A molecular study of *Mycoplasma gallisepticum* field isolates from poultry in southern Africa

by

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LIST OF ABBREVIATIONS AND DEFINITIONS

bp	(nucleotide) base pair
DGGE	denaturing gradient gel electrophoresis
ELISA	enzyme-linked immunosorbent assay
GSH	glutathione
GTS	gene-targeted sequencing
ITS	intergenic spacer
MB	*Mycoplasma* broth
MG	*Mycoplasma gallisepticum*
MG $R_{high/low}$	*M. gallisepticum* high/ low passage strains, thus affecting the pathogenicity.
Mycoplasmas	used in literature to describe isolates belonging to the genus *Mycoplasma*
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
rDNA	ribosomal DNA
RT	room temperature
SOD	superoxide dismutase
str.	strain
spp.	species

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The name *Mycoplasma* originates from the Greek words *mykes* and *plasma*, meaning fungus and formed, respectively (Krass & Gardner, 1973). A. B. Frank was the first to use this term in 1889, however incorrectly referring to it as a fungus (Krass & Gardner, 1973). There is, perhaps, no other group of bacteria that caused so much controversy and confusion as to its identity and taxonomic status; and for good reason. *Mycoplasma* species (referred to as "mycoplasmas" in literature) are known to have the smallest genome among free-living and self-replicating organisms (Morowitz, 1985), with a G + C content as low as 23-40% (Razin & Tully, 1983). These small prokaryotic organisms lack a cell wall and are bound by a plasma membrane which is reinforced as a result of the incorporation of sterols. The use of UGA to encode tryptophan, as opposed to its use as the universal stop codon, and the utilization of cholesterol further distinguish the genus *Mycoplasma* from other prokaryotes (Razin, 1983a).

The extreme simplicity and compactness of *Mycoplasma* cells led Morowitz and Wallace (1973) to propose that mycoplasmas be placed at the root of the phylogenetic tree, representing the descendants of bacteria that existed prior to the development of the peptidoglycan cell wall. This notion was then challenged by Neimark (1986) who believed mycoplasmas originated from walled bacteria by degenerative evolution. Neimark's theory was then later supported by the introduction of sequencing the rRNA as a phylogenetic measure (Woese *et al*., 1980), which more specifically defined mycoplasmas as a group of eubacteria, phylogenetically related to gram-positive bacteria.

To date, *Mycoplasma* belongs to the phylum Firmicutes, class Mollicutes, order Mycoplasmatales and the family Mycoplasmataceae largely based on 16S rRNA analysis

(Razin *et al.*, 1998). The genus contains both non-pathogenic and pathogenic species found in a wide variety of animal hosts. Of the avian pathogens, *Mycoplasma gallisepticum* is the most virulent and continues to be a major problem in the expanding poultry industry, causing outbreaks world-wide, and leading to great economic losses (Evans *et al*., 2005; Southern African Poultry Association, 2010). The outbreaks are usually controlled with the use of attenuated vaccines when complete eradication of the pathogen is difficult to attain. Accurate and sensitive detection of the pathogenic *M. gallisepticum* strains therefore plays an essential role in the control of the outbreaks. Different methods of detection may be used, including: isolation and cultivation, serological, and molecular techniques. This literature review will further explore these techniques and their history of success in the detection of *M. gallisepticum (*abbreviated as *MG).*

1.2. PATHOGENICITY

1.2.1 PATHOGENIC Mycoplasma SPECIES IN THE POULTRY INDUSTRY

Four avian *Mycoplasma* species, namely *M. gallisepticum*, *M. synoviae*, *M. meleagridis*, and *M. iowae* are commonly recognised as the main poultry pathogens. Of these, *M. gallisepticum* is the most virulent, causing chronic infections in both chickens and turkeys (Bradbury *et al.*, 1993; Ganapathy & Bradbury, 1998). It is usually the first to colonize the respiratory tract, causing a primary infection, followed by a secondary infection caused by *Escherichia coli* (*E. coli*) or viruses which result in severe air sac infection (Liu *et al.*, 2001). *M. gallisepticum* has a wide variety of clinical manifestations, of which chronic respiratory disease of chickens and sinusitis of turkeys are the most significant. Other symptoms include synovitis and arthritis, poor performance, skeletal deformities, embryo mortalities and lowered egg production caused by oviduct infections in chickens, all contributing to economic losses (Ley, 2003).

Similar symptoms have been reported in other avian *Mycoplasma* spp. such as *M. synoviae* and *M. meleagridis,* although with less severity. *M. synoviae* infection usually occurs as a subclinical upper respiratory tract infection and synovitis in chickens and turkeys. *M. iowae,* on the other hand, causes decrease in hatchability and high embryo mortality in turkeys while *M. meleagridis* is the cause of an egg-transmitted disease in turkeys in which the primary lesion is an airsacculitis in the progeny, which leads to lower hatchability and skeletal abnormalities in young turkeys (Hong *et al*., 2005; Khan, 2002). Due to these similarities, symptoms as the sole means of diagnosis should be avoided.

1.2.2. PATHOGENIC MECHANISMS

The pathogenesis of *Mycoplasma* disease is a complex process influenced by the genetic background of both the host and the organism, environmental factors, and the presence of other infectious agents (Simecka *et al.,* 1992). The infectivity, tissue tropism, and pathogenicity among various *M. gallisepticum* strains differ significantly (Domanska-Blicharz *et al*., 2008). There are a number of attributes of mycoplasmas that are likely to affect disease pathogenicity. These include the ability to attach to the host cells, to cause cell injury, to vary phenotype at a high frequency, and to modulate and resist the host immune response.

1.2.2.1 Attachment

The attachment of *M. gallisepticum* to specific target cells via sialoglycoproteins along the respiratory epithelium is required prior to initiation of the disease processes (Glasgow & Hill, 1980). This is led by a complex multifactorial process which mediates cytadherence. Attachment is also important so as to avoid rapid clearance by innate host defence mechanisms. Furthermore, mycoplasmas are metabolically deficient, therefore the close interaction probably contributes to survival by allowing the mycoplasmas to acquire essential nutrients from the host cells (Simecka *et al.,* 1992).

The mechanisms of adherence of *M. gallisepticum* to host cells are very similar to that of the better studied *M. pneumoniae*, as both organisms belong to the same phylogenetic group and share many similar cytadhesin genes. By the use of microscopy, an apparent attachment organelle or tip structure was identified (Razin *et al*., 1980) and shown to bind to sialoglycoproteins for both species (Kahane *et al*., 1984). Three clustered genes have been identified in the *M. gallisepticum* genome as encoding for products with homology to adhesion-related molecules of *M. pneumoniae*. These are (i) the *mgc2* gene showing homology to the P30 of *M. pneumoniae* (Hnatow *et al.,* 1998), (ii) the *gapA* gene showing homology to the P1 adhesin of *M. pneumoniae* (Goh *et al.*, 1998), and lastly (iii) *mgc3* (Yoshida *et al*., 2000) which shares homology to *M. pneumoniae* open reading frame 6 (ORF6). The first two genes will be discussed further in section 1.4.3.1.2.

1.2.2.2 Cell Injury

Although adherence is important in infection, it is unlikely that infection alone can produce the wide variety of symptoms seen in *Mycoplasma* disease. Although the mechanisms involved in cell injury are not well understood for *M. gallisepticum*, it is clear that several mycoplasmas have the ability to directly cause cell injury.

Mycoplasmal parasitism of host cells may contribute to cell injury through deprivation of nutrients, alteration of host cell components and metabolites, and the production of toxic substances. A number of enzymes are produced which may play a major role in this process, such as phospholipases, proteases, and nucleases (Bhandari & Asnani, 1989). However, phospholipases and proteases could also contribute to cell membrane damage, and nucleases have been suggested to increase the chances of genetic alteration of host cells leading to autoimmune response (Vincze *et al*., 1975).

The production of hydrogen peroxide by *M. gallisepticum* and various other mycoplasmas has also been suggested to play a role in cell injury. Hydrogen peroxide released in direct proximity to the host cell membrane may lead to oxidative stress (as summarised in Figure 1.1) and has shown to cause hemolysis (Cole *et al*., 1968).

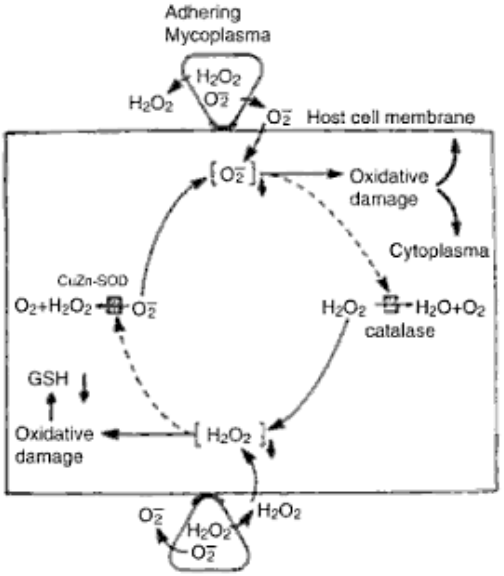


Figure 1.1: Schematic representation of the proposed mechanism for oxidative tissue damage by *Mycoplasma*. GSH, glutathione and SOD, superoxide dismutase (Razin, 2006).

Furthermore, *M. gallisepticum* is the only known avian *Mycoplasma* species shown to be invasive *in vitro* (Winner *et al.*, 2000). This not only allows the pathogen an opportunity to resist host defences and selective antibiotic therapy, but also enables the pathogen to enter the blood stream and cause systemic infections (Winner *et al.*, 2000).

1.2.2.3 Antigenic variation

The complete genome of *M. gallisepticum* R_{low} strain was sequenced by Papazisi and co-workers (2003). It was established that the genome is 996 422 base pairs long with only 742 putative genes. In order to maintain parasitism, a significant number of these mycoplasmal genes are devoted to adhesions, attachment organelles and variable membrane surface antigens directed towards evasion of the host immune system (Razin, 1997).

In general, *Mycoplasma* strains appear to be highly variable in their phenotypes. Even strains within the same species differ significantly in the ability to cause disease. Additionally, mycoplasmas can rapidly lose their virulence through passage in artificial media. Due to the lack of both light and dark repair mechanisms (Ghosh *et al*., 1977), it has been suggested that the accumulation of base pair changes occurs more frequently in *M. gallisepticum* than in other prokaryotes (Maniloff, 1978). This is consistent with the rapid evolution of the mollicutes, and allows mycoplasmas to generate diverse cell populations. Thus the presence of a large repertoire of genetic variants may provide the pathogen with the desired escape variant needed for survival in the event of sudden environmental change or when confronting the host response (Razin *et al.*, 1998).

The term “antigenic variation” is commonly used to describe the ability of microbial species to elaborate alternative forms of macromolecules recognized and distinguished by antibodies or other elements of the immune recognition (Wise *et al*., 1992). Surface organelles are typically major targets of the host antibody response (Dramsi *et al.*, 1993). Therefore, the ability of a microorganism to rapidly change the surface antigenic repertoire and consequently to vary the immunogenicity of these structures is thought to allow effective avoidance of immune recognition (Razin *et al*., 1998). It is therefore no surprise that with the absence of a cell wall, the surface proteins anchored (lipoproteins) and embedded in the cell membrane play a crucial role in the interaction with the host. No doubt these changes can also contribute to varied host binding and help the pathogen adapt to different conditions present during disease pathogenesis.

High-frequency variation in colony morphology and surface antigens has been demonstrated in *M. pulmonis*, *M. hyorhinis*, and ureaplasmas (Dybvig *et al.*, 1989). For *M. pulmonis*, structural variation of a major surface antigen, V-1, has shown to occur both *in vitro* and *in vivo* (Talkington *et al.*, 1989). Work by Watson and co-workers (unpublished data cited by Simecka *et al*., 1992) has demonstrated these variations to affect the type and severity of lung disease in mice after experimental infection.

