed C-terminus domains of other pathogenic *Mycoplasma* adhesins suggests an important role of these domains in the function of PvpA as an adhesin.

1.4.1.3 Cytadhesion gene *GapA*

The *GapA* gene is 2 895 bp-long and encodes a 105 kDa protein, GapA (Goh *et al*., 1998). The *GapA* nucleotide sequence analysis by the same authors revealed that there is 45% homology to the *M. pneumoniae* P1 gene, 46% homology to the *M. genitalium MgPa* gene and 47% homology to the *M. pirum* P1-like protein gene. *GapA* has an A-T content compared to 64, 46.6 and 72 mol%, for the P1, MgPa and the P1-like protein genes, respectively.

Similar to P1 and MgPa genes, *GapA* is a central gene in a multi-gene operon, but unlike the P1 and MgPa genes, there is only a single copy of *gapA* in the genome. GapA is a trypsin-sensitive surface-exposed protein and results in about 64% inhibition of attachment assays using the anti-GapA Fab fragments. Intraspecies strain variation in the size of GapA has been observed and has molecular weights of approximately 98.1 kDa and 110 kDa in different strains.

1.5 Molecular differentiation techniques

1.5.1 Polymerase Chain Reaction (PCR)

PCR has been widely used in many differentiation studies as it gives the desired amount of DNA that is appropriate for evaluation as well as indicating complementarity of primers to template sequences. Kiss *et al*. (1997) used PCR in differentiation of avian *Mycoplasma.* In the study, primers complementary to the 16S rRNA genes were used in detection of the organisms. The general primer pair used was GPO-3: 5'-GGG AGC AAA CAG GAT TAG AAT ACC T-3' and MGSO: 5'-TGC ACC ATC TGT CAC TCT GTT AAC CTC-3' and for the detection/exclusion of *M. gallisepticum* a different primer pair with sequences: 5'-AAC ACC AGA GGC GAA GGC GAG G-3' and 5'-ACG GAT TTG CAA CTG TTT GTA TTG G-3' was used.

Garcia *et al*. (2005) modified the standard PCR, where four generic *M. gallisepticum* PCRs were compared for analytical specificity and sensitivity using tracheal swabs. The four PCRs used are the 16S rRNA PCR, three newly developed PCRs that target surface protein genes (*Mgc2, LP and gapA*, the latter two being nested). Nested PCR requires two sets of primers used in two successive PCR runs, the second set intended to amplify a secondary target within the first run product. Another modification is the RT-PCR where a known sequence from a cell or tissues' RNA is amplified, isolated or identified. It is essentially a normal PCR preceded by reverse transcription of RNA to cDNA.

For further evaluation of diagnostic specificity, Garcia *et al*. (2005) used a licensed *M. gallisepticum* DNA Test kit (IDEXX laboratories, Inc). The kit is a nonradioactive probe-based test utilizing PCR amplification for the specific detection of *M. gallisepticum* from chicken and turkey tracheal swab samples. PCR amplification procedure utilizes two primers complementary to the *M. gallisepticum* sequence. The primers are used in conjunction with AmpliTaq®

DNA polymerase to amplify small amounts of *M. gallisepticum* DNA to provide enhancement of sensitivity. The third primer, labeled with an enzyme, is used as a sequence-specific hybridization probe to provide colorimetric detection of the amplified target DNA.

1.5.2 Restriction Fragment Length Polymorphism (RFLP)

RFLP is conducted by PCR-amplified DNA that is then cut into restriction fragments using suitable endonucleases which only cut the DNA molecule where there are recognition sequences. Restriction fragments are then separated according to length by agarose gel electrophoresis. In a study by Kiss *et al*. (1997), two restriction enzymes *Bam*HI and *Rsa*I were used for the distinction of the amplified fragments of approximately 270 bp from *Mycoplasma* isolates. The enzymes were able to differentiate between three *Mycoplasma* species: *M. synoviae*, *M. iowae* and *M. meleagridis*.

In a similar study by Garcia *et al*. (1995), a region in the 16S rRNA sequence was amplified using a single set of primers in *M. gallisepticum, Mycoplasma synoviae* and *M. iowae* DNA. The primers selectively amplified a 780 bp DNA fragment in the three organisms but did not amplify other avian *Mycoplasma* or other bacteria. The PCR product was differentiated by RFLP with the restriction enzymes *Hpa*I, *Hpa*II and *Mbo*I. The authors also concluded that preliminary results from field samples suggest that this technique could be a useful and rapid diagnostic test for the detection of these three pathogenic poultry *Mycoplasma.*

Lauerman *et al*. (1995) used RFLP in differentiation of nine species of *Mycoplasma* by cleaving them with 24 restriction enzymes. Four *(Dra*I, *Mse*I, *Rsa*I, *Tsp*509I) of the 24 enzymes cut the PCR amplicon of all nine *Mycoplasma* species and thus total differentiation of the strains was achieved.

1.5.3 Randomly Amplified Polymorphic DNA (RAPD)

RAPD is a PCR assay where segments of unknown DNA are amplified. Primers with an arbitrary sequence are designed and a PCR reaction is run followed by agarose gel analysis to observe if any DNA segments were amplified in the presence of the arbitrary primers.

Ferguson *et al*. (2005) conducted a study where a total of 77 *M. gallisepticum* field isolates from the USA, Israel and Australia, as well as ten reference strains were characterized by RAPD analysis in concert with gene-targeted sequencing. Characterization was documented by constructing RAPD groups according to the relatedness of the isolates to the reference strains. Charlton *et al*. (1999) used this technique for differentiation between 7 strains of *M. gallisepticum* by screening six commercially available primers or primer combinations for their ability to differentiate vaccine and type strains, however the primers were unsuitable for strain differentiation, a drawback that is associated with this technique. To solve this, certain combinations of primers were made and the objective of the study was achieved.

In characterization of *M. gallisepticum* isolates from turkeys, Kleven *et al*. (2004) used primers that resulted in all field isolates in the study forming patterns indistinguishable from a vaccine strain 6/85 but clearly different from the other *M. gallisepticum* strains evaluated when an RAPD gel was run. This was circumvented by analyzing the isolates with RAPD primer pair OPA13: 5'-CAGCACCCAC-3' and OPA14: 5'-TCTGTGCTGG-3' which resulted in only two of the five field isolates generating patterns similar to 6/85.

1.5.4 Amplified Fragment Length Polymorphism (AFLP)

AFLP is a highly sensitive method for detecting polymorphisms in DNA and was first described in 1993. The procedure involves digestion of total cellular DNA with one or more restriction enzymes and ligation of restriction half-site specific adaptors to all restriction fragments, selective amplification of some of these fragments with two PCR primers that have corresponding adaptors and restriction site specific sequences, as well as electrophoretic separation of amplicons on a gel matrix, followed by visualization of a band pattern.

The advantage of this method, as stated by Hong *et al*. (2005a), is that it requires a relatively small amount of DNA and has great discriminatory power and reproducibility. This was reported during their study when this method was used in differentiation of 44 strains of avian *Mycoplasma* representing eight different species. Restriction enzymes *Bgl*II and *Mfe*I were used in the digestion of genomic DNA and the MFE1 primer used was modified by adding selective nucleotide A at its 3' end to increase the selectivity of the amplification reaction and to obtain better banding pattern resolution.

It was found by the authors that the AFLP analysis provided an optimal separation and a uniform sizing of the amplified fragments of between 75 and 500 bp were used in numerical and cluster analysis for species differentiation and resulting in *M. gallisepticum* showing the highest banding pattern complexity consisting of about 90 AFLP fragments while other *Mycoplasma* species showed lower AFLP banding pattern complexity.

Hong *et al*. (2005a) also used this technique to type 34 strains of *M. gallisepticum* including vaccine strain Ts-11, 6/85 and F. A total of 10 groups with 30 distinguishable AFLP typing profiles were generated and the method was able to identify and differentiate both *M. gallisepticum* field strains from recent outbreaks and those that were epidemiologically related. It was concluded in that study that

AFLP will provide assistance in identifying the sources of *Mycoplasma* infections and also be useful in the evaluation of vaccination programs since it was successful in differentiating vaccine strains from other field strains

1.5.5 Pulse-Field Gel Electrophoresis (PFGE)

PFGE allows for separation of much larger pieces of DNA than conventional agarose gel electrophoresis. In conventional gels, the current is applied in a single direction (top to bottom) but in PFGE, the direction of the current is altered at a regular interval.

A few studies have been done to evaluate and compare the effectiveness of PFGE in *Mycoplasma* differentiation to other typing techniques. One such study was done by Marois *et al*. (2001) to type 18 strains of *M. synoviae.* All strains analyzed were successfully typed by RAPD but only 89% of the strains could be successfully typed by PFGE because of DNA degradation. Although the discriminatory power of RAPD was greater than that of PFGE, the two techniques had a discrimination index superior to 0.95 which is the threshold value for interpreting typing results with confidence.

These results correlate with those reported by Marois *et al*. (2001) where the two techniques were used to compare 21 *M. gallisepticum* strains and 5 *M. imitans* strains. The discriminatory powers in this study were also superior to 0.95 with a reproducibility of 100% for both RAPD and PFGE. It is reported, however, that the drawback of RAPD was the inconsistent band intensity complicating the interpretation of patterns, while the PFGE limit was its low typeability (86%).

Results outlined in this section make it rational to choose other typing methods over PFGE as this technique almost always shows lower effectiveness for typing of strains. Also mentioned is the degradation of DNA that takes place when using PFGE, further discouraging usage of this method. However, it might be advisable

to use this method in conjunction with other typing methods to optimize differentiation.

1.6 Previously isolated field strains of *M. gallisepticum*

A large number of *M. gallisepticum* strains have been isolated from a wide variety of habitats and it is therefore impossible to elaborate on all strains in this review. Known strains are mostly used as references during differentiation of field strains. MG-ATCC 15302, MG-A5969 and MG-PG31 were used by Marois *et al*. (2001) as reference strains in a study to differentiate between *M. gallisepticum* and *M. imitans* strains. The origins of these strains are a turkey brain (1958), chicken trachea (year not documented) and chicken (1960), respectively.

Other strains that are commonly isolated are K4029 and K4043 that were first recovered in 1995 from two commercial turkey flocks in Nebraska, K4421A from Michigan in 1997 in turkeys, K4465 isolated in 1997 from turkeys in Ohio and K4236 that was isolated in 1997 from commercial turkeys in Virginia (Kleven *et al*., 2004).

In a study by Hong *et al*. (2005b), 34 *M. gallisepticum* field strains were used from which a dendogram based on the *Mgc2* and Random Amplified Polymorphic DNA (RAPD) analysis typing was constructed**.** Using *Mgc2* to classify strains, strains were placed into 8 groups while the RAPD typing results placed strains into 11 groups.

Some field strains may differ very slightly by serology or other aspects, such as the subtypes of *M. gallisepticum*-K4997, which include subtypes starting from K4997-S1 through K4997-S10 (up to the latest isolated subtype). Strain K4997-S10 is a subculture of the vaccine strain 6/85 and K3944 is an isolate from 6/85-vaccinated turkeys.

Hong *et al*. (2005a) made a very important observation that strains isolated from the same farm are usually typed into the same groups, like the chicken strains K4997-S6 and K4997-S5, turkey strains K4997-S7 and K4997-S8, each pair isolated from a different farm in North Carolina in 2000. Another strain, MG-S6, was used successfully as a control in an experiment by Ferraz and Danelli (2003) in differentiation of vaccine strains Ts-11 and 6/85.

1.7 Control and eradication of *M. gallisepticum* infections

1.7.1 Vaccination

Interest in *M. gallisepticum* vaccines originated in the late 1970s as it became apparent that *M. gallisepticum* infection was endemic in some multiple-age, egg-laying complexes (Ley, 2003). Nowadays, vaccination is widely used in controlling *M. gallisepticum* infections in poultry. Vaccines are considered safe if they are not virulent to the host to which they are administered, as this could be a way of initiating an infection and a possible outbreak.

A disadvantage of currently available *M. gallisepticum* vaccines is that there is no convenient serological technique to accurately distinguish between vaccinated and naturally infected flocks (Ferraz & Danelli, 2003). It has been shown however, that vaccinated birds are immune to respiratory disease, airsacculitis and drops in egg production caused by *M. gallisepticum* infections and that vaccination also results in reduced levels of egg transmissibility of the organism in breeders. Both live and inactivated vaccines are used in the vaccination programs (OIE).

1.7.1.1 Inactivated vaccines

M. gallisepticum bacterins (killed organisms) are mostly used to protect pullets from infection. Bacterins are combined with an oil-emulsion adjuvant and are

administered parenterally to pullets at 12-16 weeks of age, usually subcutaneously in the neck. This suspension protected young chickens from intrasinus challenge with virulent *M. gallisepticum* and commercial egg layers from *M. gallisepticum*-induced drops in egg production (Ley, 2003). Other reports include protection of broilers and layers, from airsacculitis and reduced egg production, respectively, as cited by Ley (2003).

Vaccination with bacterins has been shown to reduce, but not eliminate colonization by *M. gallisepticum* in challenge experiments. Bacterins have been reported to be non-advisable in long-term control of infection on multiple-age production sites. Furthermore, bacterins are disadvantaged by a need for two doses for optimal protection, as well as the cost of individual bird administration. To enhance the performance of bacterins, various adjuvants and antigen delivery systems, including liposomes and iota carrageenan have been investigated. The OIE has reported that bacterins cannot prevent infection from wild-type *M. gallisepticum.* Bacterins are currently commercially produced and available.

1.7.1.2 Live vaccines

The most important live vaccines are MG-F, Ts-11 and 6/85.

1.7.1.2.1 The MG –F vaccine strain

The MG-F strain was first isolated by Yamamoto and Adler, as cited by Ley (2003). The authors first described this strain as a typical pathogenic strain. The strain has become the most utilized vaccine in preventing the colonization by most virulent *M. gallisepticum* strains (Ferraz & Danelli, 2003). It was further reported that the F strain is naturally occurring and has mild to moderate virulence in chickens but is virulent in turkeys.

The strain spreads slowly from bird to bird and little or no respiratory reaction is observed when the strain is administered to healthy chickens via the upper respiratory tract, but respiratory signs and airsacculitis may result when administered by aerosol or in the presence of other respiratory disease or other respiratory disease agents such as Newcastle disease or Infectious Bronchitis Virus. The most significant advantage of the F-strain is that vaccinated chickens are permanent carriers and therefore a single dose is adequate.

The F strain has been used extensively in multiple-age laying complexes to reduce *M. gallisepticum*-caused egg production losses. In broilers, vaccination with this strain provided some protection from airsacculitis following aerosol challenge with the virulent R strain. The biological protection by the F strain was found not to involve competition for adherence sites or blockage by prior colonization and vaccination with the F strain did not prevent colonization by the challenge strain of *M. gallisepticum*. Vaccinated pullets simply maintain the organism in the upper respiratory tract for as long as they live. The F strain can thence displace the virulent strain until no virulent strains can be detected (Ley, 2003). This vaccine strain is currently not registered for use in South Africa.

1.7.1.2.2 The Ts-11 and 6/85 vaccine strains

Ts-11 and 6/85 are commercially available as attenuated vaccines and were found to be poorly transmitted from vaccinated to unvaccinated birds. The 6/85 strain originated in the U.S.A and vaccine characteristics were described by Evans and Hafez (1992), while the development and characterization of the Australia-originated Ts-11 strain was described by Whithear (1990).

In a study by Ferraz & Danelli (2003), the two vaccines showed little or no virulence to both chickens and turkeys. Transmission can occur very poorly if birds are in very close contact, thus it is safe to say that these vaccines are safer than the F-strain. Ley *et al*. (1997b) discovered that Ts-11 could be detected by

serology via detection of antibodies in vaccinated flocks while 6/85 could not be detected using this technique.

The Ts-11 strain could not be differentiated from the F-strain until Ferraz and Danelli (2003) developed polyclonal antibodies to the *M. gallisepticum* antigens produced in chickens used in serological tests to determine antigenic variability. The most evident characteristic observed was the specific response of the vaccine-type F polyclonal antiserum to the 75 kDa peptide band of the homologous strain. It was also reported that this 75 kDa peptide is not present in the Ts-11 strain but present in the F-strain as seen in Figure 1.3 below. These results therefore show that this homology can be a drawback to the investigator in identifying a vaccine strain.

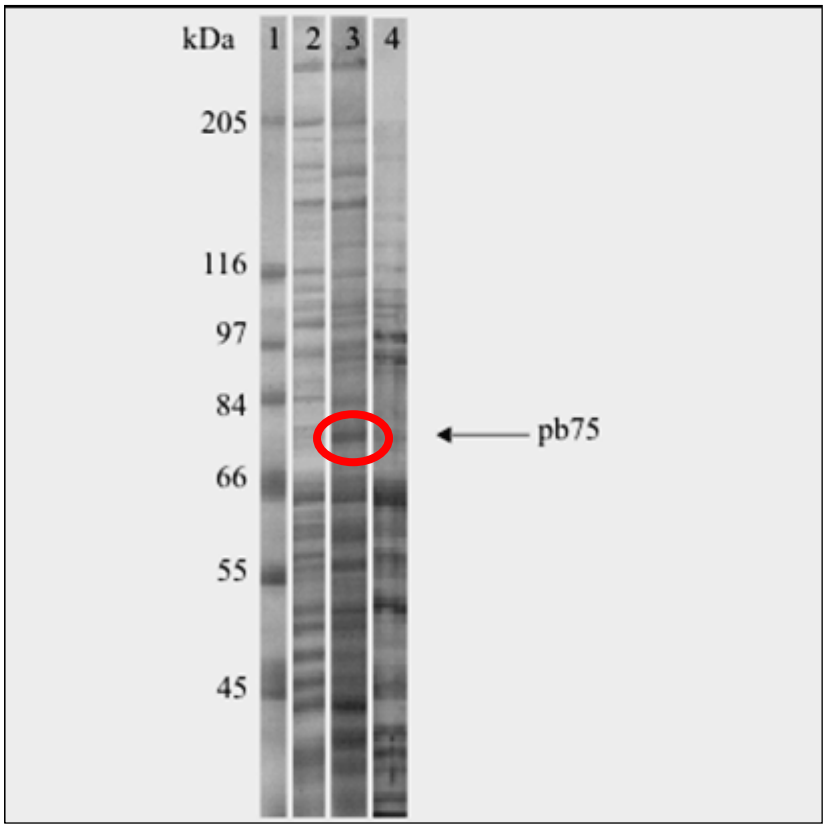

Figure 1.3: SDS-PAGE of proteins from *Mycoplasma gallisepticum* strains. Lane1,molecular mass standards (Sigma); Lane 2, MG Ts11/Mycovax Ts-11 vaccine, Rhodia-Mericux®; Lane 3, MG-F/MG-F vaccine, Coopers®; Lane 4, MG-S6 (208)/

virulent strain. The 75 kDa peptide band is marked in the figure as pb 75 and indicated by an arrow. (Ferraz & Danelli, 2003).

One of the most desirable properties of a vaccine is the ability to displace virulent wild-type strains. Such properties have been fully confirmed in the F strain. However, eradication of the live F-strain (Turner & Kleven, 1998) was achieved in 1994 when the Ts-11 strain was introduced in a commercial layer farm that had MG-F positive flocks. *M. gallisepticum* was isolated from Ts-11 vaccinated flocks but no *M. gallisepticum* could be isolated when vaccination was discontinued and also no *M. gallisepticum* was detected in non-vaccinated birds. After the last vaccinated flock was marketed, no *M. gallisepticum* was detected on the farm thus indicating a potential use of Ts-11, after the MG-F strain, for eradication of *M. gallisepticum.*

In other investigations, vaccination with Ts-11 protected broiler breeders and their embryos and also allowed better production performance. The vaccine is administered by the eye-drop route as a single dose to growing pullets 9 weeks of age or older, at least 3 weeks before expected exposure to field challenge. In vaccinated flocks, the Ts-11 vaccine strain persists for the life of the flock in the upper respiratory tract and induces long-lived immunity (Ley, 2003).

The 6/85 vaccine is administered by spray and results in little or no detectable serologic response. The vaccine is detectable in the upper respiratory tract for 4-8 weeks after vaccination. This vaccine is formulated as a freeze-dried pellet and administered as a single dose to pullets 6 weeks of age or older. The vaccine has been reported as safe for chickens and has not occurred in unvaccinated flocks.

1.7.1.2.3 Possible *M. gallisepticum* vaccines

The K5054 strain of *M. gallisepticum* was isolated from the sinus exudate of a commercial turkey flock. The flock did not exhibit the typical clinical signs of *M. gallisepticum* infection (Ferguson *et al*., 2004). To test the efficacy of K5054, the

authors used Ts-11 as a positive control while the virulent R strain was used to challenge chickens and turkeys used in the study. It was observed from the study that infection with K5054 and Ts-11 did not result in significant clinical signs or lesions indicative of *M. gallisepticum* disease. It could then be concluded that these strains are safe vaccines. The K5054 strain was consistently re-isolated from the trachea of vaccinated poults and this was indicative of the colonization and persistence of the K5054 strain in the upper respiratory tract.

Papazisi *et al*. (2002) assessed the efficacy of a modified live *M. gallisepticum* vaccine designated GT5 for the protection of chickens against infection and respiratory disease. This vaccine was constructed by the reconstitution of the avirulent high passage R (R_{high}) strain with the gene encoding GapA, a cytadhesin. The organism expressed GapA on its surface, retaining the phenotypic characteristics of the parental R_{high} strain. Birds vaccinated with GT5 were protected upon challenge with the virulent low passage R (R_{low}) strain as evidenced by the amounts of IgG. Compiled results suggested that the tracheal IgG elicited by GT5 vaccination may have been responsible for blocking the initial colonization of R_{low}, thereby resulting in protection.

1.7.2 Antibiotics

M. gallisepticum has shown sensitivity *in vitro* and *in vivo* to several antibiotics that act by inhibiting the metabolism of organisms, including macrolides, tetracyclines, fluoroquinoles and others; but is resistant to penicillin and other antibiotics which act by inhibiting cell wall biosynthesis due to their lack of cell walls. It is further reported that *M. gallisepticum* may develop resistance and demonstrate cross-resistance to commonly used antibiotics (Ley, 2003).