The mechanisms by which high-frequency variation occurs within *M. gallisepticum* still remain largely unknown; however, the *pvpA* gene has been identified to show these tendencies (Boguslavsky *et al.*, 2000) and will be further discussed in section 1.4.3.1.2.

1.3 CONTROL OF *M. gallisepticum* IN THE POULTRY INDUSTRY

The Southern African Poultry Association (SAPA) announced the gross poultry farm income for 2010 to be R22,940 billion and to be the largest segment of South African agriculture at 23% of all agriculture production. With the already large and continually expanding poultry industry in southern Africa, efficient methods of biosecurity are required for the control of outbreaks. Control of *M. gallisepticum* has generally been based on the eradication of the organism from primary breeder flocks and maintenance of the *Mycoplasma*-free status of the flocks by periodic serological monitoring (such as agglutination, hemagglutination-inhibition or commercial ELISA kits).

Vertical transmission of the organism occurs through infected eggs and horizontally by the inhalation of contaminated dust, airborne droplets and feathers resulting in the rapid spread of the disease throughout the flock by subsequent close contact (Papazisi *et al.*, 2002). In recent years, there has been an outbreak of *Mycoplasma* infection in the poultry industry. This is possibly due to the rapid expansion in the poultry industry in restricted geographical areas, resulting in a high concentration of birds of different ages and poultry sectors being in close proximity. These conditions make the maintenance of *MG*-free flocks more difficult to control and may lead to poor biosecurity (Lysnyansky *et al.*, 2005). In areas where complete eradication is difficult to attain, live vaccinations are utilized as an alternative control strategy (Whithear, 1996; Kleven, 1997).

1.3.1 *VACCINES*

The commercially available live *M. gallisepticum* vaccines include the F strain (Schering Plough, N.J), Ts-11 (Bio-properties, Australia) and 6/85 strain (Intervet America, Millsboro) (Liu *et al.*, 2001; Lysnyansky *et al.*, 2005) as discussed below:

1.3.1.1 *MG*-F strain

The *MG*-F strain was first isolated and described as a typical pathogenic, naturally occurring strain (Ley*,* 2003). It was further reported that the strain was virulent in turkeys, however only mild to moderate virulence in chickens was reported (Ferraz & Danelli, 2003). The *MG*-F strain was subsequently used in the vaccination of chickens. The advantage of the *MG*-F strain is that a single dose at one interval is needed, since vaccinated chickens remain permanent carriers. However, continuous vaccination is needed to displace field strains from multiple-aged poultry production sites. The strain also has the ability to spread slowly from bird to bird, and may run the risk of spreading to turkeys where it is able to cause infection. The F strain is however, currently not registered for use in South Africa.

1.3.1.2 Ts-11 and 6/85 vaccine strains

Ts-11 and 6/85 are attenuated vaccines and were found to be poorly transmitted from vaccinated to unvaccinated birds (Kleven *et al.*, 2004). The Ts-11 strain originated in Australia, while the 6/85 strain came from the U.S.A. The two vaccine strains show little or no virulence to both chickens and turkeys, and are thus regarded safer than the *MG*-F strain (Ferraz & Danelli, 2003). Ley and co-workers (1997b) reported that Ts-11 can be detected by serology via detection of antibodies in vaccinated flocks, while 6/85 could not be detected using this technique. However, Garcia and co-workers (2005) later pointed out that the serological response to Ts-11 remains variable. There may be a strong, minimal or even no serological response. The reasons for the varied response to Ts-11 remain unknown, however Garcia and co-workers (2005) suggested it may be related to the challenge titer and route of administration. However, even if no serum antibody is detected after vaccination with the Ts-11 strain, the birds have still shown protection against *M. gallisepticum* infection (Noormohammadi *et al.,* 2002).

The Ts-11 vaccine is administered by the eye-drop route as a single dose to growing pullets 9 weeks of age or older. It persists for the life of the bird in the upper respiratory tract of vaccinated flocks and induces long-lived immunity (Ley, 2003). The 6/85 is administered via spray as a single dose to pullets 6 weeks of age or older and is detectable in the upper respiratory tract for 4-8 weeks after vaccination (MERCK Animal health, 2009).

The F-strain is said to be the most effective vaccine strain for protection, however is not yet registered for the use in South Africa. The Ts-11 is also an efficient vaccine strain, however it needs to be maintained specifically under stipulated temperature due to its sensitivity to high temperatures. The Ts-11 stain is registered and marketed in South Africa. A disadvantage of the 6/85 strain is that it should be administered continuously for adequate protection, and for this reason is not as popular as the F and Ts-11 vaccine *M. gallisepticum* strains.

1.3.2 ANTIBIOTICS

An alternative to vaccination is the use of antibiotics. Several antibiotics that act by inhibiting the metabolism of organisms, including macrolides, tetracyclines, fluoroquinoles and others, have shown to be effective against *Mycoplasma*. However, antibiotics such as penicillin and others that acts by inhibiting cell wall synthesis are ineffective due to the fact that *Mycoplasma* species lack a cell wall (Ley*,* 2003). Tylosin and gentamicin have shown to work well against *Mycoplasma*. Tylosin for its proven efficiency and gentamicin for its broad-spectrum activity and low host cell toxicity. Tylosin can however be toxic for embryos at higher doses and results in decrese in the hatchability (Nascimento *et al.*, 2005).

There are mixed reports on the efficiency of these antibiotics in treating *M. gallisepticum* infection. A study done by Nascimento and co-workers (2005) showed these antibiotics to be successful in the eradication of *M. gallisepticum*, while more recent studies show eradication can often be complicated by persistent infections and periodic shedding under stress (Ley & Yoder, 1997). The antibiotics were shown to decrease clinical signs, however did not eliminate the infection.

Vaccination remains the better alternative for the control of *M. gallisepticum.* Antibiotics have the risk of toxicity together with the known fact that they may acquire resistance, thereby rendering the antibiotic ineffective for future use. A futile cycle between finding new effective antibiotics and the gain of resistance by the microorganisms leads to a very limited number of antibiotics remaining that can be used, possibly with even higher toxicity.

1.4 DIAGNOSIS OF *M. gallisepticum*

Several methods have been used for the diagnosis of *M. gallisepticum* infection, including serological methods, molecular techniques, and isolation and identification. The latter being the gold standard test for confirmation of diagnosis (Ley, 2008). Specific diagnosis of *Mycoplasma* is not always easy due to the limitations of diagnostic tests together with the similarities in the disease they cause. It has become increasingly important to develop methods to characterize and identify *M. gallisepticum* strains and strain variability. Reliable methods for the differentiation of *M. gallisepticum* strains play a pivotal role in understanding the epizootiology and spread of the disease. Also, the increased use of live *M. gallisepticum* vaccines requires more powerful tools to differentiate vaccine strains from circulating field isolates, pathogenic and non-pathogenic. The various methods currently employed are discussed as follows.

1.4.1 CULTIVATION TECHNIQUES: ISOLATION AND IDENTIFICATION

Direct detection of the organism by cultivation and isolation has been shown to be far from a routine procedure (Zain & Bradbury, 1996). This is mainly because *M. gallisepticum* and other avian pathogenic *Mycoplasma* species are slow-growing, relatively fastidious organisms that require one or more weeks for its growth and identification (Garcia *et al.*, 2005). Their isolation is often impaired by the overgrowth of non-pathogenic *Mycoplasma* species or other faster growing bacteria and fungi (Garcia *et al.*, 1995). Another factor to consider is the selective pressures on populations of mycoplasmas, which would differ substantially *in vivo* and *in vitro*. Thus, during passage in culture media, pathogenic attributes of strains may be lost, and mutations favouring increased cell yield and higher growth rate *in vitro* will accumulate (Miles, 1992). For this reason, the cultivation and isolation process may not always give a true indication of the *in vivo* representation.

Due to their reduced genomes and limited capacity for biosynthesis, they have complex nutritional requirements for their growth *in vitro*. These include cholesterol, amino acids, fatty acids, vitamins, nucleotides and other nutrients they would usually obtain from their hosts. The lack of many regulatory genes involved in gene expression and appropriate responses to changing environmental conditions (*in vitro*), also contribute in making this organism extremely fastidious to work with (Razin *et al.,* 1998).

Several limitations exist within morphological characterization of mycoplasmas. Not only does the size of most mycoplasmas lie at the threshold of resolution for light microscopy, but the lack of a cell wall results in a gram-negative, often pleomorphic appearance. Since phase and dark-field optics has a slightly higher resolution (approximately 0.1 µm as opposed to 0.2 µm) than bright-field optics, applications of immunofluorescence, dark-field, and phase-contrast optics are commonly used. This makes the isolation of *Mycoplasma* species a laborious, time-consuming, expensive, and often a problematic task.

1.4.2 SEROLOGICAL TECHNIQUES: DETECTION OF ANTIBODIES

Historically, serological tests are used for the conventional monitoring of *M. gallisepticum* in flocks since it is rapid, easy, and requires little expertise. Methods such as rapid plate agglutination, ELISA, and hemagglutination inhibition are available (Kleven, 1998). Although serological tests have long been the basis for *M. gallisepticum* testing, they have their drawbacks and limitations and will be discussed as follows.

Serology relies on immune responses to antigens and the subsequent detection of antibodies produced. Since seroconversion lags behind infection, it takes a minimum of 1 week after infection before enough antibodies are produced to be detected in the agglutination test, while it can take up to 3 weeks to conduct the hemagglutination inhibition test (Kempf *et al.,* 1993). Serological tests can therefore not be used for the detection of early infections.

Not only this, but there have been noted problems with the sensitivity and specificity of this method. The closely related *M. imitans* has shown to be serologically cross-reactive with *M. gallisepticum* (Bradbury *et al.,* 1993). This comes as no surprise since the two organisms

share many similarities, including the same terminal attachment organelle, and similar antigenic and phenotypic properties (Abdul-Wahab *et al.,* 1996*;* Bradbury *et al.,* 1993). This leads to the misidentification of isolates as *M. gallisepticum,* instead of its close relative *M. imitans*. It should also be kept in mind that it is possible that 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 (Ley, 2003).

Mycoplasma contains non-pathogenic species, such as *M. gallinarum* and *M. gallinaceum*, which have been found to be isolated with their pathogenic relatives. For this reason the method used needs to be able to differentiate between the species (Hong *et al.,* 2005). Additionally, due to the widespread use of live vaccines against *M. gallisepticum*, improved detection and differentiation methods are needed to distinguish the vaccine strains from circulating field strains. This shows the need for the identification not only on species level, but on strain level as well. In this respect, the specificity of serological techniques has failed to accurately distinguish between vaccinated or naturally infected flocks (Ferraz & Danelli, 2003). This distinction is vital.

1.4.3 MOLECULAR TECHNIQUES

1.4.3.1 Random amplified polymorphic DNA (RAPD)

Several molecular techniques have been developed for differentiation of *M. gallisepticum* strains, including protein profile analysis (Khan *et al*., 1987), restriction fragment length polymorphism (RFLP) (Kleven *et al.,* 1988), ribotyping (Yogev *et al*., 1988), strain-specific DNA probes (Khan *et al*., 1989) and PCR with strain-specific primers (Nascimento *et al*., 1993). However, none of these methods have been as successfully utilized in discriminating vaccine strains, in both experimental and field conditions, as RAPD (Ley *et al*., 1997a; Kleven & Fan, 1998; Geary *et al*., 1994).

However, Ferguson and co-workers (2005) reported that RAPD does not come without its problems and limitations. It is stated that due to difficulties in standardizing and unifying protocols among laboratories, the use of the RAPD method has not allowed for inter-laboratory comparisons or long-term epidemiological studies. Additionally, the RAPD technique has intrinsic problems of reproducibility due to various experimental parameters (Tyler *et al*., 1997).

1.4.3.2 Polymerase chain reaction (PCR)

PCR has become a valuable tool in the diagnosis of *Mycoplasma* species, not only for its sensitivity but for its increasing specificity (Kempf *et al*., 1993). It is a method based on the direct detection of the organism's nucleic acid. PCR has allowed the study of microbial genes, directly amplified from samples, without the need for cultivation. It is advantageous because of its sensitivity, ease, rapid turnover and relatively inexpensive application, and most importantly eliminates the need to isolate and culture.

The specificity of the method is highly flexible since it is dependent on the target. PCR methods can be developed to be species-specific by targeting unique genes to that species, or it may even be strain specific by targeting a conserved region within the strain. Several PCR assays targeting the four main avian pathogenic *Mycoplasma* species have been developed since the early 1990s (Raviv & Kleven, 2009). Earlier methods primarily targeted the 16S rDNA region (Kempf *et al*., 1993), whereas more recent methods aim at targeting the surface proteins and the more species-specific regions (Liu *et al*., 2001; Garcia *et al.*, 2005; Raviv *et al.,* 2007). Since the 16S rDNA sequences tend to be highly conserved among phylogenetically related groups such as the avian *Mycoplasma* species, PCRs that target this region therefore lack in specificity and may cross-react with other known and unknown species to give false-positives (Garcia *et al*., 2005). However, a problem with the species-specific genes, such as the surface proteins, is that they often contain high levels of intraspecific genetic polymorphism that can reduce the sensitivity of the assay.

There are many different PCR methods applied for *M. gallisepticum* detection including commercial kits, e.g. produced by IDEXX Laboratories, Genekam Biotechnology AG, and others. Since *Mycoplasma* species are known to exhibit a high degree of phenotypic variation (Domanska-Blicharz *et al.,* 2008), PCR methods have been developed to target various gene fragments, including *16S rRNA* gene, *pvpA*, *gapA*, *lipoprotein*, *mgc2* and more recently the 16S-23S intergenic spacer region.

1.4.3.2.1 16S ribosomal RNA

The *16S rRNA* gene is a highly conserved region with low levels of genetic variation, which reduces the likelihood of excluding some *M. gallisepticum* strains. Targeting this region however has its shortcomings. One of which is that the *16S rRNA* of *M. gallisepticum* has shown to be very similar to that of *M. imitans* and the PCR amplifies both organisms (Garcia *et al.,* 2005). The 16S rDNA therefore cannot differentiate between recently diverged species, let alone various strains. It thus cannot solely be used to identify *M. gallisepticum* without giving false positive results.

1.4.3.2.2 *M. gallisepticum* surface protein genes.

Before infection can occur, the *Mycoplasma* cell needs to bind to the host cell membrane-receptors. The attachment is mediated by specific interactions by proteins known as cytadhesins. The ability of *Mycoplasma* to firmly adhere to the host cells initiates the process that results in host cell alterations and pathogenesis (Goh *et al*., 1998; Winner *et al.,* 2000). Various types of surface proteins, mainly cytadhesins, found in *M. gallisepticum* are listed as follows

Mgc2 Cytadhesin

The 912-nucleotide *mgc2* gene encodes a 32.6 kD protein that was shown by Hnatow and co-workers (1998) to be clustered at the tip organelle. It exhibits 40.9% and 31.4% homology with the *M. pneumoniae* P30 and *M. genitalium* P32 cytadesins, respectively (Hnatow *et al.*, 1998). From this and other reports, it is evident that there is a family of cytadhesin genes conserved among pathogenic *Mycoplasma* species infecting widely divergent hosts (Boguslavsky *et al.*, 2000). The conservation of these genes among the different pathogenic *Mycoplasma* show their importance in the adhesion to the mucosal membranes of the host, and hence their ability to initiate infection.

The *mgc*2 gene is fairly conserved in *M. gallisepticum* and has been used for the molecular identification of isolates (Lysnyansky *et al*., 2005; Garcia *et al*., 2005). The advantage of using the *mgc2* gene-based method is the ability to differentiate between pathogenic field strains and vaccine strains by combining it with RFLP or sequencing of the DNA amplicons. Lysnyansky and co-workers (2005) used the *mgc2*-PCR-RFLP method, stating it would take

one to two days to obtain an identification starting from when DNA is extracted from the tracheal swabs. The primers used by the above authors to target the *mgc2* gene, were tested b*y* Garcia and co-workers (2005) against a wide variety of organisms (26 avian mycoplasmas, 2 avian acholeplasmas, 10 non-*Mycoplasma* bacterial DNA). The results showed the assay to be both specific and sensitive for *M. gallisepticum* strains.

GapA Cytadhesin

The *gapA* gene, characterised by Goh and co-workers (1998), is 2895 bp-long and encodes a 105 kDa protein which is trypsin-sensitive and also surface-exposed. The authors found the *GapA* to be a central gene in a multi-gene operon, and occurring as a single copy in the genome. This operon consists of three genes, from 5' to 3', the *mgc2* (as discussed above), *gapA*, and the *mgc3* (or *crmA*) respectively. In the study, the *gapA* gene shared 45% homology to the *M. pneunomiae* P1 cytadhesin gene, which unlike *gapA* is present in multiple copies throughout the genome. Similarly to the P1 protein, *gapA* has a high proline content located predominately at the carboxyl terminus. The authors suggested this region aids in the topological organization of the cytadhesin in the membrane. Goh and co-workers (1998) went further to confirm the role of *gapA* in the adherence to host cells using the chicken tracheal-ring inhibition-of-attachment assay. Anti- *gapA* Fab fragments were also shown to inhibit the attachment of *M. gallisepticum* by 64%. Intraspecies strain variation in the size of *gapA* was observed to vary approximately between 98.1 kD and 110 kD between strains (Goh *et al.,* 1998).

Garcia and co-workers (2005) tested nested primers targeting the *gapA* gene for the detection of *M. gallisepticum*. The results showed that the PCR had specifically amplified *M. gallisepticum*, when tested against a wide panel of strains, by producing a PCR product of 332 bp in samples of experimentally infected birds. Garcia and co-workers (2005) did however note that other authors occasionally experienced nonspecific PCR products of 200 bp when amplifying field samples. The 200 bp fragment was sequenced, but did not show any similarity with other sequences in Genbank (AY765219).

PvpA Cytadhesin

PvpA is a phase-variable protein localized on the terminal tip structure of the cell surface (Boguslavsky *et al*., 2000). Boguslavsky and co-workers (2000) characterised this protein and showed that the PvpA protein exhibits higher homology to the P30 and P32 proteins than Mgc2, with 54 and 52% homology respectively. PvpA is a non-lipid integral membrane protein with a surface-exposed C-terminal portion. The C-terminal has a high proline content (28%) containing identical direct repeat sequences of 52 amino acids each, designated DR-1 and DR-2. Additionally, a recurring tetrapeptide motif of Pro-Arg-Pro-X (where X is Met, Gln, or Asn) is present. The high concentration of proline residues within a surface exposed domain contributes to the protein folding, strengthening its overall conformation (McArthur & Thornton, 1991), and is known to increase the immunogenicity of the protein (Dramsi *et al.*, 1993). Boguslavsky and co-workers (2000) also documented size variations among the different *M. gallisepticum* strains as a consequence of deletions occurring within the C-terminal (Figure 1.2).

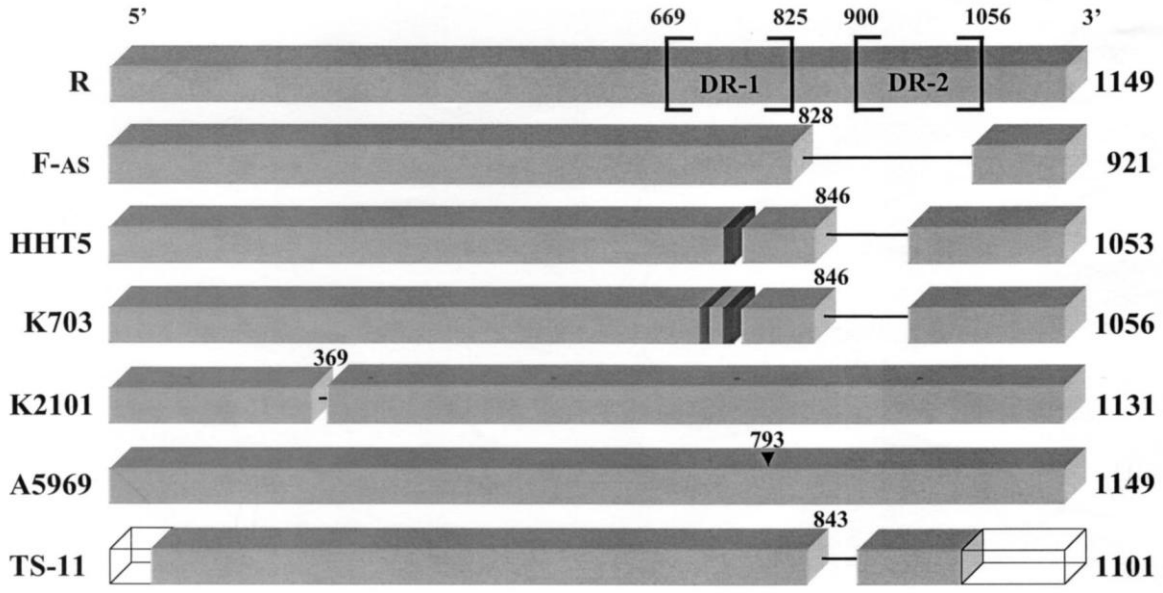


Figure 1.2: Illustration of deletions within the C terminus-encoding region of the *pvpA* gene (Boguslavsky *et al*., 2000). The *pvpA* gene from different *M. gallisepticum* strains is shown by gray rectangles. The length of each ORF (in nucleotides) is given on the right. The location of two directly repeated sequences (DR-1 and DR-2) in the C terminus-encoding region of the *pvpA* gene from strain R is shown by labelled brackets. Gaps within the *pvpA* genes represent various types of deletions in comparison to strain R. Small dark rectangles indicate nucleotide sequences within the *pvpA* gene of strain HHT5 and K703 which are not present in the R strain. The numbers at the beginning of each deletion indicate the nucleotide position. Open rectangles in the vaccine strain ts-11 represent regions which were not sequenced.

The conservation of proline-rich regions within the surface-exposed domain of various pathogenic *Mycoplasma* adhesins suggests the importance of these domains in the function of PvpA as an adhesion (Dallo *et al.,* 1996). Boguslavsky and co-workers (2000) further postulated that the variation within PvpA could possibly affect the specificity or affinity within different niches in the host where distinctive receptors may be required for optimal colonization.

Liu and co-workers (2001) conducted a study to determine the feasibility of using the variable *pvpA* gene as the target to differentiate *M. gallisepticum* strains through a PCR-RFLP assay. Semi-nested primers were designed to target the C-terminal of the *pvpA* gene. The nested primers provided increased sensitivity so as to enable diagnosis from clinical samples. The amplicon was treated with three enzymes, *PvuII, AccI* and *ScrFI*. The RFLP pattern produced was able to discern the vaccine strains apart by placing them into groups. It was also demonstrated that sequence analysis of the *pvpA* gene could further be utilized for epidemiology studies of *M. gallisepticum* outbreaks. Liu and co-workers (2001*)* concluded by suggesting that further optimization of the test is necessary to improve the sensitivity.

MGA_0319 (Surface lipoprotein Protein)

Garcia and co-workers (2005) attempted to target a relatively uncharacterized conserved surface lipoprotein that was first recognised by Nascimento and co-workers (1991), designated MGA_0319. They developed nested primers to increase the sensitivity of the assay, however the results were poor. This was later attributed to secondary structures and to the significant melting temperature differences between the external primers.

Studies targeting these regions

Garcia and co-workers (2005) conducted a study comparing the sensitivity and specificity of the various PCR targets, including the *gapA*, *mgc2*, *lipoprotein* (MGA_0319), and the *16S rRNA* gene sequences for the detection of *M. gallisepticum*. The *gapA* method was found to be the most sensitive method and detected 4ccu/reaction (colour changing units); the lipoprotein to be the least sensitive detecting 400ccu, and the *mgc2* and *16s rRNA* estimated at only 40ccu. These results were then later confirmed by a study done by Domanska-Blicharz and co-workers (2008). Both groups showed that all the methods were specific for

M. gallisepticum and could detect divergent *M. gallisepticum* strains successfully, with the exception of the 16S rRNA, which detected *M. imitans* in addition to *M. gallisepticum*. The *mgc2*-PCR was the more rapid and cost-effective method when compared to *gapA*-PCR, owing to the fact that the latter is a nested PCR. The *mgc2*-PCR was therefore chosen as the method of choice for its sensitivity, specificity, and relative speed.