A recent study was done by Nascimento *et al*. (2005a) to evaluate the eradication of *M. gallisepticum* and *M. synoviae* using antimicrobial drugs. Procedures included the injection of antibiotics into eggs (via the small end and the air sac

cell), infected chicken as well as day-old chicks. A total of 3 464 hens of a genetic stock of white egg fowls (leghorn) were used with age ranging from 45 to 53 weeks old and a laying production ranging from 37 to 48%.

For the treatment of infected eggs, tylosin and gentamicin sulfate were diluted in 85% saline and inoculated into each egg. Additionally, each day-old chick received a subcutaneous injection containing 0.2 mL of a Linco-Spectin solution that also contains 0.05 mL vitamin complex in its composition. It was concluded after observation of the results that the antimicrobial egg treatment scheme applied with biosecurity measures was sufficient for achieving *M. gallisepticum* and *M. synoviae*-free chickens with only one application.

Drugs of choice, tylosin and gentamicin, were used because of proven efficacy against *Mycoplasma* and broad-spectrum activity against bacteria respectively, the latter also showing low toxicity to host cells. However, tylosin can be toxic for embryos at higher doses and this results in drops in the hatchability (Nascimento *et al*., 2005a).

1.8 Closely related *Mycoplasma*

1.8.1 *Mycoplasma imitans*

M. imitans type strain 4229T was isolated in France from the turbinate of a duck, with airsucculitis and peritonitis (Abdul-Wahab *et al*., 1996), and was tentatively identified as *M. gallisepticum* by immunofluorescence and growth inhibition tests (Harasawa *et al*., 2004). Using *M. gallisepticum* as a reference strain, further biochemical and molecular analyses including SDS-PAGE profiles of cellular proteins, restriction enzymes cleavage patterns of genomic DNAs, the G+C contents, and Southern Blot hybridization using rRNA and *tuf* gene probes supported the observation that *M. imitans* strains were similar to one another but different from the strains of *M. gallisepticum.*

Comparison of the morphological, cultural and physical properties by Bradbury *et al.* (1993) between the type strain MI-4229T and MI-B2/85 demonstrated the similarity between these organisms by performing a restriction enzyme analysis of their genomic DNA. It was confirmed from the results obtained that both strains had phenotypic properties very similar to *M. gallisepticum* including the presence of an attachment organelle. Therefore, in essence, the "imitans" in *M. imitans* meaning "imitate", is derived from observations that this organism is very similar to *M. gallisepticum* in many ways.

Markham *et al*. (1999) reported that of the other *Mycoplasma* in the pnuemoniae group, *M. gallisepticum* is closest related to *M. imitans* according to the 16S rRNA gene analysis. Further results correlated to this, as the oligo 1 probe which was complementary to the GAA repeat sequence associated with pMGA genes in *M. gallisepticum* bound multiple bands in Southern blots of *M. gallisepticum* and *M. imitans* genomic DNA digested with *Eco*RI or *Bgl*II, but did not bind to genomic fragments of the other *Mycoplasma* species in the pneumoniae-group. Using antigenic analysis, rabbit anti-pMGA antisera reacted with a band of approximately 67 kDa in Western blot of *M. gallisepticum* proteins and with a single band of approximately 35 kDa in *M. imitans* but not with any proteins of other *Mycoplasma* species (Markham *et al*., 1999).

M. imitans causes ciliostasis in chicken and duck tracheal organ cultures and has an adherence structure similar to that present in *M. gallisepticum*. The organism produces respiratory disease similar but somewhat milder than *M. gallisepticum* in red-legged patridges. *M. imitans* does not produce signs or lesions when inoculated into chickens, but in a dual infection with Infectious Bronchitis virus, a synergistic effect was observed (Ley, 2003). The threat underlying *M. imitans* identification is the possible misidentification of isolates as *M. gallisepticum* and possible serologic cross-reactions in testing of field flocks.

1.8.2 Other avian *Mycoplasma* pathogens

The importance of gathering information of other related *Mycoplasma* species is due to the fact that these related species may, in one way or another be detected from the field. Knowing the background information of these species may aid in identification and species differentiation. Detection of these species could be when e.g.: universal *Mycoplasma* primers are used in a PCR assay. The other importance is due to the similarity of symptoms and clinical signs in the host that may be misinterpreted as those of *M. gallisepticum*. A few of these avian *Mycoplasma* are included in this review:

1.8.2.1 *Mycoplasma meleagridis*

M. meleagridis is a specific pathogen of turkeys. The organism causes egg-transmitted disease in which the primary lesion is an airsacculitis in the progeny. Other manifestations include decreased hatchability, skeletal abnormalities and poor growth performance. Broth cultures of *M. meleagridis* are similar to those of *M. gallisepticum*.

Ultrastructure studies of the cells showed that *M. meleagridis* does not have a tip-organelle typical of *M. gallisepticum* but has thicker fibrils in the central nuclear area. In both species, ribosomes are distributed in uniform rings around the cell peripheries. *M. meleagridis* is facultatively anaerobic with optimal growth at 37-38 °C and slight growth at 40-42 °C. With regards to antigenicity, *M. meleagridis* is unrelated to all other avian *Mycoplasma* species (Chin *et al*., 2003).

1.8.2.2 *Mycoplasma synoviae*

M. synoviae infection most frequently occurs as subclinical upper respiratory infection that leads to infectious synovitis. It may cause air sac lesions when

combined with Newcastle disease (ND), Infectious bronchitis (IB), or both. Nicotinamide adenine dinucleotide (NAD) is required for growth.

Cells appear as pleomorphic coccoid bodies of approximately 0.2 μm in diameter. The optimum temperature for growth is 37 °C and growth is best observed on agar if the plates are enclosed in an air-tight container (Kleven, 2003).

1.8.2.3 *Mycoplasma iowae*

M. iowae has been associated with reduced hatchability and embryo death in turkeys. The organism has also been shown to induce mortality in turkey and chicken embryos. Mild to moderate airsacculitis and leg abnormalities in chickens and turkeys have also been observed. Poult hatchability can be reduced by 2-5 %. Growth requirements are similar to those of other *Mycoplasma* but most importantly, an attachment organelle (as in *M. gallisepticum*) has been reported (Bradbury and Kleven, 2003).

1.8.2.4 *Mycoplasma gallinarum* and *Mycoplasma gallinaceum*

These species have not been considered to be pathogenic, however isolation has been possible from infected poults. These organisms are usually contaminants when isolation of *M. gallisepticum* or *M. synoviae* is attempted. *M. gallinarum* is primarily isolated from chickens but it may also be found in turkeys (Hong *et al*., 2005a; Kleven, 2003).

1.9 Avian *Ureaplasma*

Ureaplasmas fall in the same class as *Mycoplasma* but differ from *Mycoplasma* primarily in their ability to hydrolyze urea. Very little is known about their

pathogenicity. Artificial challenge of chickens produced no clinical signs or macroscopic lesions. However, turkeys and chickens challenged with a turkey *Ureaplasma* developed fibrinous airsacculitis and serological responses. Ureaplasmas have further been isolated from turkeys that were experiencing problems with reduced fertility (Kleven, 2003).

1.10 Conclusions

M. gallisepticum continues to be a major problem in the poultry industry. Flock monitoring is essential in detection and quantification of infection. Though useful and rapid, serological techniques are limited in their ability to classify infection as well as to establish if the flock is vaccinated or infected with the wild-type strain. An added advantage of serological methods is that many of these techniques can be performed at the site of infection by designated employees. Examples include commercially available antigens.

Molecular techniques have been repeatedly shown to be more useful than serological techniques in strain detection and differentiation. The drawbacks with molecular techniques, however, are the costs involved as well as the expertise that the investigator must have.

Vaccination has been proven to be the best method in controlling *M. gallisepticum* infection by increasing immunity of the poults against infection. The F-strain, which is currently not registered for use in South Africa, is said to be the most efficient vaccine strain for protection. The Ts-11 strain is also an efficient vaccine strain but should be maintained specifically under stipulated temperature due to its sensitivity to high temperature. The third most widely used strain is the MG-6/85. This strain is not as effective as the MG-F and MG-Ts-11 strains and does not provide adequate protection.

Furthermore, vaccination is a good alternative to antibiotic administration. Besides the toxicity of antibiotics, it is a known fact that microbes can develop resistance to antibiotics. This further leads to very limited number of antibiotics left that can be used and there is additional pressure to produce new antibiotics. The problem becomes continuous when microbes acquire resistance to these newly synthesized antibiotics.

Other studies were conducted to investigate differences between vaccine strains themselves. A band-difference found in the SDS-PAGE profiles of the MG-Ts-11 and the MG-F strains could be the cause of the antigenic variability between these strains. The band is present in the F strain but not in the Ts-11 strain, which can be hypothesized as responsible for the pathogenicity of the F-strain in turkeys but not in chickens.

During the process of pathogenesis, cytadhesins play an important role as mediators. In addition to this, they can also be used in both species and strain differentiation. These proteins display high homology in *M. gallisepticum* as well as some homology to cytadhesins of other *Mycoplasma*, including human pathogenic *Mycoplasma*. To date, no *M. gallisepticum* infection has been reported in humans. There is however, possibility of such a scenario due to the homology of the cytadhesins as well as the fact that like human *Mycoplasma*, *M. gallisepticum* is invasive to the target host cells.

Previous studies that evaluated differentiation have demonstrated that isolates from the same farms are usually the same strains, if not closely related. Isolated farms in the same geographical area will also have different isolates though with similar characteristics. This is an indication that, the biological properties of organisms can be altered by their environmental conditions and can therefore conform to the same requirements.

M. imitans is the most closely related avian *Mycoplasma* to *M. gallisepticum* and has, in many cases, been misidentified as *M. gallisepticum*. These organisms are serologically cross-reactive and therefore even harder to differentiate. In addition to the high serological relatedness, molecular relatedness is also significant. Findings are always more accurate if a system has been developed to fully differentiate between *M. gallisepticum* and *M. imitans*.

CHAPTER 2

INTRODUCTION INTO THE PRESENT STUDY.

The literature has always indicated that *Mycoplasma gallisepticum* is a continuous problem in the poultry industry, leading to economic losses worldwide (Ferguson *et al*., 2004). An *M. gallisepticum*-infected chicken lays 15.7 less eggs than a healthy one (Nascimento *et al*., 2005b). These reductions led to the loss of 127 millions eggs in the USA in 1984. A similar survey was conducted in Brazil ten years later and it was found that respiratory disease caused by *M. gallisepticum* had led to a loss of 34 thousand tons of broilers.

Surveys have continued to be conducted and characterization of the organism done in different geographical areas. No such information has been documented for South African incidences; therefore the diversity of the organism is not known. Work done by Hong *et al*. (2005a) indicated that *M. gallisepticum* isolates differed from farm to farm in the same geographical area. South Africa represents a different geographical area on its own, therefore giving scope for the diversity of the organism among strains in the country and also different from those from other countries.

Serological tests performed in Southern African farms have been found to give positive results and in some cases classical *M. gallisepticum* symptoms are observed. Various tests performed on isolates have indicated that there is a different type of *M. gallisepticum* in South Africa when compared to standards set-up according to previously isolated *M. gallisepticum*. Furthermore, it is important to note that it is not clearly known if the vaccine strain MG-F, reportedly the most effective and currently not registered for use in South Africa, is indeed absent in the field. The strain is naturally-occurring in the USA and can sustain viability in the host.

The stated lack of information on the different aspects of *M. gallisepticum* led to the objectives of this study which were to firstly investigate the presence of this organism in Southern Africa. Detection would be evaluated using PCR assays that would be optimized so as to ensure adequate detection. Using the technique, differentiation of the isolates would begin at this level by construction of *M. gallisepticum*-specific assays as well as an assay that can also detect closely related species.

The second objective of the study was to accurately identify the detected isolates as well as to establish their origin. This would then establish whether the strains are endemic to Southern Africa or not. RFLP patterns of these strains were examined and therefore gave an indication of the diversity among these strains. Furthermore, it was investigated whether the isolates were *M. gallisepticum* or *M. imitans*. The clinical history of the flocks from which the strains were isolated was compared to the findings of this study and a correlation was investigated.

The final objective of the study focused on constructing a phylogenic tree as this would give an indication of whether isolates from the same area were indeed closer in lineage than to those isolated from a different geographical area. If other organisms, other than *M. gallisepticum*, were isolated using the same parameters, the tree would indicate the relatedness of such organisms to *M. gallisepticum*.

CHAPTER 3

PCR detection and RFLP analyses of *M. gallisepticum* isolates from poultry productions in Southern Africa.

3.1 Introduction

Reference to Papazisi *et al*. (2003) was made in the first chapter. The authors completed sequencing of the genome of *M. gallisepticum*. It was also mentioned that some genes are conserved while others are not. Such information provides an added advantage during differentiation, particularly when the PCR assay is being investigated.

Conserved genes are usually targeted when members of a certain genus are to be detected. This may also be limited to only closely related organisms. Species-specific genes are targeted when only a specific species of organism is investigated. In most cases the rRNA genes are targeted when a broad variety of related organisms is investigated.

In *M. gallisepticum* investigations, it is advisable to target cytadhesins in increasing specificity. Though homologous in *Mycoplasma* species, cytadhesins are quite species-specific. Nucleotides sequences of chosen genes are obtained from Genbank and primers complementary to that sequence are designed.

Though an effective technique, PCR can have certain limitations. This was observed by Mekkes and Feberwee (2005) when using real-time PCR. It was found that though differentiation was achieved between *M. gallisepticum* and other avian *Mycoplasma* such as *M. synoviae* and *M. meleagridis*, *M. imitans* could not be distinguished from *M. gallisepticum*.

Harasawa and co-workers (2004) identified an insertion sequence which is present in the intergenic transcribed spacer (ITS) region of *M. imitans*. This was confirmed by utilizing a PCR assay which further indicated the size-difference of this sequence in the ITS region of *M. gallisepticum* when compared to that of *M. imitans*. When products were being viewed on an agarose gel, the size-difference played an important role in discrimination of *M. imitans* from *M. gallisepticum*. Though a small number of isolates was used in this study, the consistency of the results obtained reassures the reliability of the assay.

Following PCR assays, RFLP profiles can further confirm differences in isolates. Restriction maps drawn for different isolates may produce differences based on which enzymes do recognize their designated restriction sites. This can be used to explain the fact that PCR products from different isolates may be subjected to the same enzymes from which products can be used to confirm the presence or absence of homology.

3.2 Objectives

This section was aimed at the development of a sensitive PCR assay that could be used to detect *M. gallisepticum* isolates at the 16S rRNA gene as well as to target an *M. gallisepticum*-specific gene in attempt to differentiate this organism from related organisms. An additional aim was to target an *M. imitans*-specific gene so as to establish the presence of this organism that is extremely similar to and often hard to distinguish from *M. gallisepticum*.

3.3 Materials and methods

The materials used were obtained from **Southern Cross Biotechnology (PTY) LTD.:** QIAamp® DNA mini kit, SuperTherm DNA polymerase (Taq); **Separations:** Agarose D-1 Low EEO; **Whitehead Scientific (PTY) LTD:** Low Melt Agarose # L-M Seive; **Biolabs:** PCR ThermoPol Buffer; **Fermentas:**

O'GeneRuler™ 100 bp DNA ladder mix, O'GeneRuler™ Loading dye solution, O'GeneRuler™ 50 bp DNA ladder, *Mva*I 2691 (*Bsm*I), *Pst*I, Buffer R; **Amersham Biosciences**: GFX™ PCR DNA and Gel band Purification Kit; **Merial South Africa:** Live attenuated *Mycoplasma gallisepticum* Ts-11 vaccine; **Inqaba Biotech:** All syntheses of primers.

3.3.1 Collection of samples

Samples were collected from serologically *M. gallisepticum*-positive flocks from various farms in South Africa and Zimbabwe. Samples were collected in the form of swabs from the trachea of positive flocks.

3.3.2 Extraction of DNA

DNA was extracted following the Qiagen QIAamp® DNA Mini Kit protocol. Elution of DNA was carried out at room temperature using the elution buffer provided in the kit. Extracted DNA was frozen and stored at -20 ^{0}C until needed.

3.3.3 Positive control

The MG-Ts-11 strain was used as the positive control. Cells were obtained from a commercial vaccine solution and genomic DNA was extracted following the Qiagen QIAamp® DNA Mini Kit protocol. Elution of DNA was carried out at room temperature using the elution buffer provided in the kit. Extracted DNA was frozen and stored at -20 ^{0}C until needed.

3.3.4 PCR assays

PCR assays were carried out based on the genes targeted and melting temperatures (Tm) of the primers designed.

3.3.4.1 Amplification of the selected genes

3.3.4.1.1 Amplification of the 16S rRNA gene

Primers complementary to the 16S rRNA gene of *M. gallisepticum* were designed and designated LMGF and LMGR as shown in Table 3.1 below. These primers were designed to detect both *M. gallisepticum* and very closely related *Mycoplasma* particularly *M. imitans*, as it is the closest in relation to *M. gallisepticum.* Figure 3.1 indicates the expected product of *M. gallisepticum* and its homology to *M. imitans* which is also expected to be detected. The arrows indicate the positions of the primers. All alignments were done at: , unless stated otherwise.

```
MG              TTTTCTGAGAGTTTGATCCTGGCTCAGGATTAACGCTGGCGGCATGCCTAATACATGCAA 60
MI              ----------------------------GATTAACGCTGGCGGCATGCCTAATACATGCAA 33
                                            **********************************

MG              GTCGATCGGATGTAGCAATACATTAGAGGCGAACGGGTGAGTAACACGTATCCAATCTGC 120
MI              GTCGATCGGATGTAGCAATACATTAGAGGCGAACGGGTGAGTAACACGTATCCAATCTGC 93
                ************************************************************

MG              CTTATAGTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGCATAACAAGTTAACTATC 180
MI              CTTATAGTGGGGGATAACTAGTCGAAAGATTAGCTAATACCGCATAACAAGTTACCTATC 153
                ********************************************************* *****

MG              GCATGAGAATAACTTTAAAGAAGCAACTGCTTCGCTATAAGATGAGGGTGCGGCATATCA 240
MI              GCATGAGAATAACTTTAAAGAAGCAACTGCTTCGCTATAAGATGAGGGTGCGGCATATCA 213
                ************************************************************

MG              GCTAGTTGGTGAGGATAATGGCCCACCAAGGCGATGACGTGTAGTTATGCTGAGAGGTAG 300
MI              GCTAGTTGGTGAGGGTAATGGCCCACCAAGGCGATGACGTGTAGTTATGCTGAGAGGTAG 273
                ************** ************************************************

MG              AATAACCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAAT 360
MI              AATAACCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAAT 333
                ************************************************************

MG              TTTTCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAACGATGAAGGTCTTTTTA 420
MI              TTTTCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAACGATGAAGGTCTTTTTA 393
                ************************************************************

MG              GATTGTAAAGTTCTTTTATTTGGGAAGAACAGTTAATAGAGTGGAAAGCTATTAATTTGA 480
MI              GATTGTAAAGTTCTTTTATTTGGGAAGAACAGTTAGTAGAGTGGAAAGCTACTAATTTGA 453
                ********************************** *************** ********
                                                   ---------------->

MG              CTGTACCATTTGAATAAGTAACGACTAACTATGTGCCAGCAGTCGCGGTAATACATAGGT 540
MI              CTGTACCATTTGAATAAGTAACGACTAACTATGTGCCAGCAGTCGCGGTAATACATAGGT 513
                ************************************************************

MG              TGCAAGCGTTATCCGGATTTATTGGGCGTAAAACAAGCGCAGGCGGATTAGAAAGTCTGG 600
MI              TGCAAGCGTTATCCGGATTTATTGGGCGTAAAACAAGCGCAGGCGGATTAGAAAGTCTGG 573
                ************************************************************
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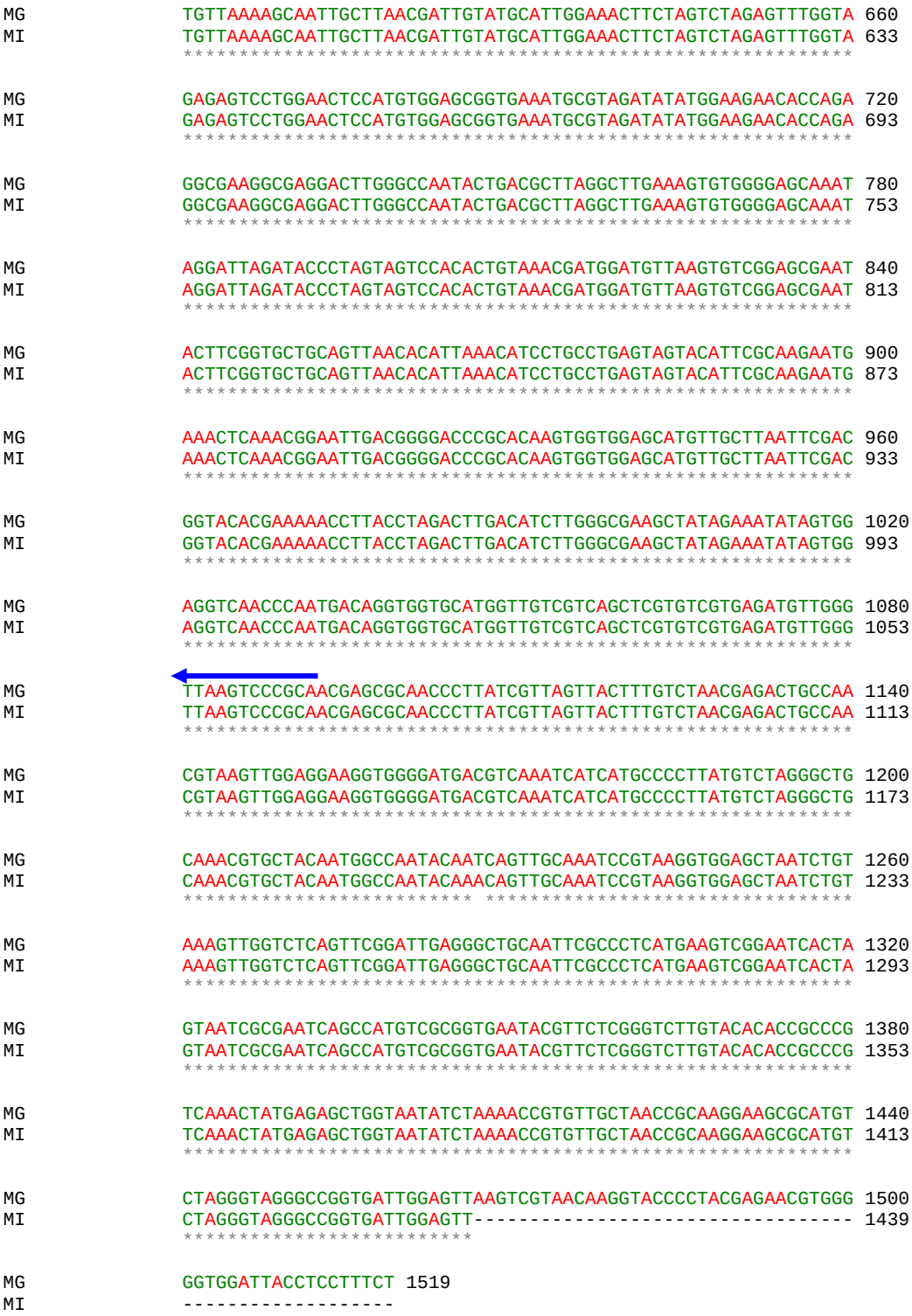

Figure 3.1: Alignment of the *M. gallisepticum* and *M. imitans* 16S rRNA gene. The arrows indicate the positions of the forward and reverse primers.

3.3.4.1.2 Amplification of the *Mgc2* gene

Primers complementary to the *Mgc2* gene of *M. gallisepticum* were designed and designated LMMF and LMMR as shown in Table 3.1 below. Figure 3.2 is an illustration of the alignment of the *Mgc2* gene sequences in 10 reference strains of *M. gallisepticum*.

3.3.4.1.3 Amplification of a gene encoding an *M. imitans* hypothetical protein.

Primers complementary to the *M. imitans* ATPase (soj) hypothetical protein and DnaA (dnaA) genes were designed and designated LMPF and LMPG as shown in Table 3.1 below. One set of primers was designed to target the sequences of both these genes.

Table 3.1: Primer sequences with their respective target genes, approximate amplicons sizes and melting temperatures (Tm).

Primer	Sequence	Target gene	Amplicon size (bp)	Tm (^{0}C)
LMGF	5'-TGCCAGCAGTCGCGGTAATACATA-3'	16S rRNA	579	64.57
LMGR	5'-TGCGGGACTTAACCCAACATCTCA-3'			64.57
LMMF	5'-TTTTACCCAGTAGTGGGTGCAG-3'	*Mgc2*	615	62.67
LMMR	5'-GTGATTAAACCCACCTCCAGC-3'			62.57
LMPF	5'-GTGCCGATTTGGATGACGAAGTTG-3'	ATPase/dnaA	292	64.57
LMPR	5'-GGTTCTTGCCCTATTATTTCAAGACTACTG-3'			64.63