Ferguson and co-workers (2005) decided to use a multi-locus sequence typing method to identify and differentiate among *M. gallisepticum* strains, referring to it as gene-targeted sequencing (GTS). They suggested it to be an improved method to the previously used RAPD, owing to its increased reproducibility and allowing rapid global comparisons between laboratories. They did this by targeting four surface-protein genes for analysis, namely the *gapA*, *mgc2*, *pvpA*, and lipoprotein designated as MGA_0319. The authors managed to characterize a total of 67 *M. gallisepticum* field isolates from the USA, Israel and Australia, and 10 reference strains. Results showed that GTS of these four surface-protein genes combined showed a better discriminatory power than RAPD analysis, thus providing an improved typing method for *M. gallisepticum* isolates.

1.4.3.2.3 16S-23S Intergenic spacer (ITS) region

Most known prokaryotes have genes coding for the different RNAs of an assembled ribosome organized into an operon as the functional transcription unit. The cistrons for rRNA molecules of the *Mycoplasma* species are most commonly organised in an operon in the order 5'-16S-23S-5S-3', while the individual rRNA genes are separated by the ITS regions. Two copies of the rRNA operon are present in both *M. imitans* and *M. gallisepticum* (Dupiellet, 1988 as cited by Harasawa *et al*., 2004). However, it was further shown by Papazisi and co-workers (2003) that the second 16S rRNA was not situated in an operon cluster and therefore does not possess the ITS region.

The size of the spacer regions may vary considerably between different species, and even among the different operons within a single cell in the case of multiple operons (Garcia-Martinez *et al.*, 1999). There are usually several functional units found within the ITS region, including *tRNA* genes, recognition sites for *ribonuclease III* and a boxA region which acts as an antiterminator during transcription. Once the rRNA has been transcribed into a monocistronic RNA transcript, the ITS regions are removed during the maturation process

involving the *ribonuclease III* enzyme (Bram *et al*., 1980). The enzyme acts by recognising and cleaving the stem-like structures formed as a result of the base pairing of the sequencing flanking the ribosomal genes (Bram *et al*., 1980). These regions therefore allow little space for mutations to occur and usually remain conserved. The reason for the apparent conservation of the rest of the ITS region among closely related strains is probably due to its location between the highly conserved ribosomal genes and due to concerted evolution of a multi-gene family (Garcia-Martinez *et al.,* 1999).

A study done by Garcia-Martinez and co-workers (1999) showed that the 16S-23S intergenic spacer region of the ribosomal genes could be used in studies of prokaryotic diversity as opposed to using the *16S rRNA* gene. Formerly, the *16S rRNA* was used extensively due its highly conserved regions interspersed with variable and hyper-variable stretches. It is therefore convenient to create primers, and there is a vast database of sequences available for the gene which makes comparison searches for close relatives more feasible.

As previously discussed, the 16S rRNA, although variable, is often not divergent enough to give good separation in close relationships and between recently diverged species (Garcia *et al.,* 2005). This problem was proposed to be solved by using the spacer region instead, having both highly variable length and sequence even within closely related species.

Harasawa and colleagues (2004) later confirmed that not only could the 16S-23S ITS region be used as an important tool for classification of *Mycoplasma* due to its variation in length and sequence from one species to another, but it could also be used to determine the relationships between genetically related species because of its high rate of divergence. The authors amplified the 16S-23S rRNA ITS region from 23 types of avian *Mycoplasma* species, including that of *M. gallisepticum* and *M. imitans*. The product included 180bp at the 3' end of the 16s rDNA to the 5'end and 115bp at the 5' end of the 23S rDNA. The nucleotide sequences of the ITS regions for the *M. imitans* 4229^{T} and *M. gallisepticum* PG31^{T} strains were 2488 nt and 645 nt, respectively, in size. An alignment of the sequences of the ITS regions showed that the first 153 nt and last 138 nt showed a high degree of conservation. However, *M. imitans* contained an operon of 1260 nt with high similarity to other transposase genes. Although other *M. imitans* strains ITS region have not yet been sequenced, it is likely that they share this property as their PCR products all shared similar sizes as seen in the gel

electrophoresis. The size of the ITS region of the *M. imitans* is relatively large compared to the other *Mycoplasma* species which mostly do not exceed 500 bp.

All sequenced 16S-23S ITS regions from *Mycoplasma* species have been shown to lack tRNA genes or even their pseudogenes, which are most commonly found in bacterial ITS regions (Harasawa *et al.,* 2004; Raviv *et al*., 2007). The ITS region of *M. imitans* 4229^{T} was sequenced and Harasawa and co-workers (2004) found that instead of the tRNA genes, the *M. imitans* genome has an ORF of 1260nt on the complementary strand of the ITS region. The amino acid sequences also showed high similarity to bacterial transposases. Of the 1260nt, 315nt (25%) showed a high identity (83%) to the P75 gene and its 3' flanking region in *M. gallisepticum*. The P75 gene in *M. gallisepticum* encodes a 75kDa protein that is recognised during natural infection (Harasawa *et al.,* 2004).

In 2007, Raviv and co-workers went further to design primers for the specific amplification of the complete *M. gallisepticum* 16S-23S ITS region segment. The primers were designed to anneal to the downstream region of the *16S rRNA* gene and the upstream region of the *23S rRNA* gene to ensure the complete amplification of the *M. gallisepticum* ITS segment. The PCR was tested against 18 different avian mollicute species that could potentially be found while swabbing. The results showed to be negative for all except the closely related *M. imitans*, which yielded a band of higher molecular weight (~2500bp) than that of the *M. gallisepticum* control (~800bp).

Raviv and co-workers (2007) also evaluated the sequence stability of the 16S-23S ITS region by sequencing several *in vitro* passages of a few reference *M. gallisepticum* species. The results showed that the ITS region was perfectly stable following a 100% identity. Another important observation was made that the Ts-11 vaccine strain's ITS sequence was unique amongst the study's strains and field isolates. This proves superior to the *mgc2* gene PCR, where several field isolates share the Ts-11 *mgc2* sequence (Raviv *et al*., 2007). This could prove to be useful in distinguishing the vaccine strain Ts-11 from field strains. The authors proposed the sequencing of the *M. gallisepticum* ITS region to be a valuable single-locus sequence typing tool for *M. gallisepticum* isolate differentiation in diagnostic cases and epizootiological studies. The study showed a discrimination index of 0.95, as opposed to that of 0.965 for multi-locus sequence typing described by Ferguson and co-workers (2005) as

previously discussed. It was suggested by Raviv and co-workers (2007) that when differences are not detected in the 16S-23S ITS region, it is necessary to continue and search for differences with alternative methods before a conclusion can be reached. The authors also noted that a single base variation was enough for isolate differentiation. Thus, when targeting this region it would be critical to use a high fidelity polymerase and also ensure high quality sequencing results with adequate coverage.

Ramírez and co-workers (2008) later showed that using the 16S-23S ITS region of various avian *Mycoplasma* species for phylogenetic analysis conflicted with that based on the 16S rDNA and was therefore not helpful for phylogenetic studies. However, the 16S-23S ITS region was shown to be valuable for determining species since there was high inter-species variation between all 23 avian *Mycoplasma* species, and in addition there was low intra-species variation *in M. gallisepticum*. The authors recommended the region to be very useful as additional information in the description of a new species.

1.5 CONCLUSION

With the dramatic expansion of poultry production in recent years, more accurate, rapid, feasible and high-throughput methods are needed for the diagnosis of *M. gallisepticum,* in order to monitor and control outbreaks. This is emphasized with the use of live vaccines administered in the poultry industry, as they offer a better alternative than antibiotics. Several methods have been used for the diagnosis of *M. gallisepticum* infection, including serological methods, isolation and identification and molecular techniques.

Serological tests have long been the basis for *M. gallisepticum* testing and commonly used for periodic monitoring of *M. gallisepticum* in poultry flocks, however they are known to have problems with both sensitivity and specificity. Serological tests are thus limited in their ability to classify infection as well as to establish if the flock is vaccinated or infected with a wild-type strain.

Although cultivation of *M. gallisepticum* colonies is considered the gold standard test for confirmation of diagnosis, it has been shown to be problematic due to the fastidious nature of *M. gallisepticum* together with various other factors. These factors include overgrowth by contaminants and the selective pressures on *Mycoplasma* populations during cultivation which would differ substantially *in vivo* and *in vitro.* Passage in culture media may also result in the loss of pathogenic attributes of strains, and mutations favouring increased cell yield and higher growth rate *in vitro* would accumulate.

Molecular techniques, such as PCR, can however directly detect the organism's nucleic acid. This gives a more accurate *in vivo* representation of the organism. Molecular techniques are regarded as a valuable tool for the diagnosis of *M. gallisepticum* as they can be more sensitive and discriminatory for the differentiation of *M. gallisepticum* strains. The PCR method is very flexible in both its sensitivity and specificity, depending on the target region chosen. The *16s rRNA* is notorious for giving false positives as a result of the lack of specificity due to highly conserved regions within phylogenetically related groups of bacteria. Since the *16S rRNA* gene of *M. gallisepticum* is so similar to that of *M. imitans*, even sequencing of this region is unsuccessful in distinguishing between these species.

Garcia and co-workers (2005) reported the *mgc2*-PCR to be the superior of the available PCRs at the time, offering the best sensitivity and specificity in the fastest turnover time. In the same year, Ferguson and co-workers (2005) developed a GTS method integrating sequence data from multiple surface-protein-encoding genes of *M. gallisepticum*. These included the *mgc2, gapA, pvpA* and lipoprotein designated MGA_0319. The method showed better discriminatory power to the previously used RAPD analysis, thus allowing for rapid global comparisons between laboratories and increased reproducibility. In addition, the study yielded a large amount of sequence data by analysing 67 *M. gallisepticum* field isolates from the USA, Israel and Australia, and 10 reference strains. This allows for good comparison studies of field isolates from less specialized laboratories in various regions.

The 16S-23 ITS region was suggested by Harasawa and co-workers (2004) as being used for the classification of *Mycoplasma* species, owing to their higher rates of divergence over the *16S rRNA* genes. Raviv and co-workers (2007) further suggested this region to be a valuable single-locus sequencing typing tool for *M. gallisepticum* isolate differentiation.

Although this method showed a high discrimination index, the multi-locus sequencing described by Ferguson and co-workers (2005) remained the superior of the methods. Ramírez and co-workers (2008) later showed the 16S-23S ITS region could not be used for phylogenetic analysis, but was valuable as a discriminatory tool between *Mycoplasma* species.

1.6 INTRODUCTION TO THE PRESENT STUDY

Although *M. gallisepticum* has been well characterised in some countries, very little information has been documented on the southern African isolates, especially in terms of molecular data. This deficiency in information has led to poor biosecurity in the poultry industry. One of the objectives of this study was therefore to investigate the presence and diversity of *M. gallisepticum* in southern Africa by screening various poultry farms and attempting isolation and cultivation of these fastidious organisms. Further characterization of *MG* field isolates would be carried out using GTS.

The second main objective is to investigate the occurrence of “failed vaccinations” reported in the field, despite the proper cold chain of administration. Field evidence throughout southern Africa suggested that vaccines were not offering sufficient protection and the birds were succumbing to *MG* infection (Bragg 2010, personal communication). This could suggest the development of a novel pathogenic strain, or simply the selection of an uncharacterised pathogenic *MG* strain. It is also known that mycoplasmas have a great capacity for antigenic variation, and *MG* cytadhesins such as the variable PvpA protein is recognized by the chicken immune system (Boguslavsky *et al*., 2000; Levisohn & Kleven, 1995). It is thus proposed that by *MG* altering its antigenic profile, it allows effective avoidance of immune recognition and antibodies produced as a result of vaccination with live and inactivated *MG* vaccines. In this study, *in silico* methods will be used to establish whether field isolates found in southern Africa contain a different antigenic profile compared to that of the vaccine strain, so as to explain the evasion of the host immune response.