```
k730 mgc2   63  GATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACC 124
f mgc2      63  GATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACC 124
k703 mgc2   63  GATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACC 124
k503 mgc2   63  GATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAACACC 124

hf51 mgc2   125 AAAAGATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATTGAGCCA 186
r mgc2      125 AAAAGATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATTGAGCCA 186
s6 mgc2     125 AAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATTGAGCCA 186
a5969 mgc2  125 AAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATTGAGCCA 186
ts 11 mgc2  125 AAAAGATGGTTGAATCCCTTGGAATAATCGAAGAACAAAATAAAACAGAAGCGATTGAGCCA 186
685 mgc2    125 AAAAGATGGTTGAATCCCTTGGAATAATCGAACAACAAAATAAAACAGAAGCGATTGAATCA 186
k730 mgc2   125 AAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAAACAGAAGCGATTGAGTCA 186
f mgc2      125 AAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAAACAGAAGCGATTGAGTCA 186
k703 mgc2   125 AAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAATCAGAAGCGATTGAGTCA 186
k503 mgc2   125 AAAAGATGGTTGAATCCCTTGGAATAATTGAACAACAAAATAAAACAGAAGCGATTGAGTCA 186

hf51 mgc2   187 ACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 248
r mgc2      187 ACTG---------------AAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 233
s6 mgc2     187 ACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 248
a5969 mgc2  187 ACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 248
ts 11 mgc2  187 ACTACATCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 248
685 mgc2    187 ACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCTGGTGTTAA 248
k730 mgc2   187 ACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCTGATGTAAA 248
f mgc2      187 ACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCTGATGTAAA 248
k703 mgc2   187 ACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCTGATGTAAA 248
k503 mgc2   187 ACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCTGATGTAAA 248

hf51 mgc2   249 TGTAGCTAATAACCCTCAGATAGGGATCAATCAACCAGGATTTAATCAACCTCAGATTAATC 310
r mgc2      234 TGTAGCTAATAACCCTCAGATGGGGATCAATCAACC--------------TCAGATTAATC 280
s6 mgc2     249 TGTAGCTAATAACCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATTAATC 310
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ts 11 mgc2  249 TGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCTCAGATTAATC 310
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f mgc2      249 TGTAGTTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATCAATC 310
k703 mgc2   249 TGTAGCTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATCAATC 310
k503 mgc2   249 TGTAGCTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCTCAGATCAATC 310

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r mgc2      281 CGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGTGCTTTGGTGGCCCAATGCAA 342
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r mgc2      343 CCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAATGCC 404
s6 mgc2     373 CCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAATGCC 434
a5969 mgc2  373 CCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAATGCC 434
ts 11 mgc2  373 CTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCACAAATGGGAGGAATGCC 434
685 mgc2    371 ------------------------------------------------------------C 371
k730 mgc2   373 CCTAACCACATGGGGATGCGACCAGGGTTTAACCAAATGCCGCCACAGATGGGTGGGATGCC 434
f mgc2      373 CCTAACCACATGGGGATGCGGCCAGGGTTTAACCAAATGCCGCCACAAATGGGTGGGATGCC 434
k703 mgc2   373 CCTAACCACATGGGGATGCGACCAGGGTTCAACCAAATGCCGCCACAGGTGGGTGGGATGCC 434
k503 mgc2   373 CCTAACCACATGGGGATGCGGCCAGGGTTTAACCAAATGCCGACACAGATGGGTGGGATGCC 434

hf51 mgc2   435 ACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 493
r mgc2      405 ACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 463
s6 mgc2     435 ACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 493
a5969 mgc2  435 ACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 493
ts 11 mgc2  435 ACCTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 493
685 mgc2    372 ACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCA---CAAATGGGAGGAA 430
k730 mgc2   435 ACCTAACCACATGGGGATGCGACCAGGGTTTAACCAAATGCCACCTAACCAAATGGGTGGAA 496
f mgc2      435 ACCTAACCACATGGGGATGCGGCCAGGGTTTAACCAAATGCCACCTAACCAAATGGGTGGAA 496
k703 mgc2   435 ACCTAACCACATGGGGATGCGACCAGGGTTTAACCAAATGCCACCTAACCAAATGGGTGGAA 496
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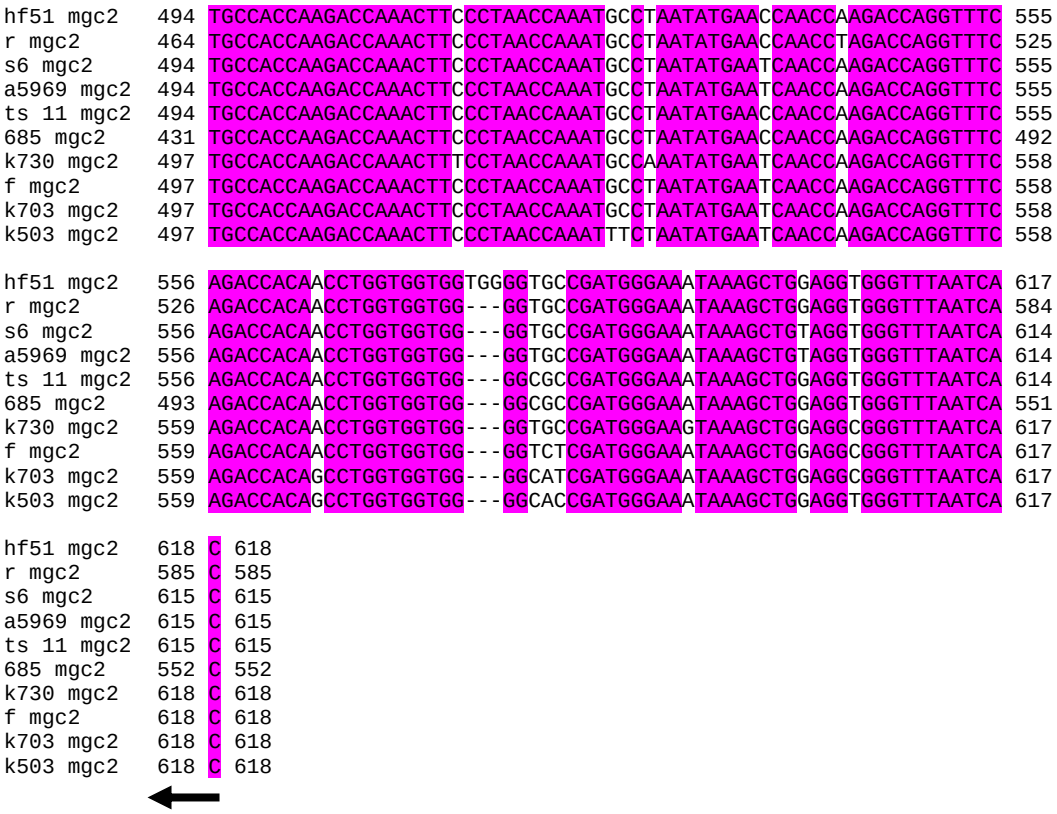


Figure 3.2: Alignment of the *Mgc2* cytadhesion gene sequences in ten strains of *M.gallisepticum.* The arrows indicate the positions and directions of the LMMF/R set of primers. The alignments were carried out using the DNAssist® Version 2.2 program (www.dnassist.com).

3.3.4.2 PCR amplification of the 16S rRNA, *Mgc2* and *M imitans* genes.

Each amplification reaction was made by addition of 5 µl of extracted DNA as template, 0.5 µl of each primer (100 µM), 1 µl of a set of dNTPs (100mM), 5 µl of 10X concentration PCR buffer, 0.5 µl of Taq DNA polymerase (250 u/ml) and made up to a final volume of 50 µl with sterile distilled water. Distilled water was substituted for DNA in all negative controls.

Reactions were thermocycled on Mastercycler Personal (Eppendorf®). The initial denaturing step was carried out at 94 ^{0}C for 5 min, followed by the denaturing step at 94 ^{0}C for 30 s, annealing at 58 ^{0}C (49 ^{0}C for the repeated amplifications of the *M. imitans* genes) for 30 s and extension at 72 ^{0}C for 90 s

for a length of 30 cycles and the final extension step was carried out at 72 ^{0}C for 5 min.

PCR products were stained with a loading dye and ran on a 1 % agarose gel at 90 mV. Ethidium bromide was added to the gels for viewing under UV illumination.

3.3.5 RFLP analyses

Restriction maps for each of the ten strains in Figure 3.3 were determined using the NEBcutter V2 website (tools.neb.com/**NEBcutter**2/index.php). These ten strains were used as reference strains because of their likeliness to be isolated from the field, following literature reviewed. A typing diagram was therefore constructed as part of this study and is shown in Figure 3.3 below. The letters A, B and C in the diagram indicate the three groups that these strains can be categorized under, subsequent to the choice of enzymes.

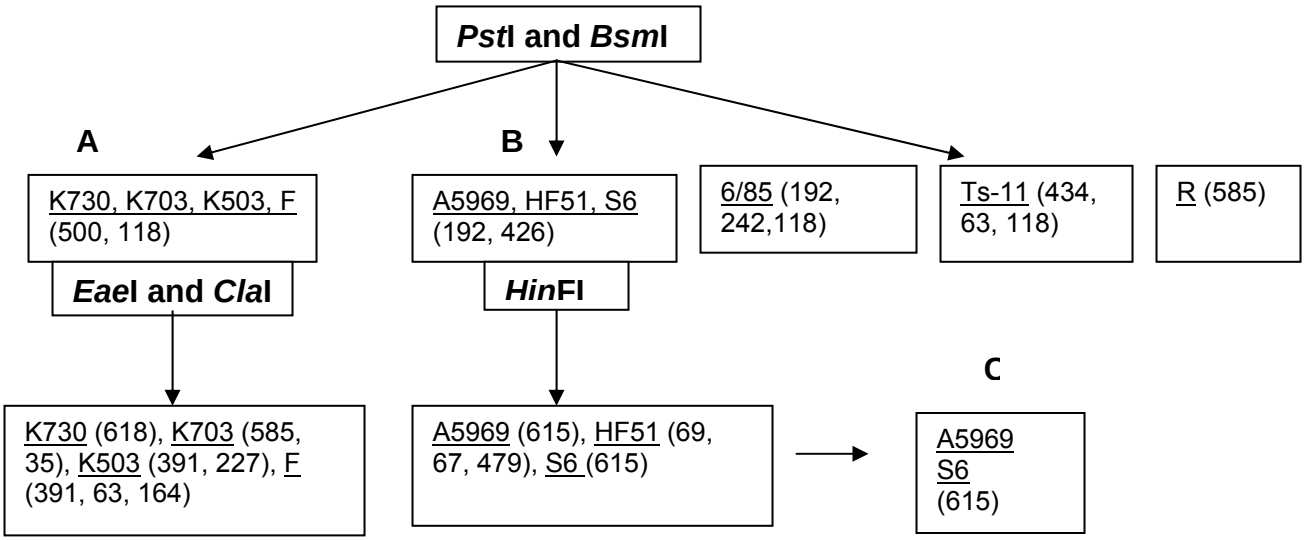


Figure 3.3: RFLP typing of the 10 reference strains of *M. gallisepticum*. A, B and C indicate typing groups that can be constructed. Strains are underlined, restriction enzymes are in bold and the sizes resulting from actions of enzymes are bracketed.

3.3.5.1 The RFLP reaction of the Ts-11 vaccine strain

Ts-11 was used as a positive control for reactions and was digested with *Bsm*I. The reason for the utilization of Ts-11 as a positive control was to rapidly ascertain whether the flock was vaccinated with the Ts-11 strain or was naturally infected with wild-type strains. Based on the restriction map constructed for Ts-11, *Pst*I is unable to cut the Ts-11 strain and this is the reason that led to exclusion of this enzyme from the RFLP reaction of Ts-11.

To perform the RFLP analysis, 1 µl of Buffer R and 0.5 µl of *Bsm*I (10 u/µl per enzyme concentration) were added to 8.5 µl of the *Mgc2*-PCR product of Ts-11. The reaction was incubated at 37 ^{0}C for 2 hours. The resulting product was stained with a loading dye and a 2 % low melt agarose gel containing ethidium bromide was run at 90 mV and viewed under UV illumination.

3.3.5.2 The RFLP reactions of the field strains

Field strains that were positive with the *Mgc2*-PCR were double-digested with *Bsm*I and *Pst*I and then compared to the groups in Figure 3.3. A 0.5 µl volume of each enzyme was added to 8 µl of the PCR product with 1 µl of Buffer R. The reaction was incubated at 37 ^{0}C for 2 hours and the product was stained with a loading dye, ran on a 2 % low melt agarose gel with ethidium bromide at 90 mV and viewed under UV illumination.

3.4 Results

3.4.1 Results of the PCR assays of the 16S rRNA genes.

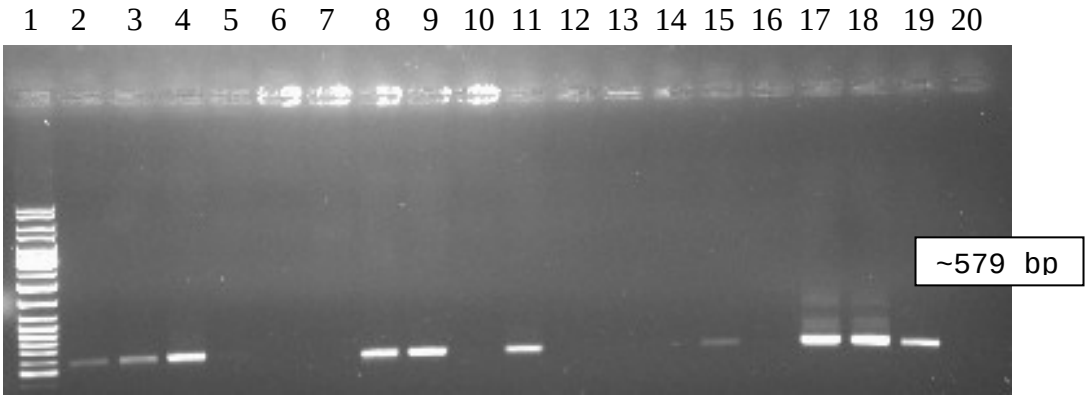
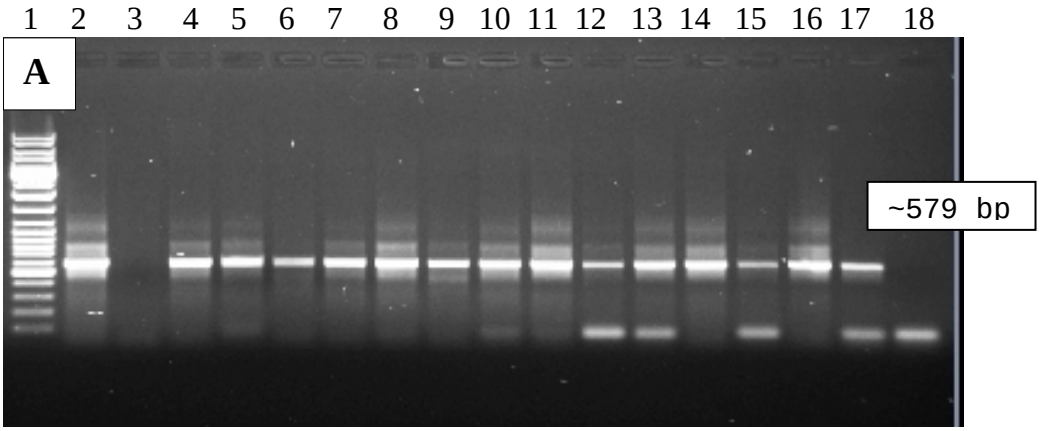
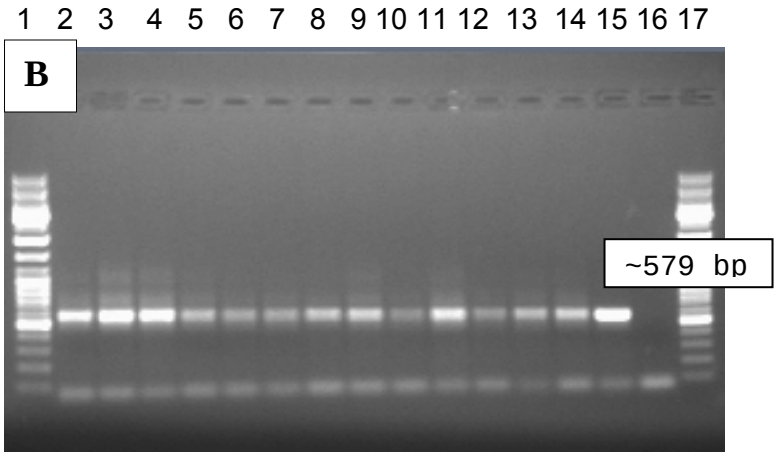


Figure 3.4: Amplification of the 16S rRNA gene of isolates pooled from various farms in South Africa. Lane 1: 100 bp marker, Lanes 2-18 indicate various samples collected. Lanes 19 and 20 indicate the positive and negative controls, respectively.

Zimbabwean samples





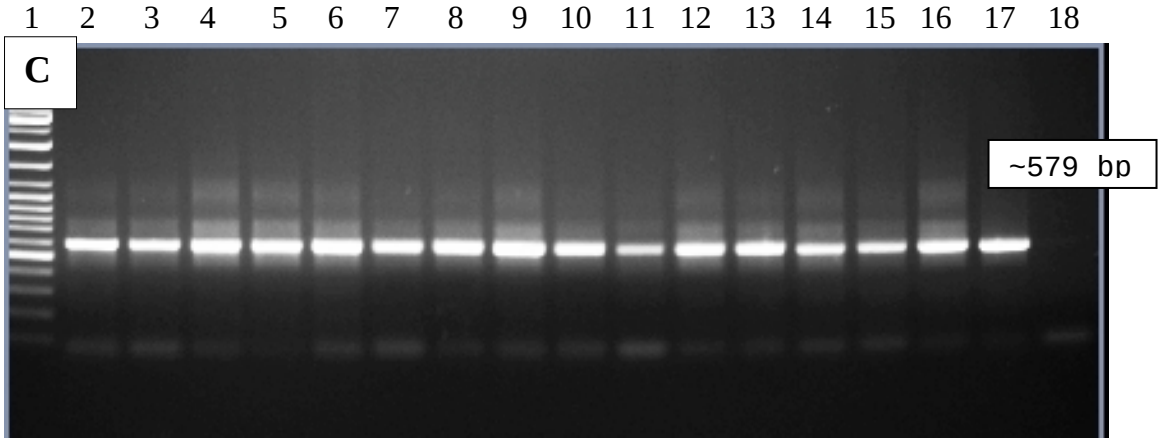


Figure 3.5: Amplifications of the 16S rRNA gene of the samples isolated from various farms in Zimbabwe. **(A)**: Lane 1: 100 bp marker, lanes 2-16: samples tested, lanes 17 and 18: positive and negative controls, respectively. **(B)**: Lanes 1 and 17: 100 bp markers, lanes 2-14: samples tested, lanes 15 and 16: positive and negative controls, respectively. **(C)**: Lane 1: 100 bp marker, lanes 2-16: samples tested, lanes 17 and 18: positive and negative controls, respectively.

3.4.2 Results of the PCR assays of the *Mgc2* gene.

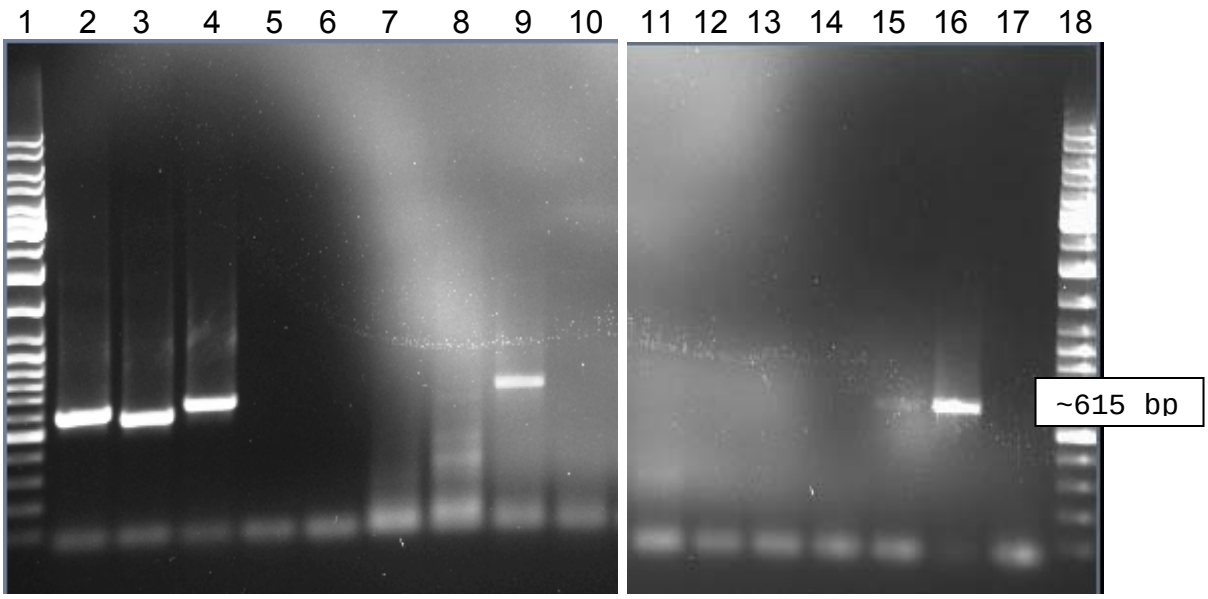
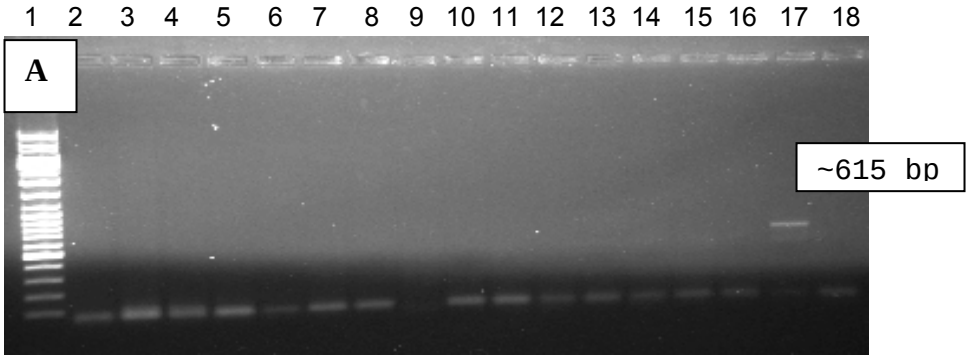
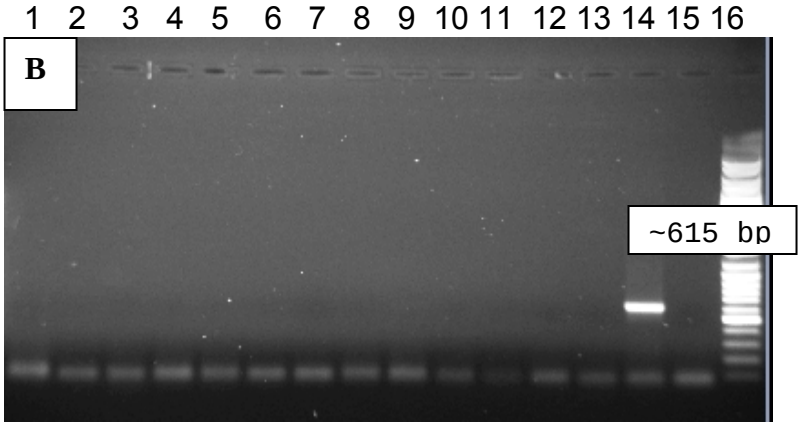


Figure 3.6: Amplification of the *Mgc2* gene of samples isolated from South Africa. Lanes 1 and 18: 100 bp marker, lanes 2-15 represent the samples tested. Lanes 16 and 17: positive and negative controls respectively. The two gel photographs represent one big gel. Detection was observed in lanes 2, 3, 4, 9 and 14; and these were designated Amplicons 1-5.

Zimbabwean samples:





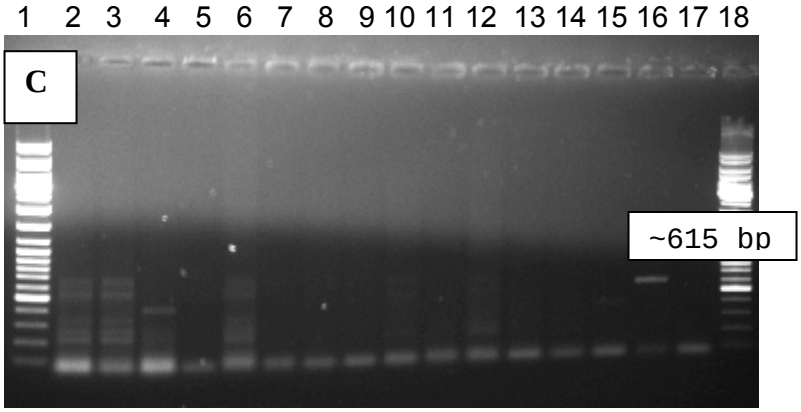


Figure 3.7: Amplifications of the *Mgc2* gene of samples isolated from Zimbabwe. **(A)**: Lane 1: 100 bp marker, lanes 2-16: tested samples and lanes 17 and 18: positive and negative controls, respectively. **(B)**: Lane 16: 100 bp marker, lanes 1-13: tested samples and lanes 14 and 15: positive and negative controls, respectively. **(C)**: Lanes 1 and 18: 100 bp markers, lanes 2-15: tested samples and lanes 16 and 17 represent the positive and negative controls, respectively.

3.4.3 Results of the PCR assays for the amplification of the gene encoding the *M. imitans* hypothetical protein.

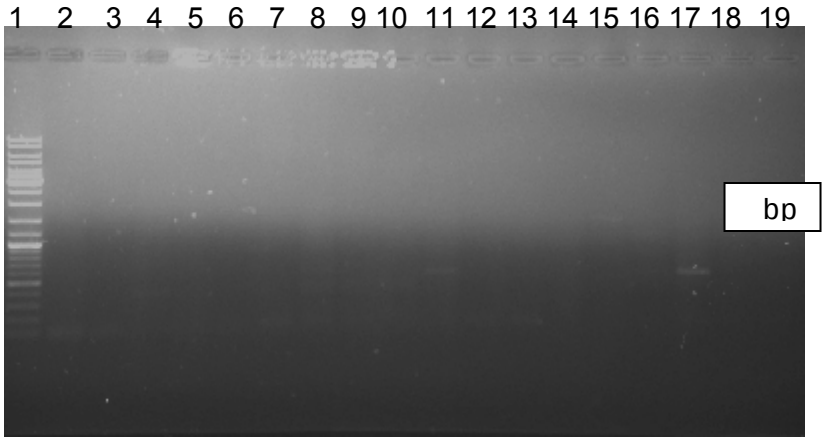


Figure 3.8: Amplfications of the gene encoding the hypothetical protein of *M. imitans*. Lane 1 represents the 100 bp marker while lane 19 indicates the negative control. Lanes 2 to 18 have been loaded with samples from South Africa.

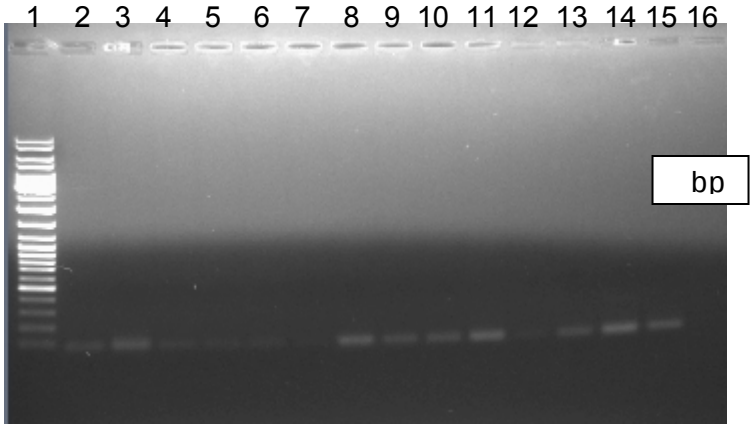


Figure 3.9: Amplifications of the gene encoding the *M. imitans* hypothetical protein from the Zimbabwean samples. Lane 1: 100 bp marker, lanes 2-15: samples tested and lane 16 is the negative control.

3.4.4 Results of the restriction digests performed on the *Mgc2* amplicons.

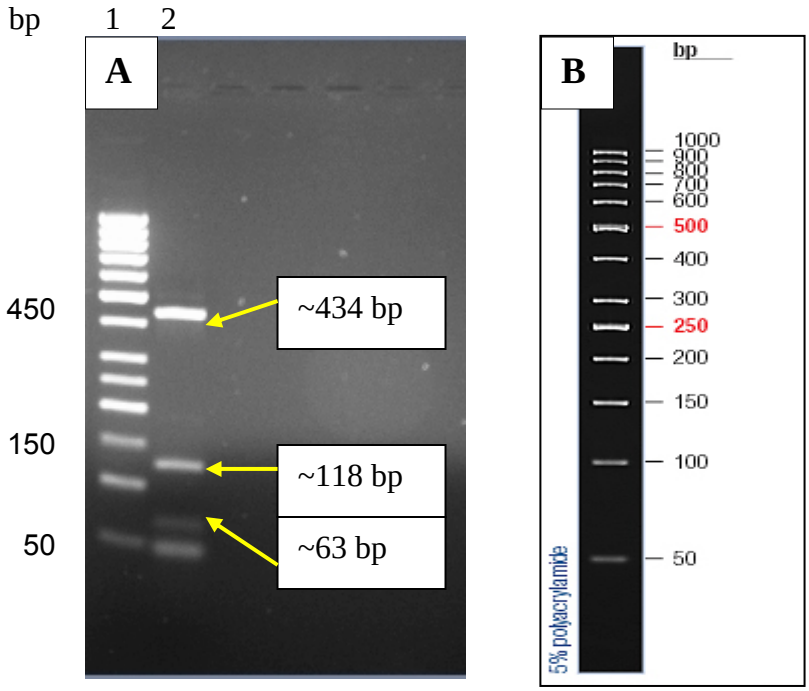


Figure 3.10: **(A)** Lane 2: RFLP profile of the positive control, the Ts-11 vaccine strain after digestion of the *Mgc2* gene with *Bsm*I. Lane 1 represents a 50 bp marker while Figure **B** illustrates the band sizes of the utilized marker.

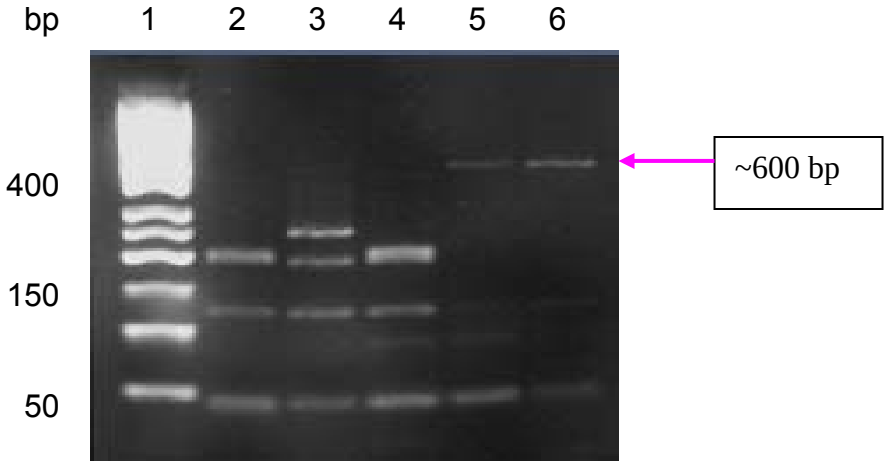


Figure 3.11: RFLP analyses of the *Mgc2* amplicons. The digestion was performed with *Pst*I and *Bsm*I. Lane 1 indicates a 50 bp DNA marker while lanes 2-6 indicate the amplicons obtained in Figure 3.6.

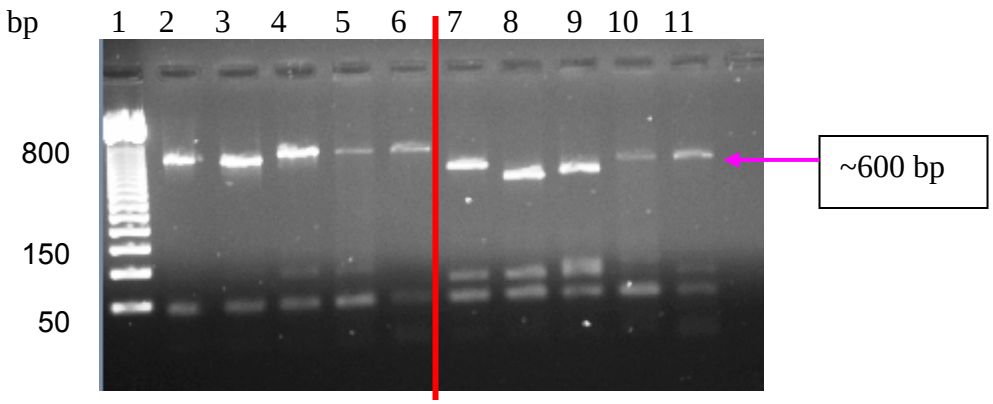


Figure 3.12: RFLP analyses of the *Mgc2* amplicons. Lane 1 indicates a 50 bp DNA marker. Lanes 2-6 indicate amplicons after double-digestion with *Cfr*I and *Cla*I, while lanes 7-11 indicate digestion with *Hin*FI. These amplicons were obtained in Figure 3.6.

Table 3.2: RFLP results**.** Obtained approximate fragment sizes (bp) of the five *Mgc2* amplicons after digestion with various enzymes.

Enzyme/Amplicon	***Pst*I & *Bsm*I**	***Cfr*I and *Cla*I**	***Hin*FI**
TS-11	63, 118, 434	ND	ND
Sample 1	130,200	600	60,540
Sample 2	130,200,260	590	60,530
Sample 3	130,200	600	60,540
Sample 4	600	610	610
Sample 5	600	610	610

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3.5 Discussions of results

The first aim of this Chapter was to establish and optimize a PCR assay that can adequately detect *M. gallisepticum* isolates at both the level of the 16S rRNA gene and that of the *Mgc2* gene. The aim was achieved when the positive control was consistently detected and the bands seen on a gel was of the expected size.

Figure 3.1 indicates the positions of the primers and also shows the many conserved regions on the 16S rRNA gene. This is indicative of the high possibility for the detection of the *M. imitans* isolates that may be present in the field. These primers detected almost all the isolates under test only leaving out a few. Therefore, at the high level of conservation, *M. gallisepticum* or *M. imitans* have

been detected. In comparison to this, only six isolates (figure 3.6) could be detected at the level of the *Mgc2* gene. This might mean that only these are *M. gallisepticum* while the rest of the samples are only closely related species. Due to positive serological tests obtained from the farms and institutes that provided samples, it is assumed that the other samples (16S positive but *Mgc2* negative) are *M. imitans* as cross-reactivity has been reported between *M. gallisepticum* and *M. imitans* (Harasawa *et al*., 2004).

It would be expected of any *M. gallisepticum* strain to be detected at the level of the *Mgc2* gene due to the high *Mgc2* homology among the strains (figure 3.2), also as reported by Boguslavsky *et al*. (2000) and Garcia *et al*. (2005). However, as reviewed in Chapter1, Hong and co-workers (2005a) found that the *M. gallisepticum* isolates differed according to the different areas they were found in. This might suggest that the samples that were not detected (figure 3.6 and figure 3.7) with the pair of *Mgc2* primers designed are *M. gallisepticum* with a different *Mgc2* gene with relation to those isolated outside Africa.

In addition to this, *Mycoplasma* have been reported to be more susceptible to mutations than other bacteria (Woese *et al*., 1985) while *M. gallisepticum* was found to have a defective DNA repair system by Ghosh *et al*. (1997). It is in the interest of *M. gallisepticum* to frequently change the surface antigens as this allows the organism to evade the host immune system and facilitate its survival when adhered to the host respiratory tract. The haemagglutinin, Mgc2, a protein encoded for by *Mgc2*, is among the cytadhesins that are reported to undergo changes (Nascimento *et al*., 2005b). This fully suggests the possibility of changes that the *Mgc2* nucleotide might posses among *M. gallisepticum* isolates.

Attempts were made to detect the gene that encodes the hypothetical protein of *M. imitans* (figure 3.8 and 3.9). This would prove the other possibility of the isolates being *M. imitans.* The attempts were unsuccessful due to the lack of a positive control that would be utilized to set-up a sensitive PCR assay for the

detection of the gene. Due to the constraints of time the control could not be awaited any further. However, there are very faint bands visible in some of the lanes in figure 3.9, that are absent in the negative control. This perhaps suggests the possibility of detection under optimized conditions.

Recently, three novel *Mycoplasma* species were isolated from South Africa by Botes and co-workers (2005) from ostriches, proving great potential of different *Mycoplasma* to those already isolated in other countries and possibly continents. The results obtained in this section may also suggest a novel avian *Mycoplasma* that can neither be detected with the *Mgc2* gene PCR as it is specific for *M. gallisepticum*, nor can it be detected with the hypothetical protein-encoding gene as it is specific for *M. imitans*.

Besides being detected with the 16S rRNA gene primers, the samples were collected from flocks that were serologically positive for *M. gallisepticum* with corresponding clinical signs. Therefore, a highly related species might be present but may not necessarily be *M. imitans*. This was a similar case in a study done by Bradbury and co-workers (1993), which led to the first isolation of *M. imitans*.

Figure 3.10 illustrated the RFLP pattern of Ts-11. The pattern was exactly the same as the theoretical digestion in Figure 3.3. Due to expected results being obtained, it shows that the reaction was well optimized and executed. The five amplicons obtained in Figure 3.6 were also digested as seen in Figure 3.11. It is observed from these profiles that the enzymes used are not a good typing system for samples 1 and 3 as the profiles obtained do not equal the total size of the amplicon obtained. The system is also not helping in profiling samples 4 and 5 as no digestion is observed. Sample 2, however, was digested in such a way that the obtained bands do indeed add up to the total approximate sample size allowing some type of profiling of this sample. Though the profile of the sample does not fall in any of the groups of the reference strains in Figure 3.3 it is more similar to that of the 6/85 strain.

Figure 3.12 shows digestion with *Cfr*I and *Cla*I. It was observed that no digestion took place in the presence of theses enzymes. However, digestion was observed in a reaction with *Hin*FI. The profiling from *Hin*FI can give an indication that there is a certain nucleotide sequence that this enzyme recognizes in the *Mgc2* as seen in samples 1 to 3 which have similar profiles.

The RFLP analyses thus provided an interesting addition to the detected *M. gallisepticum* isolates, in that they showed different profiles among the isolates themselves as well as being different from the Ts-11 strain. This is an indication of the diversity of the organism in South Africa and the assurance that wild-type *M. gallisepticum* is present in South Africa and not just vaccinated flocks are present (atleast with Ts-11).

3.6 Conclusions

A sensitive PCR was developed to detect *M. gallisepticum* at the level of the *Mgc2* gene. This was achieved and RFLP analyses were performed. Observations made from the RFLP profiles indicate differences among the isolates. This proves the presence and diversity of *M. gallisepticum* in South Africa. Furthermore, the results show that the isolates are wild-type *M. gallisepticum* as they are different from those of the Ts-11 strain which is widely used for vaccination in South Africa.

The 16S rRNA gene PCR was able to detect samples that were not detected with the *M. gallisepticum*-specific PCR, suggesting the presence of different *M. gallisepticum* isolates, *M. imitans* isolates or novel but closely related *Mycoplasma* species, as supported by literature.

3.7 Future research

It will be vital to optimize the detection of the other isolates not just at the level of the 16S rRNA gene, but also at the level of a specific gene that will characterize the isolates. Such a system will ascertain whether the organisms are indeed novel or just different. A positive control should be obtained for the correct optimization of the PCR amplification of the genes encoding the *M. imitans* hypothetical protein.

CHAPTER 4

Identification of the PCR-detected samples and the DGGE profiling of the 16S rRNA gene amplicons.

4.1 Introduction

Molecular techniques are believed to be accurate in identification of organisms as these techniques target genetic characteristics. Though phenotypic characteristics such as morphology still play a vital role in identification, most investigators are switching to molecular techniques as the basis of identification. Some of the most widely utilized molecular techniques were mentioned in Chapter 1 of this dissertation.

Denaturing Gradient Gel Electrophoresis (DGGE) is one of the most important molecular techniques used not only in identification, but also in differentiation of closely related species. The technique is PCR-based and utilizes DNA melting properties to separate fragments differing by as little as 1 bp substitution and mismatch. The principle is that a duplex DNA migrates in a polyacrylamide gel until it reaches stable domain to unwind (Brossette and Wartell, 1994).

Sometimes when the PCR assays of environmental DNA are investigated, the product can be a mixture of differing, but almost similar DNA sequences that represent many of the microbial organisms present in the environment. In such a case, the products will be of similar size (bp), making conventional separation by agarose gel electrophoresis result only in a single band that is largely non-descriptive. DGGE can overcome this limitation by separating PCR products based on sequence differences that result in differential denaturing characteristics of the DNA.

McAuliffe and co-workers (2005) stated that this method is based on the prevention of migration of DNA fragments following strand separation caused by chemical denaturants. It is further reported that though the technique has been extensively used for diversity analyses in microbial diversity, it has not been widely used for the identification and differentiation of pathogenic bacterial diversity. Using DGGE, these authors were able to differentiate 67 *Mycoplasma* species of human and veterinary origin and this showed improvement on current tests as diagnosis of *Mycoplasma* infection could be made directly from clinical samples in a period less than 24 hours.