CHAPTER 2

ISOLATION AND CULTIVATION OF *M. gallisepticum* FIELD ISOLATES IN SOUTHERN AFRICA

2.1 INTRODUCTION

In vitro cultivation is one of the methods used for the identification of *M. gallisepticum*, and through which many other techniques may be carried out for definite identification. However, mycoplasmas are known to be both nutritionally demanding and fastidious *in vitro* (Levisohn & Kleven, 2000); no doubt as a result of their reduced genomes, limited capacity for biosynthesis, and their lack of many regulatory genes involved in gene expression and appropriate responses to changing environmental conditions (Razin *et al.,* 1998).

A disadvantage of culture is that once mycoplasmas are isolated from their host, they tend to die rather rapidly if they are not placed in a suitable medium and environment (Zain & Bradbury, 1996). Thus, the way in which the sample is handled between collection from the host and inoculation into growth medium may be vital for the survival of the live organisms. Zain and Bradbury (1996) showed that swabs dipped in *Mycoplasma* broth (MB) and kept at 4°C remained more viable than dry swabs. Swabs pre-wet with MB and stored before sampling could be used without any ill effects on *Mycoplasma* recovery as long as they were stored at 4°C and for no more than 48 hr. Furthermore, they discovered wet charcoal swabs to be the most efficient in transporting *Mycoplasma* isolates, remaining viable for the longest period. The charcoal is believed to absorb toxic metabolites produced by the organisms (Cooper, 1957) and toxic ions produced by the irradiation of the swabs. They concluded the time taken between sampling and culturing of the swab, and the temperature at which it is held can be critical for successful isolation, particularly where small numbers of organisms are involved.

To overcome the assimilative deficiencies of the mycoplasmas, complex media are used for their cultivation, originally described by Frey and co-workers (1968). The media generally contains a protein digest and a meat-infusion base supplemented with serum, yeast fraction, glucose and bacterial inhibitors to retard growth of contaminant bacteria and fungi (Hong *et al*., 2005). Due to the variations occurring in the yeast extract and more importantly the serum fraction, the media is undefined. It is known that nutrient and cultural conditions may critically affect biochemical, physiological, and morphological attributes of mycoplasmas and influence genetic adaption of strains. Morphology may also be markedly affected by the nutritional quality and osmotic strength of the medium and importantly by the lipid content of the membrane (Rodwell & Mitchell, 1979).

Another factor to consider is the selective pressures on populations of mycoplasmas, which would differ substantially *in vivo* and *in vitro*. Wise and co-workers (1992) highlighted that mycoplasmas occur as rapidly mutating populations and diversification may occur in very short periods, even during growth from a single organism to a colony. This ability to diversify may play an important role for infection or disease. Host environments as well as those used for *in vitro* cultivation may be selective for populations. It is argued by Wise and co-workers (1992) that sampling the host population by cultivation may select particular phenotypes. Thus, during passage in culture media, pathogenic attributes of strains may be lost, and mutations favouring increased cell yield and higher growth rate *in vitro* will accumulate. This then limits attempts to correlate phenotypes determined *in vitro* with properties of the organism *in vivo*. These are all factors to bear in mind when attempting to isolate and cultivate mycoplasmas.

2.2 OBJECTIVES

This chapter was aimed at the isolation and cultivation of *M. gallisepticum* isolates from various poultry farms in southern Africa. Due to the undefined media used, both a stereo-microscope as well as a scanning electron microscope was used to determine if the colony morphology of the control isolates conformed to the typical growth as reported in literature (Shifrine *et al*., 1962; Kleven, 2003). This was done to test each batch of cultivation media. Morphologically-conforming colonies from field isolates were screened for *MG* using PCRs targeting the 16S-23S ITS region and a partial region of the *mgc2* cytadhesin gene. The *mgc2*-PCR has previously been shown to be both highly specific and sensitive for the detection of *MG* (Garcia *et al*., 2005).

2.3 MATERIALS AND METHODS

2.3.1 *REAGENTS AND CHEMICALS*

The reagents and chemicals used were of molecular biological and analytical grade, unless stated differently and were obtained from the following companies.

Amersham Biosciences: GFX™ PCR DNA and Gel Band Purification Kit; **Applied Biosystems**: BigDye terminator v3.1 Kit; 2720 Thermal Cycler; **DIFCO**: Bacto ™ Amies transport medium; **Fermentas**; O'GeneRuler™ Express DNA Ladder, O'GeneRuler™, Orange loading dye, Deoxynucleoside triphosphates (dNTPs); **Highveld Biological (PTY) LTD**: Filtered and gamma-irradiated swine serum; **Merck**: Phenol red, Glucose; **Merial South Africa**: Live attenuated *Mycoplasma gallisepticum* Ts-11 vaccine; **New England Biolabs®**: Taq DNA Polymerase with ThermoPol Buffer; **Roche**: Ampicillin; **Sigma-Aldrich®**: *Mycoplasma* agar base, *Mycoplasma* broth, Thallium (I) acetate (99%); **Thermo Scientific**: NanoDrop™ 1000 Spectrophotometer v3.7; **Whitehead Scientific (PTY) LTD**; Agarose D1 LE, QIAamp® DNA mini kit, All synthesis of primers; **J. Bradbury**, **University of Liverpool, UK**: Freeze-dried samples of *MG* strains, PG31(18.11.99) and A514 (21.5.94).

2.3.2 Mycoplasma ISOLATES

2.3.2.1 Controls: Live freeze-dried reference *M. gallisepticum* strains, PG31 and A514, were kindly donated by Professor Janet Bradbury (University of Liverpool). These strains were revived and used to establish the cultivation methods. Following DNA extraction as described below, the isolates furthermore served as positive controls in the various PCRs.

2.3.2.2 Field isolates: Samples were taken from various poultry farms in South Africa suspected of *MG* infection. Swabs were taken from the choanal cleft, oropharynx, oesophagus or trachea of the chickens.

Various precautionary methods were employed to increase the recovery of the isolates during transport. Swabs were pre-dipped in *Mycoplasma* broth and once the sample was collected, the swabs were stored in Amies transport media, transported on ice and cultured immediately on arrival. Names of farms on which samples were collected have been omitted due to client confidentiality privileges.

Farm A: The broiler flock (7 weeks of age), previously tested serologically positive for *MG*, was treated with antibiotics 3 weeks prior to collection. Samples were isolated from the trachea and cleft regions, and transport within 32 hr.

Farm B: Samples were taken from chicks whose progenitors were confirmed to be *MG* positive. Samples were isolated from trachea and cleft regions, and transported within 24 hr.

Farm C: Samples were isolated from Hy-line layers (29 weeks of age) showing signs of respiratory distress a few days prior sampling. High mortality was recorded in one of the flocks treated with quinalone (Quinabic), however the flock that was sampled was not treated. The supplier who did the rearing of these birds confirmed they were positive for *Mycoplasma*.

Farm D: Samples were isolated from multi-aged layers showing signs of respiratory distress. The flock had been treated with quinolone (Baytril) prior to sampling. Samples were transported within 6 hr.

Farm E: Samples were collected from multi-aged layers (23 weeks of age) previously treated with tiamulin (Denagard) and/or fosfomycin and tylosin (Fosbac plus T), and transported within 4 hr.

2.3.3 CULTIVATION OF MYOPLASMA ISOLATES

The medium to culture and isolate *M. gallisepticum* was originally described by Frey *et al.* (1968). It is made up of two parts, namely part A and part B. Part A consists of *Mycoplasma* broth (25.5 g), or *Mycoplasma* agar base (36 g) (Sigma-Aldrich) in distilled water (700 mL). Part B consists of gamma-radiated swine serum (150 mL), 25% (w/v) fresh yeast extract (100 mL), 10% (w/v) glucose solution (10 mL), 5% (w/v) thallous acetate (10 mL), ampicillin (1 mg/mL), and 0.1% phenol red (20 mL) in the case of the broth (OIE Terrestrial Manual, 2008).

Part A is adjusted to a pH of 7.8 and autoclaved at 121°C at 1 atmosphere pressure for 15 min. Part B is filter sterilized (0.2 µm) and added to Part A once cooled. Media can be stored at 4°C for up to 2 weeks.

Fresh yeast extract contains labile components not present in commercial preparations of dehydrated, enzymatic digests of yeast cells and may be readily prepared with 25% (w/v) *Saccharomyces cerevisiae* in distilled water. This was heated until boiling point, cooled and centrifuged for 20 min at 3000 x *g* at 4°C. The supernatant fluid was decanted and adjusted to pH 8.0 with 0.1 M NaOH, upon filter sterilization (0.45 µm). Swine serum was gamma-radiated to avoid inhibition of *Mycoplasma* growth by natural antibodies present in the serum. Additionally, serum was heated for 56°C for 30 min to inactivate any host complement which may be present.

The media contained certain inhibitors such as thallium acetate and ampicillin to prevent the growth of bacterial contaminants. Thallium acetate (0.5-1 g/L) prevents the growth of many gram-negative and some gram-positive bacteria, while *Mycoplasma* are generally resistant to these concentrations (OIE Terrestrial Manual, 2008). Ampicillin (1 mg/L) specifically inhibits cross-linking of peptidoglycan chains in the cell walls of both gram-positive and gram-negative bacteria. *Mycoplasma* species are however resistant to this antibiotic due to their lack of a cell wall.

Swab samples were streaked on to *Mycoplasma* agar and inoculated into *Mycoplasma* broth. Inoculated plates were incubated at 37°C in a modular incubator chamber (Billups-rothenberg $_{inc}$). The humidity was increased for optimal growth by the inclusion of damp cotton wool, and the chamber was flushed with CO_2. Broth media was placed in falcon tubes (Lasec) and the caps were sealed tightly to prevent spurious changes in pH. Dilution series of up to 10^{-3} were made of the inoculated broth solutions to dilute out any possible inhibitors such as antibiotics or host defense proteins. Broth medium was examined daily for acidity, indicated by the change of phenol red from red to yellow. Broths were subcultured on to solid medium after 7-10 days or earlier, even if no colour change was observed. This is due to arginine-hydrolysing *Mycoplasma* species capable of masking the acid colour change produced by *MG*.

Mycoplasma colonies on solid medium typically have a “fried-egg” appearance, are clear and very small (Kleven, 2003). Contaminating bacterial colonies are more often pigmented (OIE Terrestrial Manual, 2008). If contamination was seen early in the broth medium, it was passed through a 0.45 µm filter. Since *Mycoplasma* species lack a cell wall, they are more

"flexible" and able to pass through the filter. This was observed in a study by Chen (1977) where *Mycoplasma* cells could easily pass through a 0.22 µm pore-size filter membrane, thereby contaminating cell cultures. This disadvantage in cell culture was used as an advantage in *Mycoplasma* cultivation. Cultures were kept for at least 21 days before being discarded as negative for growth. Colonies were examined using a stereo-microscope (Nikon AZ-100, Tokyo, Japan).

2.3.4 SCANNING ELECTRON MICROSCOPY (SEM) OF CONTROL ISOLATES

Cells were fixed for SEM following the same procedure used by Swart and co-workers (2010). Briefly the procedure entails: Blocks of agar (5mm x 5mm x2mm) containing the cell colonies were fixed by adding 6% (v/v; 0.1 mL. L^{-1}) gluteraldehyde in sodium phosphate buffer (pH 7.2) and incubated for 2 hours. After incubation, cells were washed with the same buffer to remove excess aldehyde fixative. Post-fixation was performed with 0.5% (m/v) buffered osmium tetroxide and incubated for 1 hour. The cells were washed to remove excess osmium tetroxide. Cells were dehydrated by an ethanol sequence 50%, 70%, 95% for 10 min per step and followed by 100% (× 2) for 30 min. Drying was performed by using a critical point dryer. Thereafter specimens were mounted on stubs, coated with gold and viewed with SEM (Shimadzu SSX-550 Superscan, Tokyo, Japan).

2.3.5 DNA EXTRACTION

DNA was extracted from cells cultivated in *Mycoplasma* broth for 3 days at 37°C. The cells were centrifuged at 5000 x *g* for 5 min, the supernatant discarded, and 180 µL of Buffer ATL and 20 µL Proteinase K stock solution (QIAGEN) was mixed thoroughly with the cells. Samples were incubated at 56°C for 10 min until proteins and the trilamilar membrane had been lysed. Buffer AL (200 µL) was added to the sample mixture and incubated at 70° for 10 min to denature Proteinase K which could interfere with any upstream applications. The dielectric constant of the solution was decreased with the addition of 200 µL ethanol (96-100%), thereby dehydrating the DNA molecules. The sample mixture was transferred on to an assembled QIAamp Mini Spin Column and Collection tube and incubated for 1 min at room temperature (RT). The sample was passed through the column by centrifugation (6000 x *g* for 1 min), and the DNA absorbed on to the silica membrane due to the presence of high concentrations of chaotrophic salts in the buffer. The flow-through from the column was discarded and the salts were removed by washing with 500 µL alcohol-based Buffer AW1

and Buffer AW2, respectively (× 2). The first wash step was carried out at 6000 x *g* for 1 min, while the second was carried out at 20 000 x *g* for 3 min to remove any excess ethanol that may interfere with upstream processes. The micro spin column was transferred to a sterile DNase-free 1.5 mL microcentrifuge tube, 200 µL of a low-ionic strength solution (TE buffer) was added to the centre of the membrane and incubated at RT for 1 min. The assembled column and microcentrifuge tube was centrifuged at 6000 x *g* for 1 min to elute the purified DNA. DNA was stored at -20°C.

2.3.6 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS

Each PCR reaction consisted of 2.5 mM MgCl; 0.2 mM dNTPs; 0.5 µM of each primer, 5 µL of template, 1.5 units of Taq Polymerase (5 U/µL) and sterile Milli-Q (MILLIPORE) water to the volume of 50 µL. Negative controls were performed which did not contain DNA template. In regards to whole-cell PCR (for Section on universal 16S rDNA), a single colony was picked and incubated at 96°C for 10 min in the Milli-Q water fraction. After which, the rest of the reaction mix was added. Reactions were thermocycled on a 2720 Thermal Cycler (Applied Biosystems). All PCRs were previously optimized during my Honours studies (Moretti, 2009, unpublished data). The various PCRs are as follows:

Universal bacterial 16S rDNA

Universal prokaryotic primers 8F (5'- AGAGTTTGATCCTGGCTCAG -3') and 1525R (5'- AAGGAGGTGATCCAGCC-3') previously described by Beumer and Robinson (2005) were used to amplify the 16S rDNA, producing a band of approximately 1500 base pairs (bp). Initial denaturation was performed at 96°C for 5 minutes (min), followed by 35 cycles of denaturation at 96°C for 30 s; annealing carried out at 50°C for 30 s and extension at 72°C for 90 s. A final elongation step of 72°C for 7 min was performed to allow complete elongation of product. The universal 16S rDNA PCR was mainly used to identify bacterial contamination while establishing the techniques.

Partial *mgc2* gene

Primers mgc2-2F (5'- CGCAATTTGGTCCTAATCCCCAACA -3') and mgc2-2R (5'- TAAACCCACCTCCAGCTTTATTTCC -3') previously described by Hnatow and co-workers (1998) were used to amplify a highly conserved 237-303 bp region of the *mgc2* gene specific

for *M. gallisepticum*. The amplification reaction was performed at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s; 58°C for 30 s and 72°C for 30 s, with a final elongation of 72°C for 5 min. Garcia and co-workers (2005) showed this PCR to be both highly specific and sensitive, and so was used to screen for *M. gallisepticum*.

16S-23S Intergenic Spacer (ITS) Region

Primers IGSRG-F (5'-GGGATGACGTCAAATCATCATGCC-3') and IGSRG-R (5'-TAGTGCCAAGGCATCCACC-3') previously described by Harasawa and co-workers (2004) were designed to amplify the 16S-23S ITS region of various avian *Mycoplasma* species including *M. imitans*. Based on the size of the PCR product, *M. gallisepticum* (~940 bp) can be distinguished from *M. imitans* (~2780 bp) and most other avian *Mycoplasma* (<800 bp). DNA amplification was achieved with an initial denaturation at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s; 60°C for 30 s, 72°C for 1 min, and a final elongation of 72°C for 5 min.

Another primer set, ISR-F (5'- CGTTCTCGGGTCTTGTACAC-3') and ISR-R (5'-CGCAGGTTTGCACGTCCTTCATCG-3') was used to amplify the 16S-23 Intergenic spacer region. This primer set was first described by Ramírez and co-workers (2008) and designed to amplify most avian-associated mycoplasmas. As with the previous primer set, various sized amplicons (approximately 400-2700 bp) are produced for the different mycoplasmas, *M. gallisepticum* (~900 bp) and most other *Mycoplasma* (approximately 400-600 bp). The amplification reaction was performed at 96°C for 5 min, followed by 35 cycles of 96°C for 30 s; 60°C for 30 s and 72°C for 2 min, with a final elongation of 72°C for 5 min.

2.3.7 ANALYSIS OF PCR AMPLICONS

PCR amplicons were electrophoresed and visualized on 1-2% agarose gels containing ethidium bromide (0.3 µg/mL). Agarose gels were prepared and electrophoresed in TAE buffer (0.1 M Tris, 0.05M EDTA [pH 8.0] and 0.1 mM glacial acetic acid) at 90 V for 35 min. The electrophoresed products were visualized with a ChemiDoc XRS (Bio-Rad Laboratories) under short wavelength ultra violet (UV) light.

2.3.8 PURIFACTION OF DNA FROM AGAROSE GELS

The GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare) was used to purify PCR products and the protocol was followed as recommended. To purify the PCR products of interest from nonspecific amplification products, or undesired products, the reaction products were separated by gel electrophoresis and cut out of the agarose gel prior to purification.

The weight of the agarose gel band was determined and 10 µL of Capture buffer type 3 was added for every 10 mg of gel. The agarose was dissolved at 60°C in the chaotrophic buffer, freeing the DNA for binding to the silica membrane. No more than 700 µL of the Capture buffer type 2 sample mixture was transferred on to the GFX MicroSpin column at one time. The samples were incubated at RT for 1 min so as to allow absorption of DNA to the column, followed by centrifugation at 16 000 x *g* for 30 s (Eppendorf 5415D). The flow-through from the column was discarded and 500 µL of Wash buffer type 1 was added to the GFX MicroSpin column containing the bound DNA. The assembled column and collection tubes were centrifuged at 16 000 x *g* for 30 s. After removal of contaminants by ($\times 2$) alcohol-based washes, the GFX MicroSpin column was transferred to a sterile DNase-free 1.5 mL microcentrifuge tube, 50 µL of Elution buffer type 6 was added to the centre of the membrane and incubated at RT for 1 min. The assembled column and sample collection tube were centrifuged at 16 000 x *g* for 1 min to elute the purified DNA.

2.3.9 SEQUENCING OF PCR PRODUCTS

Purified PCR products were quantified using NanoDrop^{TM} 1000 Spectrophotometer v3.7 (Thermo Scientific) and subject to sequencing using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). Each amplification product was sequenced in both directions with the forward and reverse amplification primers. Sequencing was performed in 10 µL reactions of appropriate DNA template (see Table 2.1), 3.2 ρmoles of the corresponding primers in separate reactions, 1 µL terminator ready premix and 2 µL PCR reaction buffer (5x). The reagents were mixed well and spun down by brief centrifugation. The reagent-containing tubes were placed in a 2720 Thermal Cycler (Applied Biosystems) set at 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min and ending with a cooling set held at 4°C.

Table 2.1: Indicating the amount, in nanograms (ng), of template needed for sequencing based on the size of the cleaned PCR product to be sequenced.

PCR product size (base pairs)	Amount Template Added (ng)
100-200	1-3
200-500	3-10
500-1000	5-20
1000-2000	10-40

Post-reaction cleanup was performed manually according to the BigDye Protocol. Milli-Q water (10 µL) was added to PCR sample containing the fluorescently labeled DNA fragments and transferred to a clean 1.5 mL microcentrifuge tube. DNA was precipitated from solution by the addition of 60 µL pure ethanol and 5 µL ethylenediaminetetraacetic acid (EDTA) (pH8.0, 0.125 M) was added in order to remove unincorporated BigDye terminators (preventing "BigDye blobs"). DNA was pelleted by centrifugation at 20 000 x *g* for 15 min and the supernatant completely discarded. The pellet was washed twice with a cold 70% ethanol solution (100 µL), centrifuging at 20 000 x *g* for 5 min each time. The supernatant was removed and the samples were dried completely in a Speedvac Concentrator (SAVANT). Sequencing was performed with the capillary sequencer 3130xl ABI Genetic Analyzer (Applied Biosystems) at the University of the Free State, Department of Microbial, Biochemical and Food Biotechnology.

Complete overlapping of complementary sequences, editing and consensus construction was performed using Geneious Pro v5.4.4 (Drummond *et al*., 2011). Analyzed sequences were compared with known sequences in the GenBank Database using a nucleotide-nucleotide BLAST analysis tool (Altschul *et al*., 1990).

2.3.10 STORAGE OF ISOLATES

Mycoplasma species were isolated in pure culture when possible and stored in liquid nitrogen. This was done by growing the cells up in broth solution, centrifuging at 13 200 x *g* for 20 min, discarding the supernatant and resuspending in fresh broth to make a 10x

concentrate. Glycerol (20%) was added in 1:1 ratio with the sample, and stored in a cryotube in liquid nitrogen.

2.4 RESULTS AND DISCUSSION

2.4.1 MORPHOLOGICAL STUDY OF CONTROL ISOLATES

The live *MG* control isolates, PG31 and A514 were used to establish the cultivation techniques and test the quality of the media, owing to the variability of nutrients found in the serum and yeast extract. Each time new media was prepared from a fresh stock of serum, the batch was tested using these low-passage *MG* control strains. As seen below in Figure 2.1, PG31 was inoculated on to the *Mycoplasma* agar by the spread plate method and viewed using a stereo-microscope. Although the colonies did not comply with the typical "fried-egg" appearance of *Mycoplasma*, they did appear as tiny, smooth, circular, translucent masses in the range of 0.1-0.3 mm in diameter. The colonies were of various sizes, some being smaller than that of 0.1mm in diameter. Although it is typical for mycoplasmas to show the "fried-egg" appearance, it is not definite (Jordan *et al*., 1982).

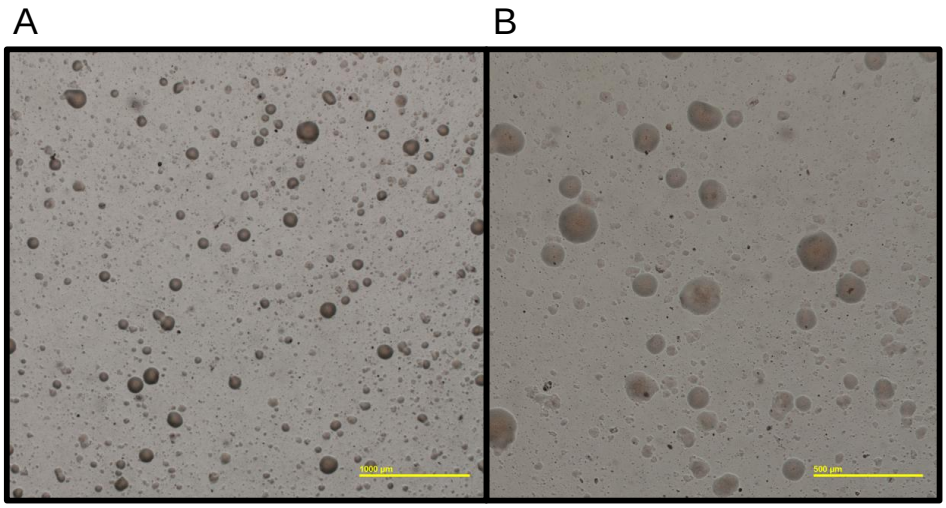


Figure 2.1: *M. gallisepticum* control strain, PG31 was grown overnight in *Mycoplasma* broth, of which a 100 µL was inoculated on to *Mycoplasma* agar using the spread plate method. **(A)** The samples were incubated for 18 days and viewed using a stereo-microscope. **(B)** Increased magnification while viewing the colonies.