A total of 37 *Mycoplasma* species of veterinary significance, including *M. gallisepticum*, were differentiated by McAuliffe and co-workers (2003). The results show that DGGE could differentiate between 85 % of the organisms. *M. gallisepticum* could simply be differentiated from the other avian *Mycoplasma* used as it gave a unique band. The very closely related organism, *M. imitans*, was however not used in the study. The utilization of the organism would have provided a valuable input as it would point out whether both *M. gallisepticum* and *M. imitans* could be differentiated.

4.2 Objectives

This Chapter is aimed at identifying the detected isolates in Chapter 3 to strain level. Further investigations will ascertain if the results obtained correlate to the serological observations. Alignments will also be performed to find differences in the sequences of the detected isolates. Samples will be further evaluated for analyses of the number of organisms that have similar gene sequences to *M. gallisepticum* and *M. imitans*. Lastly, phylogenetic trees will be drawn to show distances between the isolates.

4.3 Materials and Methods

4.3.1 Sequencing of the Amplicons

Sequencing reactions were carried out at **Inqaba Biotech, South Africa.**

4.3.2 DGGE analyses

Primers used

For quantification of the bacterial diversity in the samples the universal bacterial primer pair 341F-GC: 5'-CGC CCG CCG CGC GCG GCG GGC GGG CCT ACG GGA GGC AGC AG-3' and 517R: 5'-ATT ACC GCG GCT GCT GG-3' was used. The expected size of the product was ~200 bp. The gel percentage prepared was thus 8 % (Table 4.1)

Amplicons from the 16S rRNA gene PCR (Chapter 3) obtained with the primer pair LMGF/LMGR were used.

Table 4.1: Percentage of acrylamide/bisacrylamide needed for a particular amplicon size range.

Gel percentage	Base pair separation
6 %	300-1000 bp
8 %	200-400 bp
10 %	100-300 bp

PCR assays

The PCR parameters of the primer pair 341F-GC/517R PCR were as follows: the initial denaturing step was carried out at 95 ^{0}C for 5 min, followed by the denaturing step at 95 ^{0}C for 45 s, annealing step at 55 ^{0}C for 45 sec and extension at 72 ^{0}C for 25 cycles while the final extension step was carried out at 72 ^{0}C for 10 min.

The DGGE preparations

All reagents were obtained from **BioRad:** Acrylamide/bisacrylamidesolution, Deionized formamide, Urea, Ammonium persulfate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), TAE buffer, Glacial Acetic Acid, Tris.

Preparation of stock solutions:

- **50x TAE**
    - 242 g Trs-HCl
    - 57.1 ml Glacial Acetic Acid
    - 18.6 g EDTA
    - Fill with distilled water up to 1 L
    - Filter and store at room temperature.

- **40 % Acrylamide/Bis (37:35:1)**
    - 38.93 g acrylamide
    - 1.07 g bis-acrylamide
    - Dissolve in 100 ml distilled water
    - Filter and store at 4 ^{0}C in the dark.

- **Deionized formamide**
    - 100 ml formamide
    - Add ~5 g of Mixed Red Resin
    - Shake overnight protected from light
    - Filter and keep at 4 ^{0}C in the dark.

- **10 % APS**
    - Dissolve 1 g APS in 10 ml distilled water
    - Filter and dispense in aliquots
    - Store at -20 ^{0}C until needed.

Preparations of working 0 and 80% urea-formamide (UF) solutions.

- **0 % UF (50 ml)**
    - 10 ml 40 % acrlyamide/bis
    - 1 ml 50x TAE
    - 39 ml distilled water.

- **80 % UF (50 ml)**
    - 10 ml 40 % acrylamide/bis
    - 1 ml 50x TAE
    - 16 ml formamide
    - 16.8 g urea
    - Fill to 50 ml with distilled water.

The solutions can be stored at 4 ^{0}C until needed.

Table 4.2: Gradient urea-formamide (UF) solutions

% UF	0 % stock solution volume (ml)	80 % UF stock solution volume (ml)
10 %	8.75	1.25
20 %	7.5	2.5
30 %	6.25	3.75
40 %	5	5
50 %	3.75	6.25
60 %	2.5	7.5
70 %	1.25	8.75

Both low and high density solutions were prepared by mixing the appropriate volumes of each 0 and 80 % stock solutions in two 15 ml tubes. A stacking 0 % UF solution was prepared separately in another tube. It was insured that all gradient delivery system components were ready prior to casting. Appropriate volumes of APS (63 µl) and TEMED (7 µl) were added to the gradient solutions.

The gel was cast with the wheel, following the manual instructions. The APS and TEMED were added to the stacking solution, mixed and carefully delivered between the glasses with a Pasteur pipette to avoid contact with the gradient solution. When the solution reached the top, a comb was placed and the system was left for at least an hour to allow polymerization.

The tank was filled with 7 L of 1x TAE. Once the gel had polymerized, the comb was removed. The gel was transferred to the tank and the wells washed. The buffer was pre-heated to 60 ^{0}C before the samples were loaded. The gel was run at 200 V for 3 h. The gel was carefully removed and stained with ethidium bromide solution for 15 min. After staining, the gel was washed with distilled water and viewed under UV illumination.

The bands were excised from the gel with a sterile blade and transferred into a sterile tube. Elution of DNA was carried out with 50 µl of distilled water and incubation at 50 ^{0}C overnight.

4.4 Results

4.4.1 BLAST results of PCR-detected isolates

The *Mgc2* PCR amplicons

Table 4.3: BLAST results of the strains with the highest homology to the *Mgc2* amplicon*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY556279.1	*Mycoplasma gallisepticum* strain K5033FTK00 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556278.1	*Mycoplasma gallisepticum* strain K5033ATK00 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556276.1	*Mycoplasma gallisepticum* strain K5027BTK00 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556275.1	*Mycoplasma gallisepticum* strain K5011TK00 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556271.1	*Mycoplasma gallisepticum* strain K4705CK99 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556255.1	*Mycoplasma gallisepticum* strain K4311TK96 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556254.1	*Mycoplasma gallisepticum* strain K4280CK96 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556253.1	*Mycoplasma gallisepticum* strain K4246TK96 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556229.1	*Mycoplasma gallisepticum* strain S6 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
AY556227.1	*Mycoplasma gallisepticum* strain A5969 Mgc2 *(mgc2)* gene, partial cds	929	1205	100%	0.0	100%
U34842.1	*Mycoplasma gallisepticum* MGC2 precursor *(mgc2)* gene and MGC1 precursor *(mgc1)* gene, complete cds	929	1205	100%	0.0	100%

*The “amplicon” represents the PCR product obtained in Figure 3.6, lane 4 of the gel. This amplicon will be designated Amplicon 2 henceforth.

Table 4.4: BLAST results of the strains with the highest homology to the *Mgc2* amplicon*

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AY556287.1	*Mycoplasma gallisepticum* strain K5111ATKY01 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556263.1	*Mycoplasma gallisepticum* strain K4465TK97 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556262.1	*Mycoplasma gallisepticum* strain K4423BTK97 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556261.1	*Mycoplasma gallisepticum* strain K4421ATK97 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556252.1	*Mycoplasma gallisepticum* strain K4236TK96 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556244.1	*Mycoplasma gallisepticum* strain K4043TK95 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556243.1	*Mycoplasma gallisepticum* strain K4029TK95 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556241.1	*Mycoplasma gallisepticum* strain K3944TK95 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%
AY556231.1	*Mycoplasma gallisepticum* strain 685 Mgc2 *(mgc2)* gene, partial cds	841	841	99%	0.0	99%

*The “amplicon” represents the PCR product obtained in Figure 3.6, lane 3 of the gel. This amplicon will be designated Amplicon 1 henceforth.

The 16S rRNA gene PCR amplicons results:

Three of the detected samples gave expected results that indicated that they are either *M. gallisepticum* or *M. imitans* isolates as seen in Table 4.5, Table 4.6 and Table 4.7 below:

Table 4.5: BLAST results of the strains with the highest homology to the 16S rRNA gene amplicon*.

(*The “amplicon” represents the PCR product obtained in Figure 3.4, lane 10 of the gel. This amplicon will be designated Sample 1 henceforth.)

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
L35043.3	*Mycoplasma gallisepticum* strain A5969 genomic large direct repeat and flanking sequences	1046	1876	69%	0.0	99%
AE015450.1	*Mycoplasma gallisepticum* strain R complete genome	1046	2092	69%	0.0	99%
M22441.1	*M.gallisepticum* 16S small subunit ribosomal RNA	1046	1046	69%	0.0	99%
L24103.1	*Mycoplasma imitans* 16S ribosomal RNA (16S rRNA) gene	1046	1046	69%	0.0	99%
L08897.1	*Mycoplasma gallisepticum* (strain A5969) 16S-, 23S-, 5S ribosomal RNA (rrsA, rrlA, rrfA) genes	1035	1035	69%	0.0	98%
AM075207.1	*Mycoplasma gallisepticum* partial 16S rRNA gene, isolate MG-V25-2	1031	1031	68%	0.0	99%
U09788.1	*Mycoplasma testudinis* ATCC 43263 16S rRNA gene, partial sequence	893	893	69%	0.0	94%
U44765.1	*Mycoplasma alvi* 16S ribosomal RNA (rrn) gene, partial sequence	880	880	69%	0.0	94%
AY531655.1	*Mycoplasma amphoriforme* strain A39 16S ribosomal RNA gene, partial sequence	876	876	69%	0.0	93%
AY531656.1	*Mycoplasma amphoriforme* strain M5572 16S ribosomal RNA gene, partial sequence	869	869	68%	0.0	93%

Table 4.6: BLAST results of the strains with the highest homology to the16S rRNA amplicon*.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
L35043.3	*Mycoplasma gallisepticum* strain A5969 genomic large direct repeat and flanking sequences	959	1717	92%	0.0	99%
AE015450.1	*Mycoplasma gallisepticum* strain R complete genome	959	1919	92%	0.0	99%
M22441.1	*M.gallisepticum* 16S small subunit ribosomal RNA	959	959	92%	0.0	99%
L24103.1	*Mycoplasma imitans* 16S ribosomal RNA (16S rRNA) gene	959	959	92%	0.0	99%
AM075207.1	*Mycoplasma gallisepticum* partial 16S rRNA gene, isolate MG-V25-2	953	953	91%	0.0	99%
L08897.1	*Mycoplasma gallisepticum* (strain A5969) 16S-, 23S-, 5S ribosomal RNA (rrsA, rrlA, rrfA) genes	948	948	92%	0.0	99%
U09788.1	*Mycoplasma testudinis* ATCC 43263 16S rRNA gene, partial sequence	806	806	92%	0.0	94%
U44765.1	*Mycoplasma alvi* 16S ribosomal RNA (rrn) gene, partial sequence	793	793	92%	0.0	93%
AY531655.1	*Mycoplasma amphoriforme* strain A39 16S ribosomal RNA gene, partial sequence	789	789	92%	0.0	93%
AY531656.1	*Mycoplasma amphoriforme* strain M5572 16S ribosomal RNA gene, partial sequence	782	782	92%	0.0	93%
M23940.1	*M.pirum* 16S ribosomal RNA small subunit	750	750	92%	0.0	92%

*The “amplicon” represents the PCR product obtained in Figure 3.5A, lane 3 of the gel. This amplicon will be designated Sample 2 henceforth.

Table 4.7: BLAST results of the strains with the highest homology to the 16S rRNA amplicon*.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
L35043.3	*Mycoplasma gallisepticum* strain A5969 genomic large direct repeat and flanking sequences	1044	1882	95%	0.0	99%
AE015450.1	*Mycoplasma gallisepticum* strain R complete genome	1044	2088	95%	0.0	99%
M22441.1	*M.gallisepticum* 16S small subunit ribosomal RNA	1044	1044	95%	0.0	99%
L24103.1	*Mycoplasma imitans* 16S ribosomal RNA (16S rRNA) gene	1044	1044	95%	0.0	99%
AM075207.1	*Mycoplasma gallisepticum* partial 16S rRNA gene, isolate MG-V25-2	1033	1033	95%	0.0	99%
L08897.1	*Mycoplasma gallisepticum* (strain A5969) 16S-, 23S-, 5S ribosomal RNA (rrsA, rrlA, rrfA) genes	1033	1033	95%	0.0	99%
U09788.1	*Mycoplasma testudinis* ATCC 43263 16S rRNA gene, partial sequence	891	891	95%	0.0	94%
U44765.1	*Mycoplasma alvi* 16S ribosomal RNA (rrn) gene, partial sequence	878	878	95%	0.0	94%
AY531655.1	*Mycoplasma amphoriforme* strain A39 16S ribosomal RNA gene, partial sequence	874	874	95%	0.0	94%
AY531656.1	*Mycoplasma amphoriforme* strain M5572 16S ribosomal RNA gene, partial sequence	865	865	95%	0.0	93%
M23940.1	*M.pirum* 16S ribosomal RNA small subunit	835	835	95%	0.0	92%

*The “amplicon” represents the PCR product obtained in Figure 3.5B, lane 5 of the gel. This amplicon will be designated Sample 3 henceforth.

However, two samples consistently gave similar results that have the highest homology to unexpected results as can be seen in Table 4.8 and Table 4.9 below:

Table 4.8: BLAST results of the strains with the highest homology to the 16S rRNA amplicon*.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
EF012270.1	*Psychrobacter phenylpyruvicus* strain NCTC 10526 16S ribosomal RNA gene, partial sequence	933	933	97%	0.0	98%
DQ406835.1	*Psychrobacter phenylpyruvicus* isolate YNLB41C-74-270505 16S ribosomal RNA gene, partial sequence	933	933	97%	0.0	98%
CP000713.1	*Psychrobacter* sp. PRwf-1, complete genome	928	4640	97%	0.0	98%
AF005192.1	*Moraxella phenylpyruvica* 16S ribosomal RNA gene, partial sequence	928	928	97%	0.0	98%
DQ191162.1	*Psychrobacter* sp. X 159 16S ribosomal RNA gene, partial sequence	917	917	97%	0.0	97%
EF204254.1	*Psychrobacter phenylpyruvicus* isolate F162 16S ribosomal RNA gene, partial sequence	900	900	94%	0.0	98%

*The “amplicon” represents the PCR product obtained in Figure 3.5C, lane 8 of the gel. This amplicon will be designated Pheny 1 henceforth.

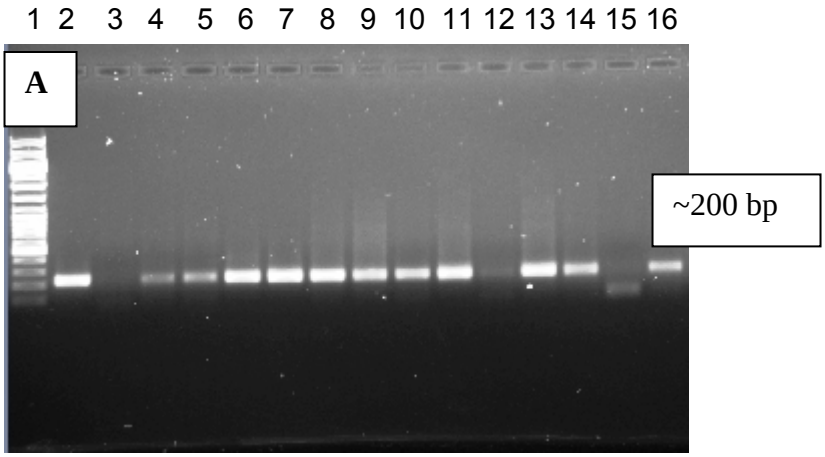
Table 4.9: BLAST results of the strains with the highest homology to the 16S rRNA amplicon.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
DQ406835.1	*Psychrobacter phenylpyruvicus* isolate YNLB41C-74-270505 16S ribosomal RNA gene, partial sequence	1035	1035	99%	0.0	99%
CP000713.1	*Psychrobacter* sp. PRwf-1, complete genome	1029	5148	99%	0.0	99%
AF005192.1	*Moraxella phenylpyruvica* 16S ribosomal RNA gene, partial sequence	1029	1029	99%	0.0	99%
DQ191162.1	*Psychrobacter* sp. X 159 16S ribosomal RNA gene, partial sequence	1018	1018	99%	0.0	98%
AJ609273.1	Psychrobacter arenosus 16S rRNA gene, type strain R7T	996	996	99%	0.0	98%
EF632922.1	Uncultured bacterium clone Par-w-85 16S ribosomal RNA gene, partial sequence	990	990	99%	0.0	98%
AM403658.1	*Psychrobacter* sp. 206(116zx) 16S rRNA gene, strain 206(116zx)	990	990	99%	0.0	98%
DQ447390.1	Uncultured bacterium clone RBbac164 16S ribosomal RNA gene, partial sequence	990	990	99%	0.0	98%
AY771725.1	*Psychrobacter alimentarius* isolate S3-15 16S ribosomal RNA gene, partial sequence	990	990	99%	0.0	98%
AY771724.1	*Psychrobacter glacincola* isolate S3-14 16S ribosomal RNA gene, partial sequence	990	990	99%	0.0	98%
AY573042.1	*Psychrobacter* sp. ARCTIC-P6 16S ribosomal RNA gene, partial sequence	990	990	99%	0.0	98%

*The “amplicon” represents the PCR product obtained in Figure 3.5C, lane 12 of the gel. This amplicon will be designated Pheny 2 henceforth.

The rest of the randomly selected samples could not be sequenced as they were found to have mixed templates. This is an indication of similar organisms that were present together in the field and were isolated as such from the host. This finding therefore led to choosing DGGE which could indicate the number of organisms with similar gene sequences that were present in those samples. These mixed amplicons were obtained from PCR assays of results shown in: Figure 3.4 (lanes 4, 8, 11, 17 and 18), Figure 3.5a (lanes 2, 4, 5, 6 and 11), Figure 3.5b (lanes 2, 3, 9 and 14) as well as Figure 3.5c (lanes 2, 3, 11 and 13).

4.4.2 Results of the PCR amplifications with the universal primers:



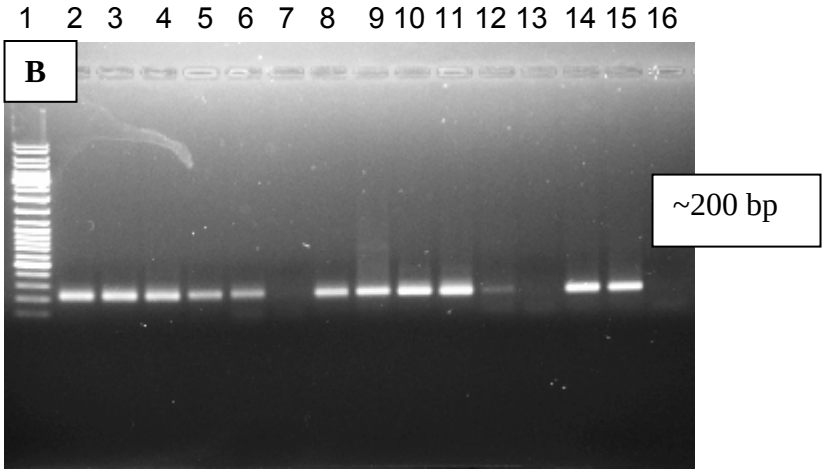


Figure 4.1: PCR amplifications with the Universal primers. The first lanes on both **A** and **B** represent a 100 bp marker while the rest of the lanes represent randomly selected samples. The last lane of **B** is the negative control.

4.4.3 Results of the DGGE analyses:

The DGGE analysis of the universal primers amplicons:

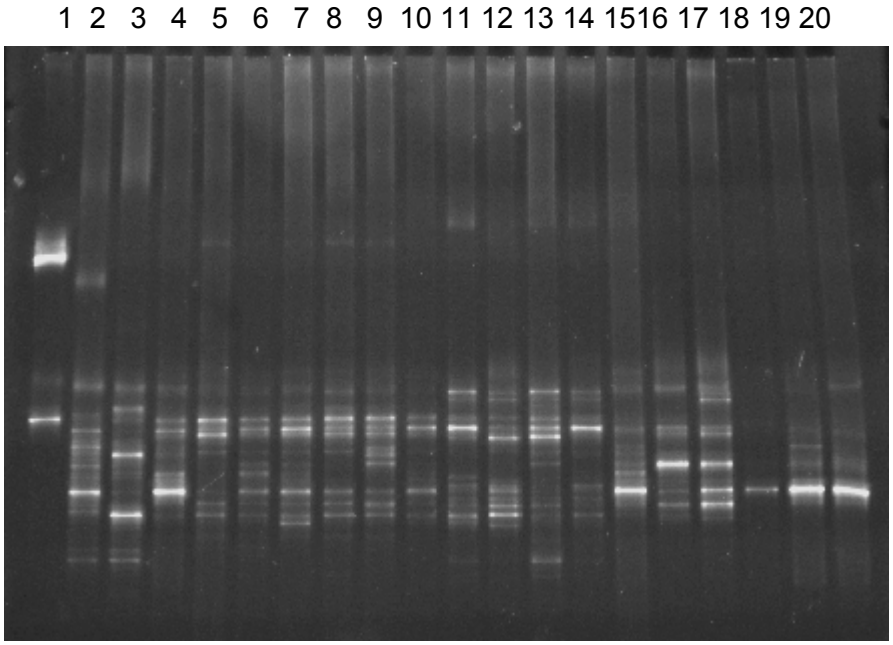


Figure 4.2: The DGGE analysis of the universal primers amplicons.

The DGGE analysis of the amplicons obtained from the 16 rRNA gene PCR.