Colonies of the *MG* control isolates were also viewed using Scanning Electron Microscopy (SEM). As seen below in Figure 2.2, the colonies appear to "age" and peel off the agar, although this might be an artefact due to the preparation process.

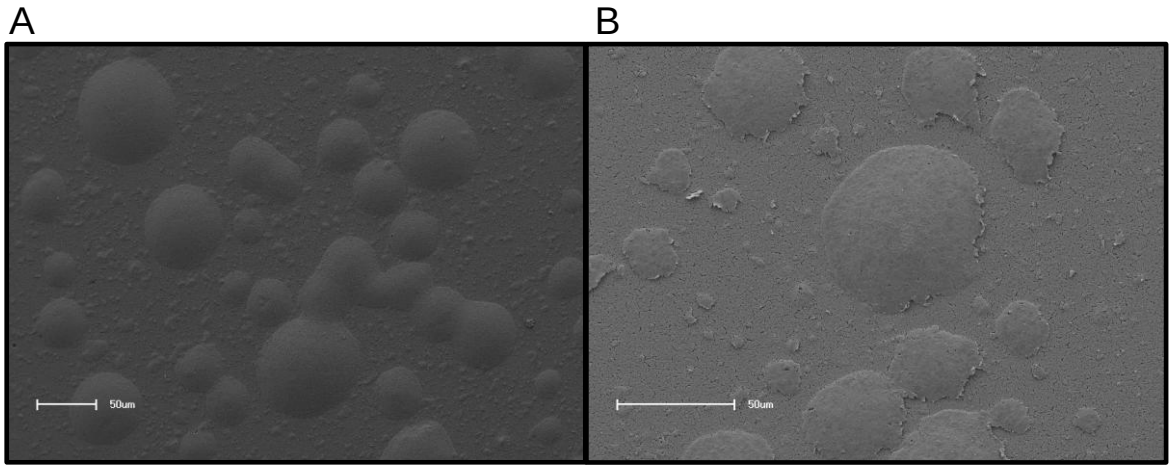


Figure 2.2: *M. gallisepticum* control strain, A514 was grown overnight in *Mycoplasma* broth, of which a 100 µL was inoculated on to *Mycoplasma* agar using the spread plate method. Samples were incubated for (**A**) 14 days and (**B**) 28 days and viewed using a SEM.

A higher magnification of the margin of a colony can be seen in Figure 2.3 below. The control *M. gallisepticum* PG31 strain is compared to the morphology of the S6 strain described by Shifrine and co-workers (1962).

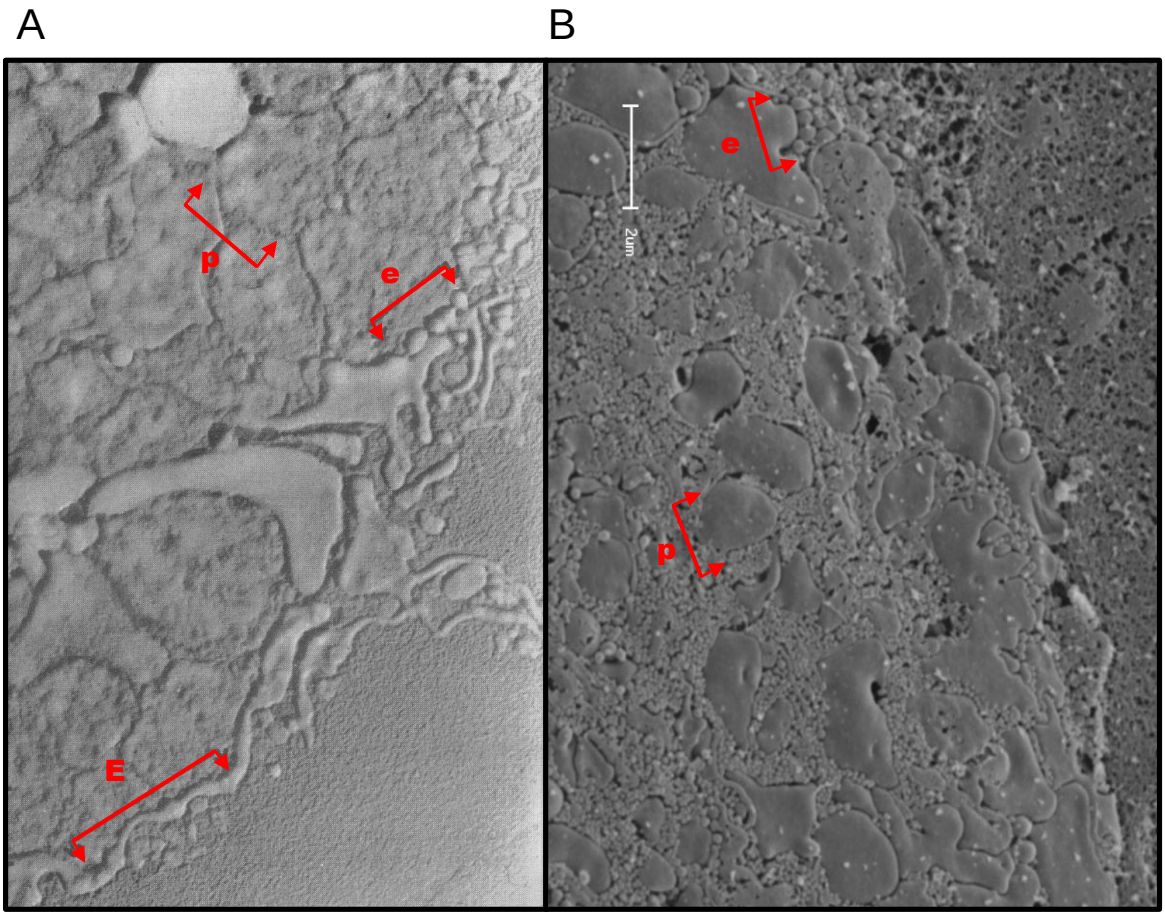


Figure 2.3: Micrograph (SEM) of margin of a colony from *M. gallisepticum* (**A**) S6 strain grown between 16-72 hrs (Shifrine *et al*., 1962) and (**B**) PG31 control strain grown for approximately 14 days. Both have hexagonal platycytes (**p**), although (**B**) appears to be multi-layered and more developed towards the centre of the colony, mostly likely owing to its age. Exoblasts (**E**) can be seen protruding from platycytes near the margin of the colony in (**A**) and resulting in elementary cells (**e**), while mostly only elementary cells can be seen in (**B**).

It should be noted that the colony of the S6 strain was also much younger (between 16-72 hrs) as compared to the control strain in this case which was cultivated for 2 weeks. Most likely owing to this age difference, the platycytes appear to be multi-layered and more developed towards the centre of the colony for the older PG31 strain. Exoblasts, only present in colonies older than 12 hrs (Shifrine *et al*., 1962), can be seen protruding from the platycytes predominantly near the margin of the colony. It is from these exoblasts that elementary cells develop. They are the minimal reproductive units (0.1-0.5µm). Elementary cells are, however capable of developing directly from platycytes (Shifrine *et al.*, 1962). The platycytes and elementary cells around the periphery of a colony occur in a single layer, while those near the centre tend to pile up. It is speculated that this may be the cause for the

central papillae often observed in mycoplasmal colonies, resulting in the “fried-egg” appearance. The role of the different types of cells play during infection, when mycoplasmas invade a host, and whether both types are present in lesions of diseased animals, is unknown at this stage.

2.4.2 STUDY OF FIELD ISOLATES

2.4.2.1 Farm A

Of the 12 swab samples taken from Farm A, only 3 of which changed the *Mycoplasma* broth yellow, indicating the presence of acid production (Figure 2.4). Nonetheless, all 12 broths were plated out on to *Mycoplasma* agar since arginine-hydrolyzing *Mycoplasma* may inhibit the presence of glucose-fermenting *Mycoplasma*. Despite this, only the broths that changed yellow grew faintly on the plates with a “fried-egg” appearance, as seen in Figure 2.6A.

Figure 2.4: Photograph illustrating the change in colour of *Mycoplasma* broth from red to yellow with the production of acid from the fermentation of glucose.

DNA was extracted from all the broth samples and screened using the *mgc2*-PCR highly specific for *M. gallisepticum*. As seen in Figure 2.5, both the positive and negative controls were in order while all samples were negative for *M. gallisepticum*.

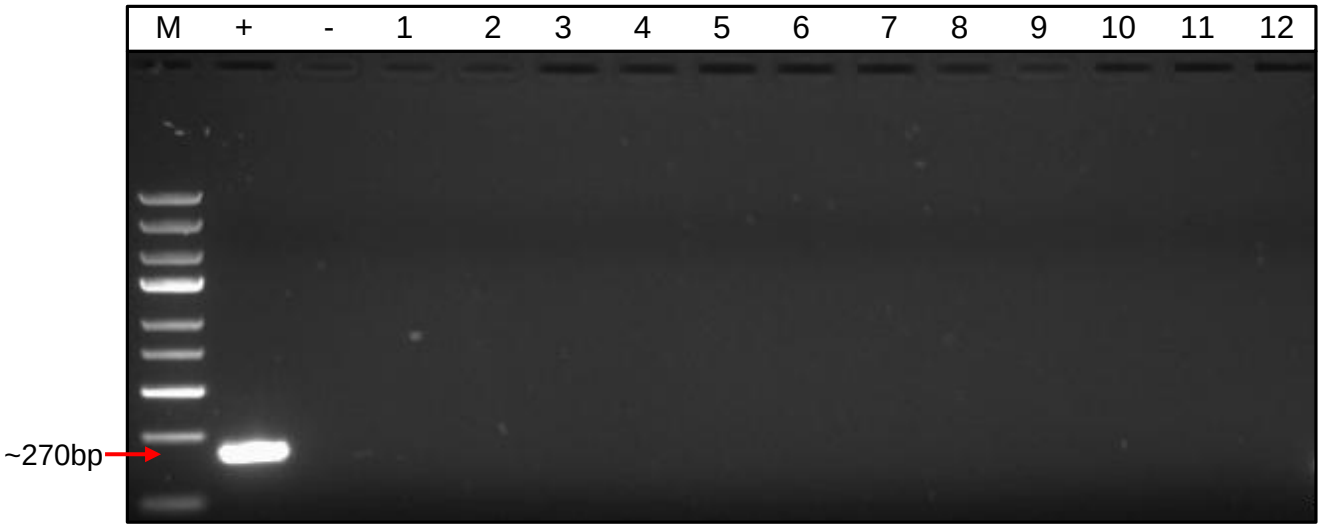


Figure 2.5: PCR amplification products using primer pair mgc2-2F and mgc2-2R (Hnatow *et al.,* 1998) to amplify the partial *mgc2* gene highly specific and sensitive for *M. gallisepticum*. Samples 1-12 (indicated as 1-12) were all negative, while the positive control (+) was *M. gallisepticum* (strain A514) of approximately 270 bp. The negative control (-) showed there to be no contamination. Lane M represents the molecular marker (O'GeneRuler^{TM} Express DNA ladder). Fragments were separated on a 2% gel (w/v) stained with ethidium bromide and visualized with UV illumination.