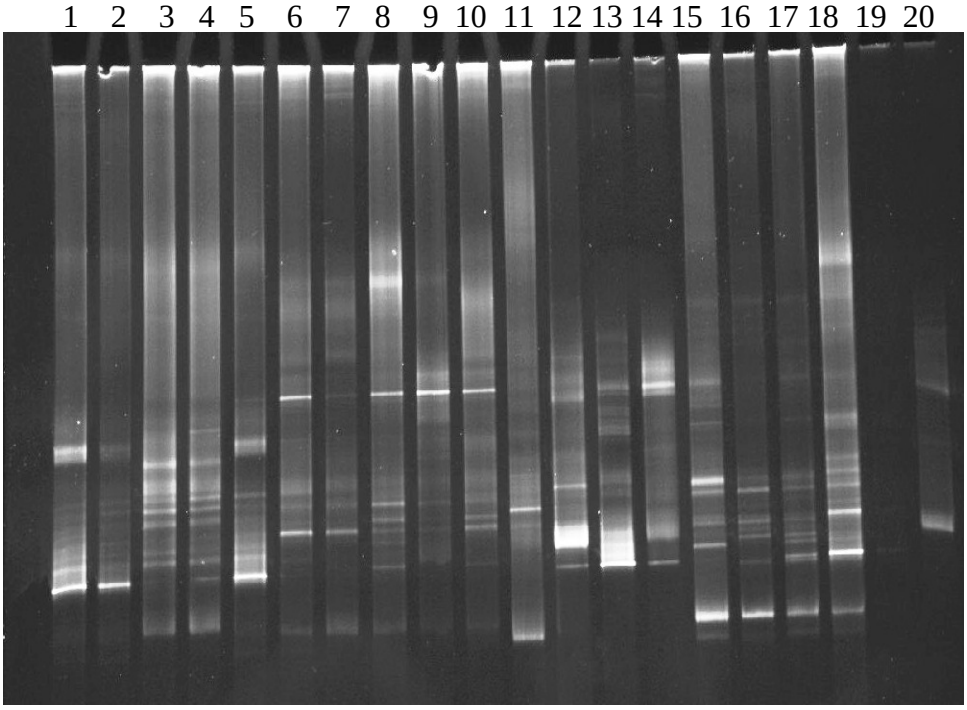


Figure 4.3: The DGGE analysis of the amplicons from the 16S rRNA gene. The amplicons were used in the same form as they were obtained in chapter 3.

The primers used in Chapter 3 were not GC-clamped. In the absence of a GC-clamp, double-stranded DNA that represents one organism is denatured, resulting in two strands of the DNA. Each strand is then represented as a band on the DGGE gel. Thus a GC-clamp helps keep the two strands together during the DGGE reaction.

Due to this, Figure 4.3 might over-represent the number of microorganisms that were detected. Following this, a 40bp GC-clamp of the sequence: 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3' was added to the 5'-end of the LMGF primer (Table 3.1). A PCR assay to ascertain whether the primers would be able to detect the samples under test was run and the results can be seen in Figure 4.4 below:

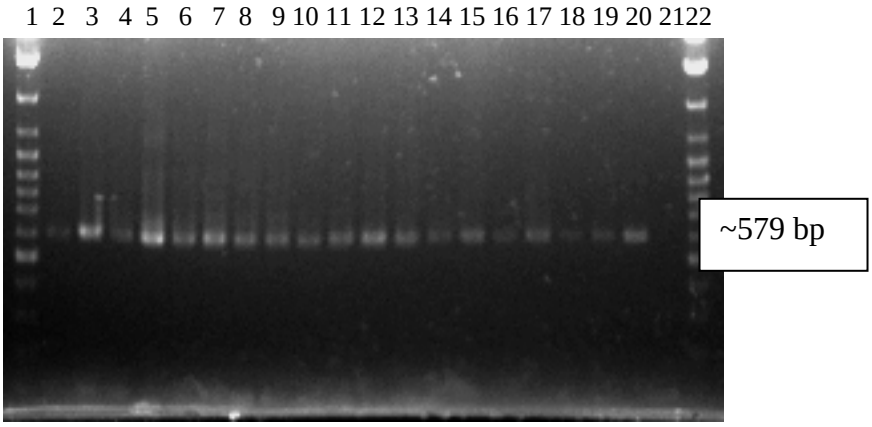


Figure 4.4: Amplifications of the 16S rRNA gene with a GC-clamp attached to the forward primer. Lanes 1 and 22 are the 100 bp markers, lanes 2-19 are the isolates under test while lanes 20 and 21: positive and negative controls, respectively.

Following the amplifications in Figure 4.4, a DGGE analysis of the amplicons was performed as can be seen in Figure 4.5 below:

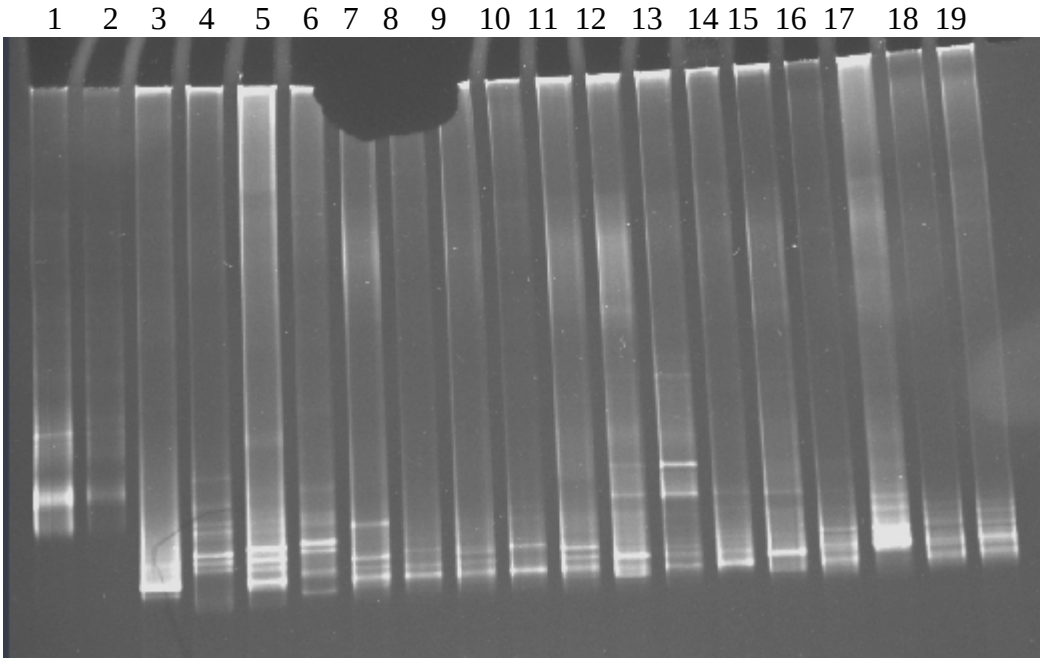


Figure 4.5: The DGGE analysis of the 16S rRNA gene. The amplicons were achieved with a forward primer that had a GC-clamp attached to it. Lane 1 represents the positive control (Ts-11 vaccine) while the other lanes represent the detected amplicons.

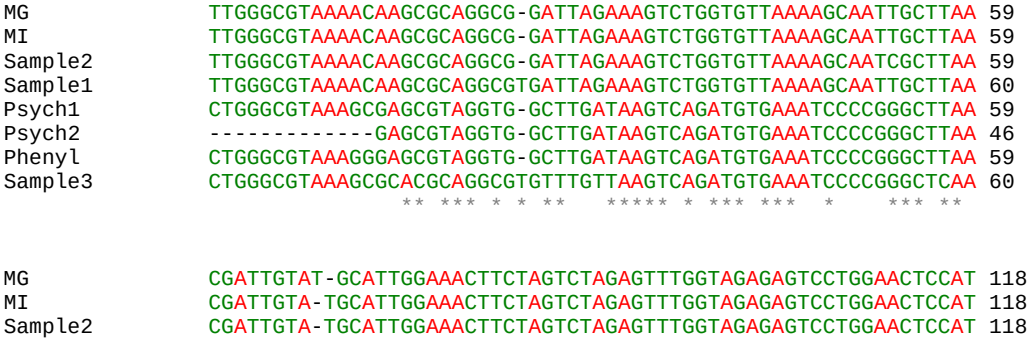
4.4.4 The alignments of the 16S rRNA gene of the sequenced *M. gallisepticum* isolates

```
R             CTGTACCATTTGAATAAGTAACGACTAACTATGTGCCAGCAGTCGCGGTAATACATAGGT 540
MI            CTGTACCATTTGAATAAGTAACGACTAACTATGTGCCAGCAGTCGCGGTAATACATAGGT 513
Sample1       ------------------------------------------------------------
Sample2       -------------------------------TTGCCAGGCCAGTGCGGTAATACATAGGT 29
Sample3       -------------------------------TTTCCCACCCCGTGCGGTAATACATAGGG 29

R             TGCAAGCGTTATCCGGATTTATTGGGCGTAAAACAAGCGCAGGCG-GATTAGAAAGTCTG 599
MI            TGCAAGCGTTATCCGGATTTATTGGGCGTAAAACAAGCGCAGGCG-GATTAGAAAGTCTG 572
Sample1       ---------------------TTGGGCGTAAAACAAGCGCAGGCGTGATTAGAAAGTCTG 39
Sample2       TGCAAGCGTTATCCGGATTTATTGGGCGTAAAACAAGCGCAGGCG-GATTAGAAAGTCTG 88
Sample3       TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGTGTTTGTTAAGTCAG 89
                                **********  *   ********  * **   ***** *

R             GTGTTAAAAGCAATTGCTTAACGATTGTA-TGCATTGGAAACTTCTAGTCTAGAGTTTGG 658
MI            GTGTTAAAAGCAATTGCTTAACGATTGTA-TGCATTGGAAACTTCTAGTCTAGAGTTTGG 631
Sample1       GTGTTAAAAGCAATTGCTTAACGATTGTA-TGCATTGGAAACTTCTAGTCTAGAGTTTGG 98
Sample2       GTGTTAAAAGCAATCGCTTAACGATTGTA-TGCATTGGAAACTTCTAGTCTAGAGTTTGG 147
Sample3       ATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCG 149
               *** ***  *    *** ***    * * *****  ** ***   *  ** **** * *

R             TAGAGAGTCCTGGAACTCCATGTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 718
MI            TAGAGAGTCCTGGAACTCCATGTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 691
Sample1       TAGAGAGTCCTGGAACTCCATGTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 158
Sample2       TAGAGAGTCCTGGAACTCCATGTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCA 207
Sample3       TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCG 209
              ***** *   * *** **** *** ***************** ** **** *** ***

R             GAGGCGAAGGCGAGGACTTGGGCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAA 778
MI            GAGGCGAAGGCGAGGACTTGGGCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAA 751
Sample1       GAGGCGAAGGCGAGGACTTGGGCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAA 218
Sample2       GAGGCGAAGGCGAGGACTTGGGCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAA 267
Sample3       GTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAA 269
              * **********    * *** * ** ********* ***   ***** ***********

R             ATAGGATTAGATACCCTAGTAGTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG- 837
MI            ATAGGATTAGATACCCTAGTAGTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG- 810
Sample1       ATAGGATTAGATACCCTAGTAGTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG- 277
Sample2       ATAGGATTAGATACCCTAGTAGTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG- 326
Sample3       ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCC 329
              * ***************** ********* * **********     *  * *   * **

R             --AATACTTCGGTGCTGCAGTTAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAA 895
MI            --AATACTTCGGTGCTGCAGTTAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAA 868
Sample1       --AATACTTCGGTGCTGCAGTTAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAA 335
Sample2       --AATACTTCGGTGCTGCAGTTAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAA 384
Sample3       TTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAA 389
                 *  * * * * * * ** **** * ****     * ***** * *****   *****

R             GAATGAAACTCAAACGGAATTGACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAAT 955
MI            GAATGAAACTCAAACGGAATTGACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAAT 928
Sample1       GAATGAAACTCAAACGGAATTGACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAAT 395
Sample2       GAATGAAACTCAAACGGAATTGACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAAT 444
Sample3       GGTTAAAACTCAAATG-AATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAAT 448
              *  * ********* * ********** ********** ************ * *****


R             TCGACGGTACACGAAAAACCTTACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATAT 1015
MI            TCGACGGTACACGAAAAACCTTACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATAT 988
Sample1       TCGACGGTACACGAAAAACCTTACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATAT 455
Sample2       TCGACGGTACACGAAAAACCTTACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATAT 504
Sample3       TCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAG-AACCTTGTAGAGATAC 507
              **** *  ** **** ********** * *********    *  *   * **** ***
```

Figure 4.6: Alignments of the 16S rRNA gene nucleotides of the sequenced samples with the R-strain of *M. gallisepticum* and the *M. imitans* gene.

4.4.5 Alignments of the *Mycoplasma* isolates with the *Pscychrobacter* isolates based on the sequenced region of the 16S rRNA gene.

Further alignments were performed with the inclusion of isolates that were found to be *Psychrobacter*. This was done for the investigation of the relatedness of *M. gallisepticum* and *M. imitans* to *Psychrobacter*. The relatedness became imperative due to the ability of the primers that were designed to detect *M. gallisepticum* and *M. imitans* also being able to detect *Psychrobacter*. The *Psychrobacter* species, *P. phenylperuvicus* was also included as a reference in the alignments as both the isolates that were found to be *Psychrobacter* seemed to have the highest similarity to it.



```
Sample1         CGATTGTA-TGCATTGGAAACTTCTAGTCTAGAGTTTGGTAGAGAGTCCTGGAACTCCAT 119
Psych1          CCTGGGAACTGCATCTGATACTGTCAGGCTAGAGTAGGTGAGAGGGAGGTAGAATTTCAG 119
Psych2          CCTGGGAACTGCATCTGATACTGTCAGGCTAGAGTAGGTGAGAGGGAGGTAGAATTTCAG 106
Phenyl          CCTGGGAACTGCATCTGATACTGTTGGGCTAGAGTAGGTGAGAGGGAGGTAGAATTCCAG 119
Sample3         CCTGGGAACTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAG 120
                *    * *  ****  ** ***      ** ****     **** *   * *** * **

MG              GTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGAGGCGAAGGCGAGGACTTGG 178
MI              GTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGAGGCGAAGGCGAGGACTTGG 178
Sample2         GTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGAGGCGAAGGCGAGGACTTGG 178
Sample1         GTGGAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGAGGCGAAGGCGAGGACTTGG 179
Psych1          GTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGATGGCGAAGGCAGCCTCCTGG 179
Psych2          GTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGATGGCGAAGGCAGCCTCCTGG 166
Phenyl          GTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCTTCTGG 179
Sample3         GTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGG 180
                *** ****************** ** ** * *** ***   *********       ***

MG              GCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATAGGATTAGATACCCTAGTA 238
MI              GCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATAGGATTAGATACCCTAGTA 238
Sample2         GCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATAGGATTAGATACCCTAGTA 238
Sample1         GCCAATACTGACGCTTAGGCTTGAAAGTGTGGGGAGCAAATAGGATTAGATACCCTAGTA 239
Psych1          CATCATACTGACACTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTA 239
Psych2          CATCATACTGACACTGAGGTTCGAAAGCGTGGGTAGCAAACAGGATTAGATACCCTGGTA 226
Phenyl          CATAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA 239
Sample3         ACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTA 240
                     * ****** ** ***   ***** ***** ****** *************** ***


MG              GTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG---AATACTTCGGTGCTGCAGT 295
MI              GTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG---AATACTTCGGTGCTGCAGT 295
Sample2         GTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG---AATACTTCGGTGCTGCAGT 295
Sample1         GTCCACACTGTAAACGATGGATGTTAAGTGTCGGAGCG---AATACTTCGGTGCTGCAGT 296
Psych1          GTCCACGCCGTAAACGATGTCTACTAGTCGTTGGGGAACTTGATTCCTTAGTGACGCAGC 299
Psych2          GTCCACGCCGTAAACGATGTCTACTAGTCGTTGGGGAACTTGATTCCTTAGTGACGCAGC 286
Phenyl          GTCCACGCCGTAAACGATGTCTACTAGTCGTTGGGTCCCTTGAGGACTTAGTGACGCAGC 299
Sample3         GTCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGC 300
                ****** * **********     *    *   *        *    *   *   * **

MG              TAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAAGAATGAAACTCAAACGGAATT 355
MI              TAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAAGAATGAAACTCAAACGGAATT 355
Sample2         TAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAAGAATGAAACTCAAACGGAATT 355
Sample1         TAACACATTAAACATCCTGCCTGAGTAGTACATTCGCAAGAATGAAACTCAAACGGAATT 356
Psych1          TAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG-AATT 358
Psych2          TAACGCAATAAGTAGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG-AATT 345
Phenyl          TAACGCAATAAGTAGACCGCCTGGGGAGTACGGTCGCAAGGTTACCACTCAAATG-AATT 358
Sample3         TAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATG-AATT 359
                **** *  ***     * ***** * *****   ******  *   ******* * ****

MG              GACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAATTCGACGGTACACGAAAAACCT 415
MI              GACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAATTCGACGGTACACGAAAAACCT 415
Sample2         GACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAATTCGACGGTACACGAAAAACCT 415
Sample1         GACGGGGACCCGCACAAGTGGTGGAGCATGTTGCTTAATTCGACGGTACACGAAAAACCT 416
Psych1          GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT 418
Psych2          GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT 405
Phenyl          GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTACTTCGATGCAACGCGAAGAACCT 418
Sample3         GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCT 419
                ******* ********** ************ * *** ***** *  ** **** *****

MG              TACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATATAGTGGAGGTC-----AACCCA 470
MI              TACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATATAGTGGAGGTC-----AACCCA 470
Sample2         TACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATATAGTGGAGGTC-----AACCCA 470
Sample1         TACCTAGACTTGACATCTTGGGCGAAGCTATAGAAATATAGTGGAGGTC-----AACCCA 471
Psych1          TACCTGGTCTTGACATATCTAG-AATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCTA 477
Psych2          TACCTGGTCTTGACATATCTAG-AATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCTA 464
Phenyl          TACCTGGTCTTGACATATCTAG-AATCCTGCAGAGATGGGGAAGTGCCTTCGGGAATTTG 477
Sample3         TACCTGGTCTTGACATCCACAG-AACCTTGTAGAGATACGAGGGTGCCTTCGGGAACTGT 478
                ***** * ********     *  *   *  *** **      * *        **

MG              ATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC-G 529
MI              ATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCC-G 529
Sample2         ATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCCG 530
```

```
Sample1         ATGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTAAGTTCCCCC 531
Psych1          GAA------------------------------------------------------- 480
Psych2          GAA------------------------------------------------------- 467
Phenyl          AATACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTCAGATGTTGGGTTATGTCCCGC 537
Sample3         GAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAGATGTTGGGTAAGTCCCCCC 538


MG              CAACG- 534
MI              CAACG- 534
Sample2         CAAACC 536
Sample1         CCAAAA 537
Psych1          ------
Psych2          ------
Phenyl          AACGAG 543
Sample3         CAAATT 544
```

Figure 4.7: Alignments of the *Mycoplasma* isolates with the *Psychrobacter* isolates. Samples 1-3 represent isolates that had the highest homology for both *M. gallisepticum* and *M. imitans* while Psych 1 and Psych 2 represent isolates that had the highest homology for *Psychrobacter.* Phenyl in the Figure represents *Psychrobacter phenylpyruvicus* that has been used as a reference species.

4.4.6 Alignments of the sequenced *Mgc2* amplicons with the *Mgc2* sequences of the ten reference strains.

```
S6              TTTTACCCAGTAGTGGGCGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
Amplicon2       ------------------------------------------------------------
A5969           TTTTACCCAGTAGTGGGCGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
HF51            TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
R               TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
TS11            TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
6/85            TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
Amplicon1       ------------------------------------------------------------
K730            TTTTACCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
F               TTTTACCCAGTAGTGGGTGCAGGTGCTGGGTTGATCGTTGTTTCTTTACTCTTGGGTTTA 60
K703            TTTTACCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
K503            TTTTACCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60


S6              GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
Amplicon2       -----------TCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 49
A5969           GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
HF51            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
R               GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
TS11            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
6/85            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
Amplicon1       --------------------AARAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 39
K730            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
F               GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
K703            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
K503            GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
                            ** ************************************


S6              CACCAAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
Amplicon2       CACCAAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATT 109
A5969           CACCAAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
HF51            CACCAAAAGATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATT 180
R               CACCAAAAGATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATT 180
TS11            CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
6/85            CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAACAACAAAATAAAACAGAAGCGATT 180
Amplicon1       CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAACAACAAAATAAAACAGAAGCGATT 99
```

```
K730            CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAAACAGAAGCGATT 180
F               CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAAACAGAAGCGATT 180
K703            CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAGCAACAAAATAAATCAGAAGCGATT 180
K503            CACCAAAAGATGGTTGAATCCCTTGGAATAATTGAACAACAAAATAAAACAGAAGCGATT 180
                ************************* ***** **  *********** ***********

S6              GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
Amplicon2       GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 169
A5969           GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
HF51            GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
R               GAGCCAACTG---------------AAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 225
TS11            GAGCCAACTACATCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
6/85            GAATCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
Amplicon1       GAATCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 159
K730            GAGTCAACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCT 240
F               GAGTCAACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCT 240
K703            GAGTCAACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCT 240
K503            GAGTCAACCACAGCAGTACCAACTGAAGAAGTTAATGTTCAAGAACCAACTCAACCAGCT 240
                **  ****                  ***********  **********************

S6              GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
Amplicon2       GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 229
A5969           GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
HF51            GGTGTTAATGTAGCTAATAACCCTCAGATAGGGATCAATCAACCAGGATTTAATCAACCT 300
R               GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACC---------------T 270
TS11            GGTGTTAATGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
6/85            GGTGTTAATGTAGATAATAATCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
Amplicon1       GGTGTTAATGTAGATAATAATCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 219
K730            GATGTAAATGTAGCTAATAATCCTCAGATGAGGATCAATCAACCAGGATTTAATCAACCT 300
F               GATGTAAATGTAGTTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
K703            GATGTAAATGTAGCTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
K503            GATGTAAATGTAGCTAATAATCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
                * *** ******* ****** ********  ********** **                *

S6              CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGGGCTTTGGT 360
Amplicon2       CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGGGCTTTGGT 289
A5969           CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGGGCTTTGGT 360
HF51            CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGGGCTTTGGT 360
R               CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGTGCTTTGGT 330
TS11            CAGATTAATCCGCAATTTATTCCTAATCCCCAACAAAGAATGAACCCACAGGGCTTTGGT 360
6/85            CAGATTAATCCGCAATTTGGTCCTCATCCCCAACAAAGAATTAACCCGCAGGGCTTTGGT 360
Amplicon1       CAGATTAATCCGCAATTTGGTCCTCATCCCCAACAAARAATTAACCCGCAGGGCTTTGGT 279
K730            CAGATCAATCCACAATTTGGTCTTAATCCCCAACAAAGAATTAATCCACAGGGTTTTGGT 360
F               CAGATCAATCCACAATTCGGTCTTAATCCCCAACAAAGAATTAATCCACAGGGTTTTGGT 360
K703            CAGATCAATCCACAATTTGGTCTTAATCCCCAACAAAAAATTAATCCACAGGGTTTTGGT 360
K503            CAGATCAATCCACAATTTGGTCTTAATCCCCAACAAAGAATTAATCCACAGGGTTTTGGT 360
                ***** ***** *****   ** * ************ *** ** ** *** * ******

S6              GGCCCAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
Amplicon2       GGCCCAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 349
A5969           GGCCCAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
HF51            GGCCCAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
R               GGCCCAATGCAACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 390
TS11            GGCCCAATGCCACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
6/85            GGCCCAATGT---------------------------------------------------- 370
Amplicon1       GGCCCAATGT---------------------------------------------------- 289
K730            GGCCTAATTCCACCTAACCACATGGGGATGCGACCAGGGTTTAACCAAATGCCGCCACAG 420
F               GGCCCAATGCCACCTAACCACATGGGGATGCGGCCAGGGTTTAACCAAATGCCGCCACAA 420
K703            GGCCCAATGCCACCTAACCACATGGGGATGCGACCAGGGTTCAACCAAATGCCGCCACAG 420
K503            GGCCCAATGCCACCTAACCACATGGGGATGCGGCCAGGGTTTAACCAAATGCCGACACAG 420
                **** ***

S6              ATGGGAGGAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA 480
Amplicon2       ATGGGAGGAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA 409
A5969           ATGGGAGGAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA 480
HF51            ATGGGAGGAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA 480
R               ATGGGAGGAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCA 450
TS11            ATGGGAGGAATGCCACCTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCA 480
6/85            -------------CACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCA 417
Amplicon1       -------------CACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCA 336
```

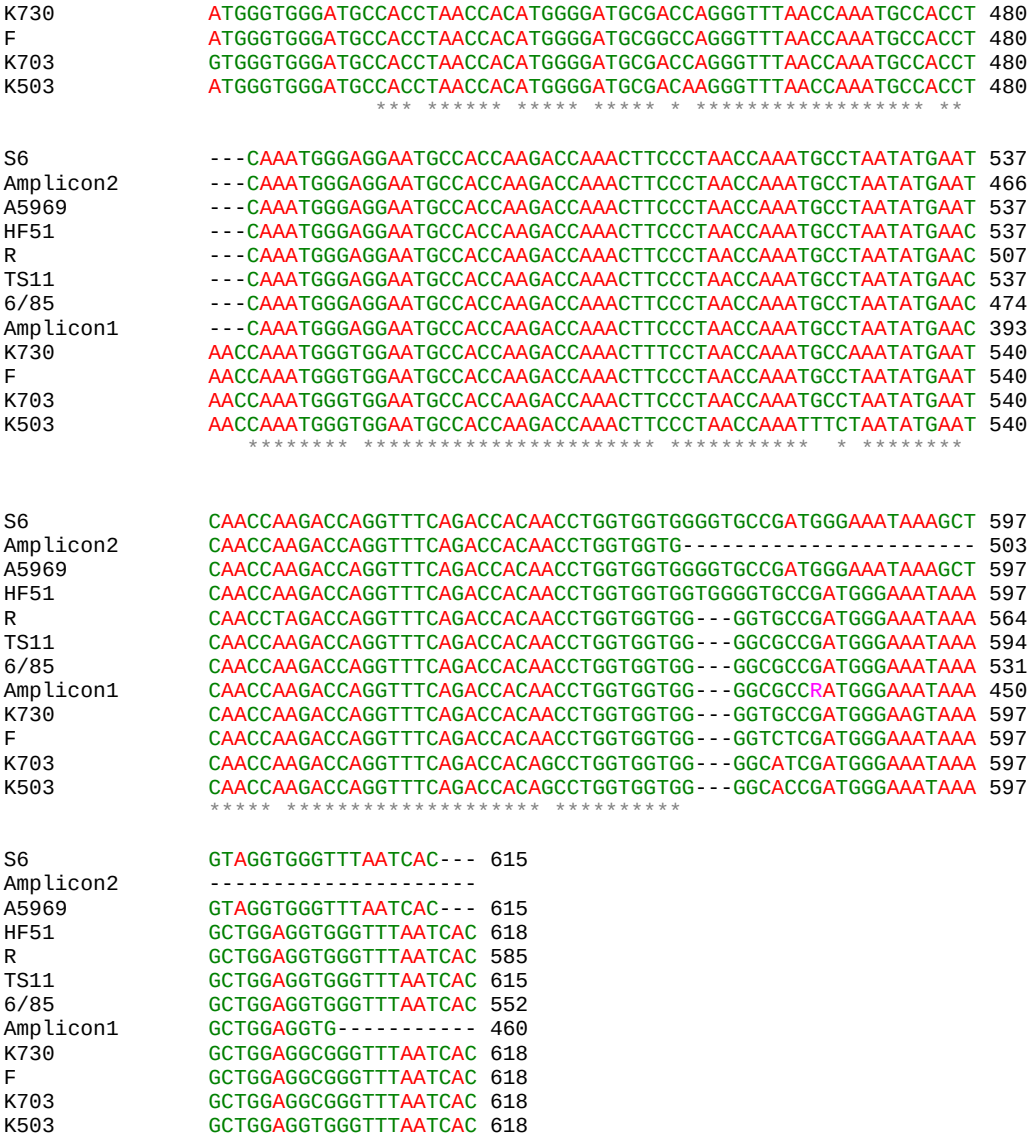


Figure 4.8: The *Mgc2* gene alignments of the reference strains with the two sequenced amplicons.

4.4.7 Phylogenetic trees

The 16S rRNA gene phylogenetic tree

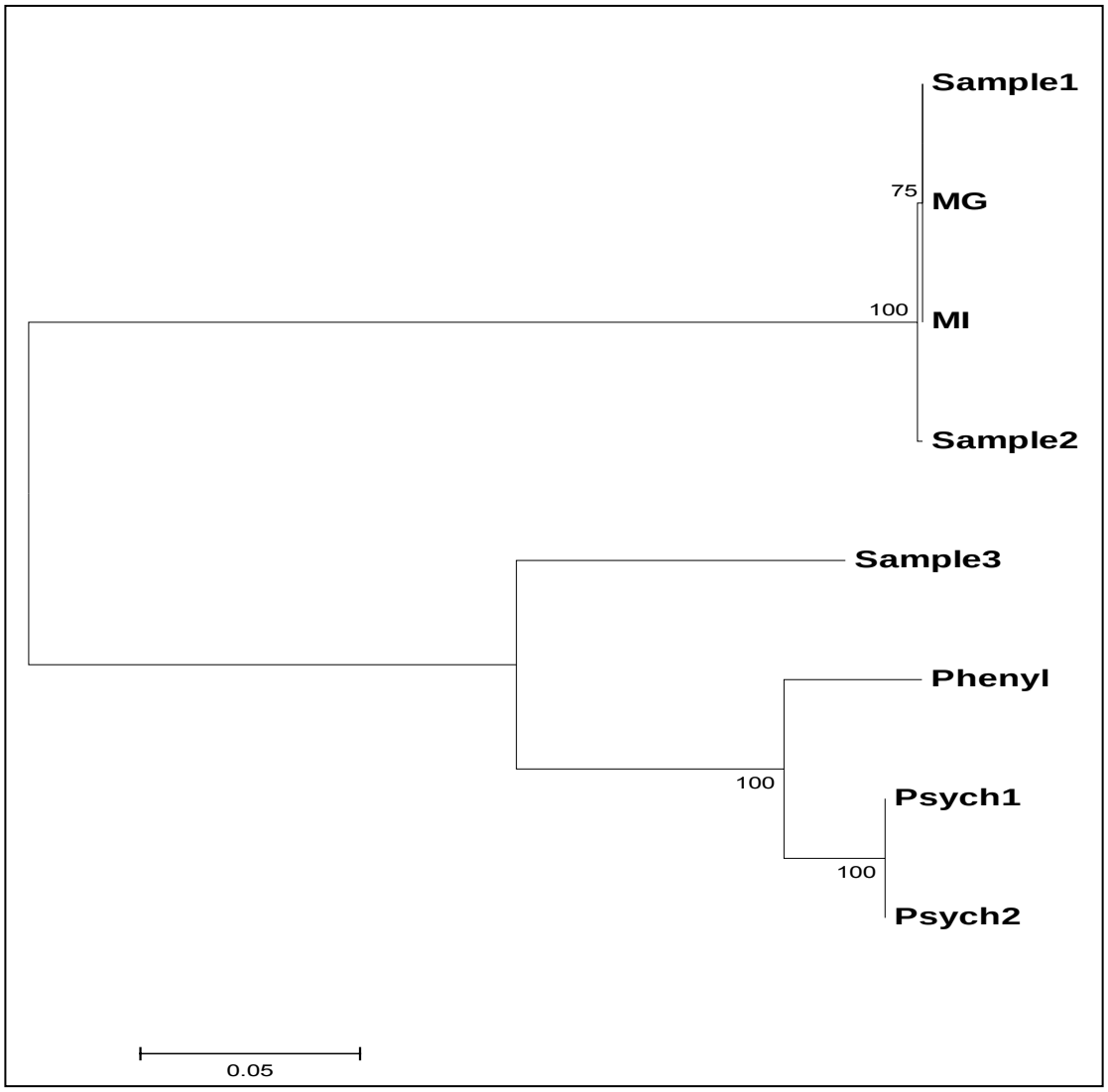


Figure 4.9: A phylogenetic tree based on the partial 16S rRNA gene sequences of the reference strains of *M. gallisepticum* (MG) and *M. imitans* (MI), samples that had the highest homology to *M. gallisepticum* and *M. imitans* (Samples 1-3) as well as those found to be *Psychrobacter* (Psych 1& 2). Phenyl represents a *Psychrobacter* reference, *P. phenylpyruvicus*. Constructed using MEGA version 3 Software.

The *Mgc2* gene phylogenetic tree.

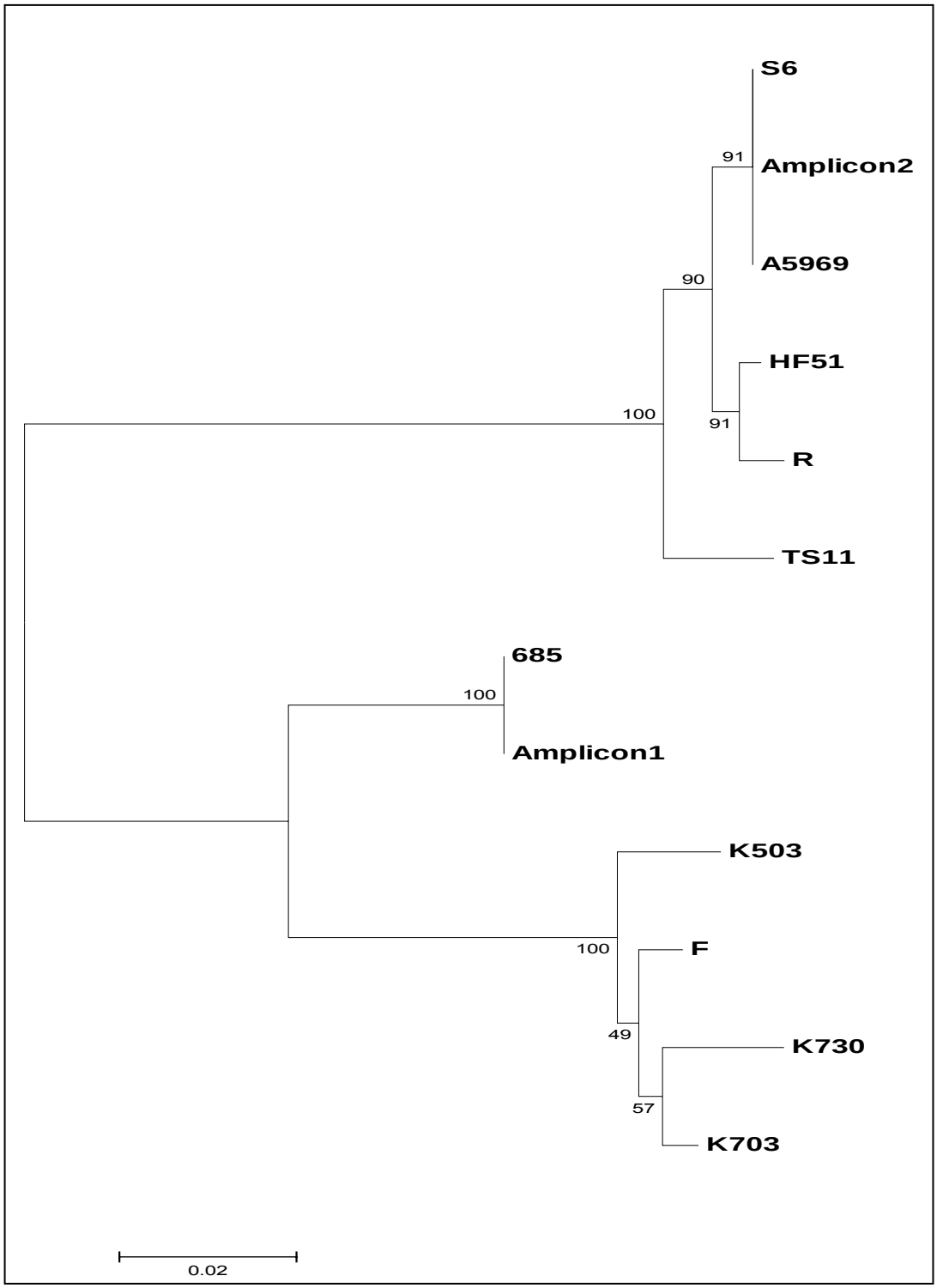


Figure 4.10: A phylogenetic tree based on the *Mgc2* gene of the 10 reference strains and two products of the *Mgc2*-PCR designated Amplicon 1 & 2. Constructed using MEGA version 3 Software.

4.5 Discussions of results.

The first aim of this Chapter was to identify the amplicons obtained from the PCR assays to strain level. The results obtained in achieving this aim are of vital importance. Three amplicons of the 16S rRNA gene PCR showed the highest homology to the same organisms. The organisms were found to be either *M. gallisepticum* or *M. imitans*, which were expected as products of this PCR. The two *M. gallisepticum* isolates that came up were the R and A5969 strains. This might indicate the possibility of the isolates obtained in this study as being either of these two strains. However, the possibility of the samples being either of those two strains only, was disproved by further investigations made in Genbank.

These investigations were to quantify the information available on *M. gallisepticum* in Genbank. It was thus found that completed sequences of the 16S rRNA gene in Genbank for *M. gallisepticum* are of the strains: R and A5969 only, while the rest are partial sequences of limited information. For this reason, any other sequence obtained by an investigator on a certain isolate will be limited to these two strains only when a search is made in Genbank, regardless of whether the strain is R, A5969 or neither. A further limitation is that any novel species or strain that is similar to *M. gallisepticum* will be recognized as being either one of the two strains or *M. imitans.*

As expected, *M. imitans* was present in the results as being one of the possible species to the amplicon obtained. However, it is not clear from Genbank which strain it is as it is only entered as a gene. It is however evident that the degree of relatedness decreases when the BLAST results start outlining other species of *Mycoplasma*. This is proof that the organisms are definitely of *M. gallisepticum* or *M. imitans* origin, or a novel species that is closely related to these two organisms than any other currently known *Mycoplasma* species.

The Figures 4.4 and 4.5 show strange and unexpected results that indicate that the highest homology of the isolates is to the Genus *Psychrobacter* with *P. phenylpyruvicus* being the most likely species. The results were thought to be strange initially due to the name *Psychrobacter* possibly indicating an extremophilic genus. The members of the genus are however psychotolerant (Bozal *et al*., 2003) and grow at temperatures of approximately -10 ^{0}C but can also grow at much higher temperatures of 40 ^{0}C. Isolation of some of these organisms has been reported from poultry (Juni & Heym, 1986) while Bowman and co-workers reported isolation from the faecal matter of a variety of bird species.

Following this information, the two isolates found to be *Psychrobacter* and the three found to be *Mycoplasma* were used to construct a Phylogenetic tree. References used were the *M. gallisepticum* strain R, the *M. imitans* isolate and the *P. phenylpyruvicus* sequences (Figure 4. 9). The results from the tree show that Sample 1, *M. gallisepticum* and *M. imitans* are very similar as they form one cluster. Joining this cluster is Sample 2 at a 100 % confidence value. This clustering supports what was found when a BLAST search was performed. The search indicated that Samples 1 to 3 are *Mycoplasma*.

Interesting to note is that Sample 3, also found to be *Mycoplasma*, is very far from the other *Mycoplasma* species on the tree. This sample is rather closer to the *Psychrobacter* isolates as it joins a *Psychrobacter* cluster at a 100 % confidence value. This suggests a *Mycoplasma* that has evolved away from the other *Mycoplasma*. It is important to note that this tree was drawn solely to show the relatedness of partial sequences of Samples 1-3 with those in Genbank and interest was only in these isolates. It is therefore very important to consider the differences that are apparent from the tree at this region, as it seems that Sample 1 is the only very closely related species to *M. gallisepticum* and *M. imitans*. The alignments outline the variable and non-variable regions that were important for the construction of the tree drawn.

The second tree (Figure 4.10) is based on the *Mgc2* gene products and the ten reference strains used. Two of the five amplicons could be properly sequenced and the BLAST results are outlined in Table 4.3 and 4.4 for Amplicon 2 and 1, respectively. The results also show a tremendous distance in the two amplicons. Amplicon 2 has clustered on higher branches in the tree while Amplicon 1 has clustered on the second branch of the tree. Important to note is also the fact that Amplicon 2 has clustered with the two wildtype pathogenic strains while Amplicon 1 has clustered with the vaccine strain 6/85. The sequence of the 6/85 strain is very particular as can be seen in Figure 4.8. There are many gaps present in the sequence of this strain and such gaps can only be seen in Amplicon 1 in the same alignment figure. Furthermore, Figure 3.6 lane 3 indicates the first detection of this isolate and it can be seen from the gel picture that the size of the band is a bit smaller (by position) that the other bands and among all the ten reference strains, strain 6/85 has the smallest *Mgc2* size. In addition to this the RFLP profile obtained for this strain (Figure 3.11 and Table 3.2) is somewhat similar to that of the 6/85 strain, strongly suggesting that this strain is 6/85 or a mutant thereof.

The results of the tree are in correlation with those in Table 4.3 and 4.4. In Table 4.3, for the identification of Amplicon 2, there are many strains from the K-series after which the only other strains are S6 and A5969 which can be clearly seen in the table as well. The K-series strains, S6 and A5969 were all isolated from the USA in various states and some of the K-series strains that came out of a search in Table 4.3 were involved in various outbreaks in the USA (Ferguson *et al*., 2005). Using GTS (Gene-targeted Sequencing) and RAPD, Ferguson and co-workers (2005) showed that the strain A6959 and S6 fall in the same group with reference to their *Mgc2* properties. The tree in Figure 4.10 correlates well with this data.

There is an evolutionary history behind the strains that came up in Table 4.4, that were listed as being similar to Amplicon 1. These strains were all isolated from

the USA and were found to have either originated from 6/85-vaccinated flocks or were found to be very similar to the vaccine strain 6/85 when they were isolated from the field. This serves as an even greater suggestion of Amplicon 1 being of 6/85 origin. When Ferguson and co-workers (2005) investigated these strains they found that the *Mgc2*-profiling of these strains was also the same as that of the 6/85 strain. Other than the similar *Mgc2*-profiling between these strains, results on other targeted genes yielded the same results. It is not clear if these strains are pathogenic, but due to their very high similarity to the vaccine strain they may also have similar properties.

The DGGE assays were used to investigate whether the amplicons from the PCR products were indeed mixed. Figure 4.2, which is the DGGE analysis of the amplicons obtained by amplification with the universal primers, shows the vast number of organisms that are present in the samples and this might also give an idea of the under-representation of the desired test-organism. The gel photographs in Figure 4.1 which are the same amplicons obtained with the universal primers, showed these samples as single bands but from using DGGE it is apparent that there are many more bands. This proved the efficacy of this method and therefore DGGE was chosen as a method of choice to ascertain whether the samples that could not be sequenced were indeed mixed. There is great scope for diversity studies in this case.

Figure 4.3 shows the DGGE analyses of the samples as they were obtained in Chapter 3. It is believed that the DNA of the amplicons was denatured and the bands hence represent single-stranded DNA. This is due to the exclusion of the GC-clamp from the assays. To make a more accurate quantification of the isolates detected with the 16S rRNA gene-PCR, a GC-clamp was included in the PCR assay and samples could still be detected with the same PCR parameters (Figure 4.4). The new amplicons were run on the DGGE gel and the number of bands was reduced (Figure 4.5). This is due to fact that a GC-clamp prevents

double-stranded DNA from separating and the single strands from presenting as many bands when a gel is viewed.

Important to note in Figure 4.5 are the two bands in lane 1 which contains Ts-11, a commercial live vaccine. In theory this would mean that Ts-11 might be mixed, while on the other hand, the most probable reason is that Ts-11 has two copies of this gene in its genome. If proven further, this hypothesis will greatly improve information on this vaccine strain. Lane 2 in the same figure has the same profile as that of the Ts-11 strain while the rest of the lanes have different profiles to these two. This is an indication that while some flocks might be vaccinated with the Ts-11 vaccine, others are infected with wild-type *Mycoplasma*. Due to time constraints, the bands in all the lanes could not be fully characterized, but the work completed has provided significant results.

4.6 Conclusions

The diversity of *M. gallisepticum* in South Africa was further proven by sequencing results of the *Mgc2* gene in addition to the RFLP analyses obtained. It has been shown in this study that isolates found in South Africa share certain characteristics with those isolated in the USA. However, lack of information on the South African isolates limits the exact identification of organisms under test.

While some *Mycoplasma* isolates seem to be related to those that have been isolated outside South Africa, a phenomenon of a totally different *Mycoplasma* isolate was strongly suggested in this study based on the phylogenetic tree.

To further prove the different *Mycoplasma* strains, biological properties in support of these findings will be required. Furthermore, to determine that the different *Mycoplasma* are indeed novel, serological tests need to be performed as they will provide more insight into the antigenic diversity of these *Mycoplasma*. This

proves the limitations in molecular techniques if used without coupling with serological techniques as well as challenge studies, emphasizing the importance of serology.

The vaccine strain Ts-11, used as a positive control in this study, might contain two copies of the amplified region of the 16S rRNA gene. This shows the limitation of the DGGE analyses as it might indicate such a case of two copies as different organisms. Alternatively, Ts-11 might indeed be mixed with an organism of the same sequence in this region.

4.6 Future research

For further characterization, organisms must be grown and challenge experiments performed. Bands from DGGE must also be characterized. However, it would be necessary for a number of reference strains of *M. gallisepticum* to be obtained for further optimization of the assay. Those reference strains can be used as markers to which the tested samples can be compared. Cloning of these amplicons is also advised.

CHAPTER 5

SUMMARY

Mycoplasma gallisepticum is the most pathogenic avian *Mycoplasma* species and leads to great economic losses worldwide (Kleven, 2003). Prevention and control of this organism has been achieved by vaccination and antibiotic administration. Ferraz and Danelli (2003) reported on the most widely used live vaccines which are MG-F, Ts-11 and 6/85. The MG-F strain is currently not registered for use in South Africa.

M. imitans is the most closely related organism to *M. gallisepticum* and was tentatively identified as *M. gallisepticum* until Bradbury and co-workers (1993) defined it as a different species. However, this species is still misidentified as *M. gallisepticum* due to serological cross-reactivity and *M. gallisepticum* specific primers also detecting *M. imitans* (Markham *et al*., 1999).

While *M. gallisepticum* has been characterized in some countries, very little information is documented on the South African isolates. It therefore became the aim of this study to investigate the presence and diversity of this organism in Southern Africa, investigate the presence of *M. imitans* as well as to establish the relatedness of the Southern African strains to those isolated outside Africa.

Samples were collected from serologically positive birds from different farms in South Africa and Zimbabwe. Two PCR (Polymerase Chain Reaction) assays were optimized, one to detect *M. gallisepticum* of a specific gene *Mgc2,* the other to detect both *M. gallisepticum* and *M. imitans*.

A total of five samples were detected with the *Mgc2*-PCR, while almost all samples could be detected with the 16S rRNA gene-PCR. This is a possible indication of a different *M. gallisepticum* isolate that can be detected on the 16S

rRNA gene level but not at the *Mgc2* level, or the isolate could indeed be *M. imitans*. In a study by Woese and co-workers (1985), the *Mgc2* gene was implicated as one of the rapidly mutating genes due to adaptation, a possible reason for the non- detection of *Mgc2* but the detection of the 16S rRNA gene.

Of the sequenced 16S rRNA gene-PCR amplicons, *M. gallisepticum* and *M. imitans* had the highest homology. However, there are only two complete sequences of the 16S rRNA genes that belong to two *M. gallisepticum* strains deposited in Genbank, thus limiting the information on the isolates detected. Restriction Fragment Length Polymorphisms (RFLPs) were performed and well optimized. Ts-11 gave the expected profile while tested samples could not be placed in any of the reference groups. It was observed, however, that the RFLP profile of one of the amplicons was similar to the 6/85 vaccine strain and subsequent results correlated with this finding. Two of the amplicons could be sequenced and further analyzed.

A phylogenetic tree showed one of the amplicons clustering away from the other *Mycoplasma* species though its sequence was found to be that of *M. gallisepticum* or *M. imitans*. In another tree, one of the amplicons showed more homology to the pathogenic strains while the other showed more homology to the vaccine strain 6/85.

The DGGE analyses showed that the amplicons consist of a mixed template which was the reason why some samples could not be properly sequenced. This might be an indication of mixed infection within the flocks. However, it was expected of the 16S rRNA to give these results. Furthermore, the DGGE results indicated that the vaccine strain Ts-11 is used to vaccinate some of the flocks, while other flocks are infected with wild-type *Mycoplasma*. The results also suggest the possibility of the presence of two copies of the amplified region of the 16S rRNA gene in this vaccine strain.

The study has confirmed the presence of *M. gallisepticum* and the possible presence of *M. imitans*. Different yet closely related *Mycoplasma* isolates are also present in South Africa. These could represent novel strains or species and warrant further investigation.

OPSOMMING

Mycoplasma gallisepticum is die mees patogeniese voël *Mycoplasma* spesie en ly tot groot ekonomiese verliese wêreldwyd (Kleven, 2003). Voorkoming en beheer van hierdie organisme is bewerkstellig deur vaksinasie. MG-F, Ts-11 en 9/85 is volgens Ferraz en Danelli (2003) die mees gebruikte lewendige entstowwe. Die MG-F stam is tans nie geregistreer vir gebruik in Suid Afrika nie.

M. imitans is die naaste verwant aan *M. gallisepticum* en was eksperimenteel geïdentifiseer as *M. gallisepticum* totdat Bradbury en medewerkers (1993) dit as 'n verskillende spesie geïdentifiseer het. Hierdie spesie word egter nog steeds verkeerdelik geïdentifiseer as *M. gallisepticum* as gevolg van serologiese kruisreaktiwiteit en omdat die *M. gallisepticum* spesifieke voorvoerders ook *M. imitans* identifiseer (Markham *et al*., 1999).

M. gallisepticum is al geïdentifiseer in sommige lande, maar baie min inligting is beskikbaar oor die voorkoms van hierdie organisme in Suid-Afrika. Dit het gevolglik die doel van hierdie studie geword om die teenwoordigheid en diversiteit van die organismes in Suider-Afrika te ondersoek, die teenwoordigheid van *M. imitans* in Suid-Afrika te ondersoek asook om die verwantskap van die Suider-Afrikaanse spesies met die wat buite Afrika geïsoleer is, te bepaal.

Monsters is versamel van serologies positiewe voëls vanaf verskillende plase in Suid-Afrika en Zimbabwe. Twee PKR reaksies was gestandaardiseer, een om die *M. gallisepticum* deur middel van amplifikasie van die *Mgc2* geen te

identifiseer en ’n tweede PKR reaksie om *M. gallisepticum* en *M. initans* te identifiseer.

’n Totaal van vyf monsters is positief geïdentifiseer deur middel van die *Mgc2*-PKR terwyl die meeste geïdentifiseer was met die 16S rRNA-PKR. Hierdie is ’n mootlike aanduiding dat ’n verskillende *M. gallisepticum* isolaat geïdentifiseer is op die vlak van die 16S rRNA maar nie op die vlak van die *Mgc2* geen nie. Die moontlikheid bestaan egter wel dat hierdie isolaat wel *M. imitans* kan wees. In ’n studie van Woese en medewerkers (1985) was die *Mgc2* geen uitgewys as een van die vinnigste muterende gene as gevolg van aanpassing en mag dalk nog ’n rede wees waarom identifikasie deur gebruik te maak van die *Mgc2* geen onsuksesvol was.

Homologie van die basisopeenvolgings van die 16S rRNA PKR produkte was die hoogste tussen *M. gallisepticum* en *M. imitans*. Daar is egter slegs twee volledige basisopeenvolgings van die 16S rRNA geen beskikbaar op die Genbank databasis wat behoort aan twee stamme van *M. gallisepticum* wat inligting oor die isolate beperk. Restriksie Fragment Lengte Polimorfisme (RFLP) analises is uitgevoer en gestandaardiseer. Die verwagte profiel is verkry met Ts-11 maar die ander monsters wat getoets is kon in geen verwysings groep geplaas word nie. Resultate het egter gewys dat een van die PKR produkte ’n RFLP profiel gehad het soortgelyk aan die van die 6/85 entstof, verdere analise het hierdie resultaat bevestig. Die basispaaropeenvolging van twee van die PKR produkte is bepaal en verder geanaliseer.

Filogenetiese analise het gewys dat een van die PKR produkte groepeer weg vanaf die ander *Mycoplasma* isolate alhoewel die basispaaropeenvolging aandui dat dit wel *M. gallisepticum* of *M. imitans* is. In ’n ander filogenetiese boom toon een van die PKR produkte homologie teenoor die patogeniese stamme terwyl die ander homologie tot die entstof stam 6/85 toon.

DGJE analise het aangetoon dat die PKR produkte uit ’n gemengde populasie bestaan het en dit kan die mootlike rede wees waarom die basisopeenvolging van hierdie monsters nie bepaal kon word nie. Dit kan dalk ook beteken dat daar ’n gemengde infeksie was in die voëls. Dit was egter verwag dat 16S rRNS resultate hierop moes gewys het. Volgens DGJE analise is die entstof stam Ts-11 gebruik om sommige van die voëls in te ent terwyl ander met die wilde tipe *Mycoplasma* geïnfekteer was. Die resultate dui ook tot die moontlike teenwoordigheid van twee kopieë van die geamplifiseerde deel van die 16S rRNA geen in die entstof stam.

Hierdie studie het die teenwoordigheid van *M. gallisepticum* en die moontlike teenwoordigheid van *M. imitans* bevestig. Verskillende, maar naby verwante *Mycoplasma* isolate, is ook teenwoordig in Suid-Afrika. Hierdie kan unieke stamme of spesies verteenwoordig en regverdig verdere ondersoek om dit te bevestig.

<u>Keywords</u>:

Denaturing Gradient Gel Electrophoresis.
***Mgc2*.**
Mycoplasma gallisepticum.
Mycoplasma imitans.
Polymerase Chain Reaction.
Restriction Fragment Length Polymerase.
South Africa.

CHAPTER 6

REFERENCES

Abdul-Wahab, O. M. S., Ross, G & Bradbury, J. M. (1996). Pathogenecity and cytadherence of *Mycoplasma imitans* in chicken and duck embryo tracheal organ cultures. *Infect and Immun* 64(2), 563-568.

Bencina, D., Mrzel, I., RoJs, O. Z., Bidovec, A. & Dovc, A. (2003). Characterization of *Mycoplasma gallisepticum* strains involved in respiratory disease in pheasants and peafowl. *Vet Rec* 152(8), 230-234.

BLAST: www.ncbi.nlm.nih.gov/blast/Blast.cgi

Boettger, C. M. & Dohms, J. E. (2006). Separating *Mycoplasma gallisepticum* field strains from nonpathogenic avian Mycoplasmas. *Avian Dis* 50, 605-607.

Boguslavsky, S., Menaker, D., Lynsnyansky, I., Liu, T., Levisohn, S., Rosengarten, R., Garcia M. & Yogev, D. (2000) Molecular characterization of the *Mycoplasma gallisepticum pvpA* gene which encodes a putative variable cytadhesin protein. *Infect and Immun* 68(7), 3956-3964.

Botes, A., Peyrot, B. M., Olivier, A.J., Burger, W. P. & Bellstedt, D. U. (2005). Identification of three novel *Mycoplasma* species from ostriches in South Africa. *Vet Microbiol* 111, 159-169.

Bozal, N., Montes, M. J., Tudela, E. & Guinea, J. (2003). Characterization of several Psychrobacter strains isolated from Antarctic environments and description of *Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov. *Int J Syst and Evol Microbiol* 53, 1093-1100.

Bradbury, J. M., Abdul-Wahab, O. M., Yavari, C. A., Dupiellet, J. P., & Bove, J. M. (1993). *Mycoplasma imitans* sp. nov. is related to *Mycoplasma gallisepticum* and found in birds. *J Syst Bacteriol* 43(4), 721-728.

Bradbury, J. M. & Kleven, S. H. (2003). Mycoplasmosis. In *Diseases of Poultry,* 11th edn: pp.719-774. Edited by Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne. Blackwell Publishing.

Brossette, S. & Wartell, R. M. (1994). A program for selecting DNA fragments to detect mutations by denaturing gel gradient electrophoresis methods. *Nucl Acids Res* 22(20), 4321-4325.

Charlton, B. R., Bickford, A. A., Walker, R. L. & Yamamoto, R. (1999). Complementary randomly amplified polymorphic DNA (RAPD) analysis pattern and primer sets to differentiate *Mycoplasma gallisepticum* strains. *J Vet Diagn Invest* 11(2), 158-161.

Chin, R. P., Ghazikhanian, G. Y. & Kempf, I. (2003). Mycoplasmosis. In *Diseases of Poultry,* 11th edn: pp.719-774. Edited by Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne. Blackwell Publishing.

ClustalW: www.ebi.ac.uk/Tools/clustalw/index.html

DNAssist Version 2.2 program: www.dnassist.com

Edward, D. G. & Kanarek, A. D. (1960). Organisms in the pleuropneumonia group of avian origin: their classification into species. *Ann New York Acad Sci* 79, 696-702.

Evans, R. D. & Hafez, Y. S. (1992). Evaluation of a *Mycoplasma gallisepticum* strain exhibiting reduced virulence for prevention and control of poultry mycoplasmosis. *Avian Dis* 36, 197-201.

Ferguson, N. M., Leiting, V. A. & Kleven, S. H. (2004). Safety and efficacy of the avirulent *Mycoplasma gallisepticum* strain K5054 as a live vaccine in poultry. *Avian Dis* 48, 91-99.

Ferguson, N. M., Hepp, D., Sun,S., Ikuta, N., Levisohn, S. & Garcia, M. (2005). Use of molecular diversity of *Mycoplasma gallisepticum* by gene-targeted sequencing (GTS) and random amplified polymorphic DNA (RAPD) analysis for epidemiological studies. *Microbiology* 151, 1883-1893.

Ferraz, N. P. & Danelli, M. M. (2003). Phenotypic and antigenic variation of *Mycoplasma gallisepticum* vaccine strains. *Braz J Microbiol* 34, 238-241.

Garcia, M., Jackwood, M. W., Levisohn, S. & Kleven, S. H. (1995). Detection of *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma iowae* by multispecies polymerase chain reaction and restriction fragment length polymorphism. *Avian Dis* 39(3), 606-616.

Garcia, M., Ikuta, N., Levisohn, S. & Kleven, S. H. (2005). Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chickens. *Avian Dis* 49, 125-132.

Ghosh, A., Das, J. & Maniloff, J. (1997). Lack of repair of ultraviolet light damage in *Mycoplasma gallisepticum*. *J Mol Biol* 116(2), 337-344.

Goh, M. S., Gorton, T. S., Forsyth, M. H., Troy, K. E. & Geary, S. J. (1998). Molecular and biochemical analysis of a 105 kDa Mycoplasma gallisepticum cytadhesin (GapA). *Microbiology* 144, 2971-2978.

Harasawa, R., Pitcher, D. G., Ramirez, A. S. & Bradbury, J. M. (2004). A putative transposase gene in the 16S-23S rRNA intergenic spacer region of *Mycoplasma imitans*. *Microbiology* 150, 1023-1029.

Hnatow, L. L., Keeler,jr., C. L., Tessmer, L. I., Czymmek, K. & Dohms, J. E. (1998). Characterisation of MGC2, a *Mycoplasma gallisepticum* cytadhesin with homology to the *Mycoplasma pneumoniae* 30-kilodalton protein P30 and *Mycoplasma genitalium* P32. *Infect and Immun* 66(7), 3436-3442.

Hong, Y., Garcia, M., Levisohn, S., Lysnyansky, I., Leiting, V., Savelkoul, P. H. M. & Kleven, S. H. (2005a). Evaluation of amplified fragment length polymorphism for differentiation of avian *Mycoplasma* species. *J Clin Microbiol* 43(2), 909-912.

Hong, Y., Garcia, M., Levisohn, S., Savelkoul, P., Leiting, V., Lysnyansky, I., Ley, D. H. & Kleven, S. H. (2005b). Differentiation of *Mycoplasma gallisepticum* and other DNA-based typing methods. *Avian Dis* 49(1), 43-49.

Juni, E. & Heym, G. A. (1986). *Psychrobacter immobilis* gen. nov., sp. nov.: Genospecies composed of Gram-negative, aerobic, oxidase-positive coccobacilli. *Int J Syst Bacteriol* 36(3), 388-391.

Kiss, I., Matiz, K., Kaszanyitzky, E., Chavez, Y. & Johansson, K. E. (1997). Detection and identification of avian mycoplasmosis by polymerase chain reaction and restriction fragment length polymorphism assay. *Vet Microbiol* 58, 23-30.

Kleven, S. H. (2003). Mycoplasmosis. In *Diseases of Poultry,* 11th edn: pp.719-774. Edited by Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne. Blackwell Publishing.

Kleven, S. H., Fulton, R. M., Garcia, M., Ikuta, V. N., Leiting, V. A., Liu, T., Ley , D. H., Opegart, K. N., Rowland, G. N. and others. (2004). Molecular characterization of *Mycoplasma gallisepticum* isolates from turkeys. *Avian Dis* 48, 562-569.

Lauerman, L. H., Chilina, A. R., Closser, J. A. & Johansen, D. (1995). Avian *Mycoplasma* identification using polymerase chain reaction amplicons and restriction fragment length polymorphism analysis. *Avian Dis* 39, 804-811.

Levisohn, S. & Kleven, S. H. (2000). Avian mycoplasmosis (*Mycoplasma gallisepticum*). *Rev Sci Tech Off Intern Epizo* 19(2), 425-442.

Ley, D. H., Berkhoff, J. E. & Levisohn, S. (1997a). Molecular epidemiological investigations of *Mycoplasma gallisepticum* conjunctivitis in songbirds by random amplified polymorphic DNA analyses. *Emerg Infect Dis* 3(3), 375-380.

Ley, D. H., Laren, J. M., Miles, A. M., Barnes, H. J., Miller, S. H. & Franz, G.(1997b). Transmissibility of live *Mycoplasma gallisepticum* vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry. *Avian Dis* 41(1), 187-194.

Ley, D. H. (2003). Mycoplasmosis: *Mycoplasma gallisepticum* infection. In *Diseases of Poultry,* 11th edn: pp.719-774. Edited by Y. M. Saif, H. J. Barnes, J. R. Glisson, A. M. Fadly, L. R. McDougald & D. E. Swayne. Blackwell Publishing.

Liu,T., Garcia, M., Levisohn, S., Yoger,D. & Kleven, S.H. (2001). Molecular variability of the adhesion-coding gene *pvpA* among *Mycoplasma gallisepticum* strains and its application in diagnosis. *J Clin Microbiol* 39(5), 1882-1888.

Markham, P. F., Duffy, M. F., Glew, M. D. & Browning, G. F. (1999). A gene family in *Mycoplasma imitans* closely related to the pMGA family of *Mycoplasma gallisepticum*. *Microbiology* 145, 2095-2103.

Marois, C., Dufour-Gesbert, F. & Kempf, I. (2001). Molecular differentiation of *Mycoplasma gallisepticum* and *Mycoplasma imitans* strains by pulse-field gel electrophoresis and random amplified polymorphic DNA. *J Vet Med* 48, 695-703.

McAuliffe, L., Ellis, R. J., Ayling, R. D. & Nicholas, R. A. J. (2003). Differentiation of *Mycoplasma* species by 16S Ribosomal DNA PCR and denaturing gradient gel electrophoresis fingerprinting. *J Clin Microbiol* 41(10), 4844-4847.

McAuliffe, L., Ellis, R. J., Lawes, J. R., Ayling, R. D. & Nicholas, R. A. J. (2005). 16S rDNA PCR and denaturing gradient gel electrophoresi; a single generic test for detecting and differentiating *Mycoplasma* species. *J Med Microbiol* 54, 731-739.

McMartin, D. A., Khan, M. I., Farver, T. B. & Christie, G. (1987). Delineation of the lateral spread of *Mycoplasma gallisepticum* infection in chickens. *Avian Dis* 31, 814-819.

MEGA Version 3: www.megasoftware.net

Mekkes, D. R. & Feberwee, A. (2005). Real-time polymerase chain reaction for the qualitative and quantitative detection of *Mycoplasma gallisepticum*. *Avian pathol* 34(4), 348-354.

Nascimento, E. R., Nascimento, M. G. F., Santos, M. W., Dias, P. G. O., Resende, O. A. & Silva, R. C. F. (2005a). Eradication of *Mycoplasma gallisepticum* and *M. synoviae* from a chicken flock by antimicrobial injections in eggs and chicks. *Acta Sci Vet* 33(2), 119-124.

Nascimento, E. R., Pereira, V. L. A., Nascimento, M. G. F. & Barreto, M. L. (2005b). Avian mycoplasmosis update. *Braz J Poult Sci* 7(1), 01-09.

Nebcutter Version 2 software: tools.neb.com/**NEBcutter**2/index.php

OIE: World Organization for Animal Health: http://www.oie.int/eng/normes/mmanual/A_00104.html

Papazisi, L., Silbart, L. K., Frasca,jr., S., Rood, D., Liao, X., Gladd, M., Javed, M. A. & Geary, S.J. (2002). A modified live *Mycoplasma gallisepticum* vaccine to protect chickens from respiratory disease. *Vaccine* 20, 3709-3719.

Papazisi, L., Gorton, T. S., Kutish, G., Markham, F., Browning, G. F., Nguyen, D., Swartzell, S., Madan, A., Mahairas, G. & others. (2003). The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain $R_{low.}$ *Microbiology* 149, 2307-2316.

Poultrymed: www.poultrymed.com

Razin, S. (1996). Mycoplasmas. In *Medical Microbiology*, 4th edn: Chapter 37. Edited by Baron, S., Peake, R. C., James, D. A., Susman, M., Kennedy, C. A., Singleton, M. J. D. & Schuenke, S. The University of Texas Medical Branch: Galveston.

Talha, A. F. S., Bisgaard, M. & Das, P. M. (Thesis, 2003). Investigation of the prevalence and significance of *Mycoplasma gallisepticum* in layer chickens in Bangladesh. The royal Veterinary and Agricultural University: Department of veterinary Microbiology, 17 Bulowsvej, DK-1870, Mymensingh, Bangladesh.

Turner, K. S. & Kleven, S. H. (1998). Eradication of live F-strain *Mycoplasma gallisepticum* vaccine using live ts-11 on a multiage commercial layer farm. *Avian Dis* 42(2), 404-407.

Whithear, K. G., Harringan, K. E. & Ghiocas, E. (1990). Safety of temperature sensitive mutant *Mycoplasma gallisepticum* vaccine. *Aust Vet J* 67(5), 159-165.

Winner, F., Rosengarten, R. & Citti, C. (2000). In vitro cell invasion of *Mycoplasma gallisepticum*. *Infect and Immun* 68(7), 4238-4244.

Woese, C. R., Stackebrandt, E. & Luwig W. (1985). What are mycoplasmas: the relationship of tempo and mode in bacterial evolution. *J Mol Evol* 21, 305-316.

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