After further isolation and incubation of the three isolates, the colonies grew more rapidly, appeared larger, more pigmented and with a slimy appearance as seen in Figure 2.6B. Additionally, they gave off a strong odour. A Gram stain confirmed the bacteria to be Gram negative with short rods (Figure 2.3C). The contamination was identified as *Pseudomonas aeruginosa* by sequencing the 16S rDNA (BLAST results were omitted as to keep the focus on the mycoplasmas, however are available on request). *Pseudomonas* is generally considered to be an opportunist pathogen in poultry, and has been found to occur with mycoplasmas resulting in concurrent infection (Peterson, 1975; Randall *et al.,* 1984). *Pseudomonas* is commonly isolated from poultry and can be found in the infra-orbital sinuses and air sacs, resulting in respiratory infections and sinusitis (Fales *et al*., 1978). *Pseudomonas* is also known to be naturally resistant to many antimicrobials. This is due to the presence of the thick cell-surface polysaccharides aiding as a barrier between the cell wall and the environment, as well as the action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes (Poole, 2004). This is most likely why the *Pseudomonas* isolates initially grew slowly, and upon passage gained resistance to the ampicillin and thallium acetate thereby accelerating its growth rate.

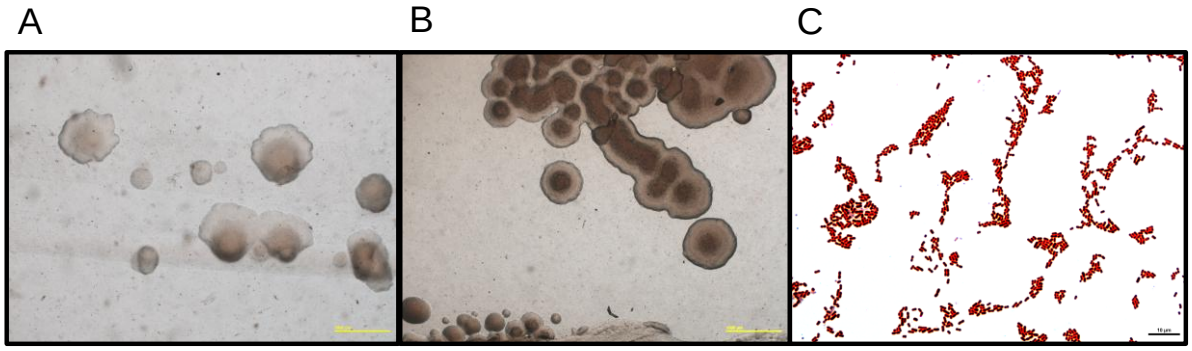


Figure 2.6: Micrograph of the morphology of bacteria isolated from Farm A. (**A**) The colonies originally appeared small, translucent and with a "fried-egg" appearance. (**B**) Upon further isolation and cultivation their growth rate increased, the colonies became larger and also more pigmented. (**C**) A Gram stained showed them to be Gram negative short rods later identified as *Pseudomonas aeruginosa*.

2.4.2.2 Farm B

Of the 12 swab samples taken from Farm B, only 6 samples showed a positive yellow colour change in the *Mycoplasma* broth. All 12 broths were plated out on to *Mycoplasma* agar, however only the 6 samples showing positive colour change grew. Two samples were identified as fungi by the presence of mycelium and their relative size under a light-microscope. Control of fungal contamination may be achieved by using amphotericin B (2.5 mg/mL). It works by binding sterol, which occurs in mycoplasmas; however has a higher affinity for ergosterol, which is the principal membrane sterol in fungi (Tully, 1983). As with the previous samples from Farm A, the remaining isolates were identified as *P. aeruginosa*. They showed the same trend in morphology (Figure 2.7), and were confirmed by sequencing the 16S rDNA.

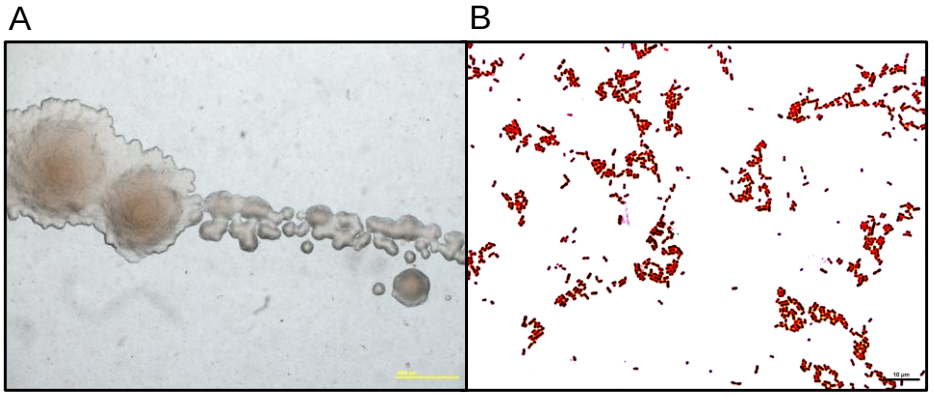


Figure 2.7: Micrographs showing the morphology of the contamination identified as *P. aeruginosa*. (**A**) The colony morphology of the isolate showing a slimy, "fried-egg" morphology. (**B**) Gram stain done on a 24 hr culture showing the Gram negative short rods.

2.4.2.3 Farm C

Of the 12 swabs samples taken from Farm C, only 2 samples resulted in a yellow colour change of the *Mycoplasma* broth. One of which was identified as a contaminating yeast by its larger size and the presence of budding under a light-microscope. All 12 broths were plated out on to *Mycoplasma* agar, with two samples showing growth. Colonies were tiny, smooth, circular, translucent and showed the “fried-egg” appearance, thereby conforming to the typical morphology of *Mycoplasma* (Figures 2.8 and 2.9).

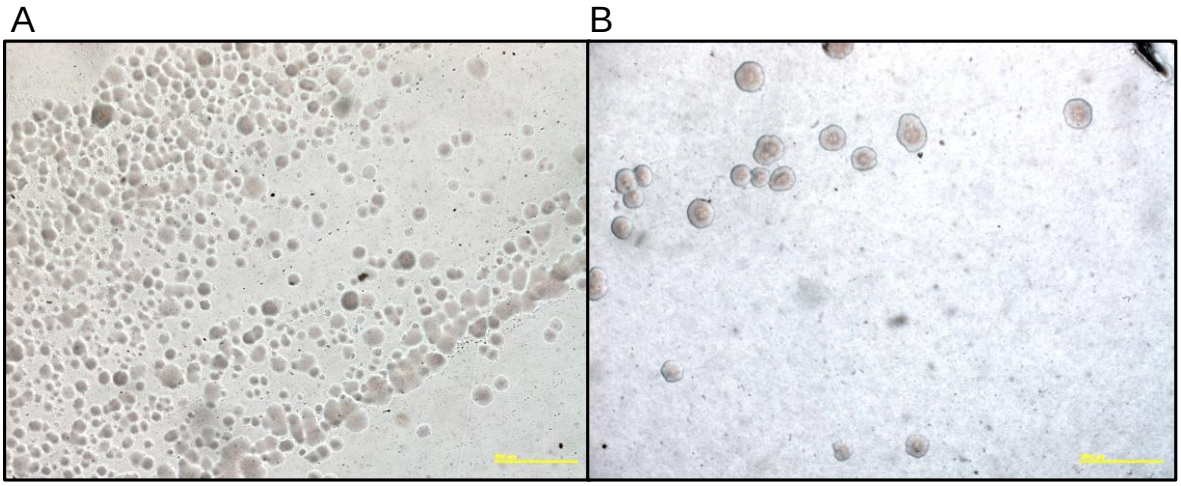


Figure 2.8: Micrograph from a stereo-microscope of the colony morphology of sample 2 identified as *M. gallinarum* (**A**) after 7 days of incubation, (**B**) and 18 days of incubation. The streak plate method was used.

DNA was extracted from these two samples and screened using the *mgc2*-PCR highly specific for *M. gallisepticum.* No PCR amplification products were seen, implying the absence of *M. gallisepticum*. On the assumption it was perhaps another *Mycoplasma* species, the 16S-23S ITS region and the 16S rDNA were sequenced. The isolate capable of glucose fermentation (sample 1) was identified as *M*. *gallinaceum*; while the isolate present in the broth remaining red (sample 2) was identified as *M. gallinarum*.

Table 2.2: Indicating nucleotide-nucleotide BLAST results for the 16S rDNA of samples 1 and 2 with isolates of highest percentage identity and their GenBank accession numbers.

SAMPLE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***IDENTITY***
1	*M. gallinaceum* str. SA	JN935893.1	100%	100%
	M. gallinaceum str. 887	JN935886.1	100%	100%
	M. gallinaceum str. ATCC 33550	JN935878.1	100%	100%
	M. gallinaceum str. 33F	JN935866.1	100%	99%
	M. verecundum str. *ATCC 27862*	JN935881.1	100%	89%
2	*M. gallinarum* str. ATCC 15319	JN935884.1	100%	99%
	M. gallinarum str. B2	FJ666137.1	100%	99%
	M. gallinarum str. PG16	NR044638.1	100%	99%
	M. columbinum str. FG295	EU859979.1	100%	95%

Table 2.3: Indicating nucleotide-nucleotide BLAST results for the 16S-23S ITS region of sample 1 and 2 with isolates of highest percentage identity and their GenBank accession numbers.

SAMPLE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***IDENTITY***
1	*M. gallinaceum*	AY729927.1	100%	100%
	M. gallinaceum str. 887	JN935886.1	100%	99%
	M. gallinaceum str. ATCC 33550	JN935878.1	100%	99%
	M. gallinaceum str. SA	JN935893.1	100%	99%
	M. gallinaceum str. 33F	JN935866.1	100%	99%
	M. glycophilum	AY729932.1	100%	89%
2	*M. gallinarum* str. ATCC 15319	AY766086.1	100%	100%
	M. gallinarum str. B2	FJ666137.1	99%	99%
	M. iners str. PG30	JN935870.1	99%	89%

Upon examination of the colonies of sample 1 (identified as *M. gallinaceum*) with a stereo-microscope, two different colony morphologies were observed: small, raised colonies (Figure 2.9Br) and larger, flattened colonies (Figure 2.9Bf).

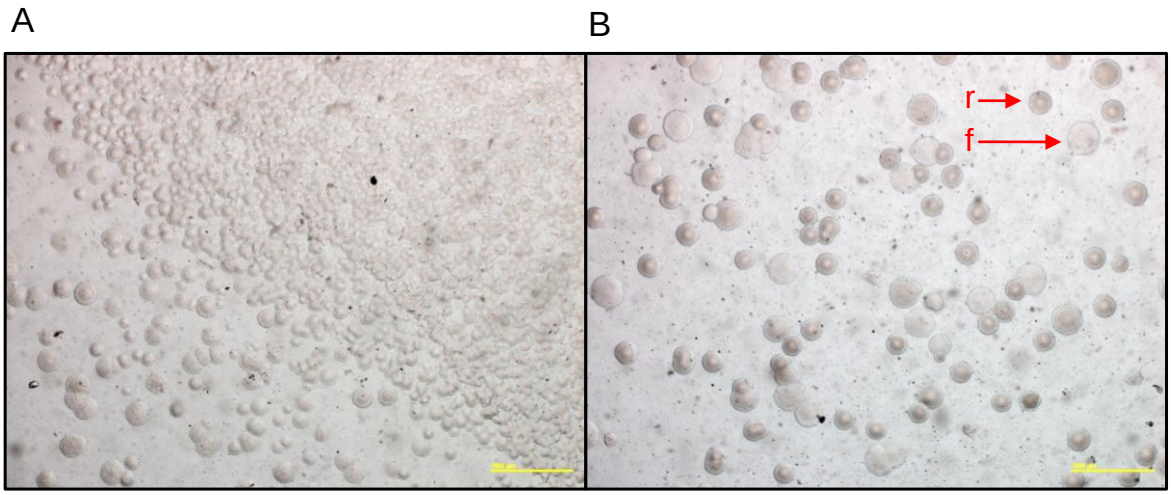


Figure 2.9 Micrograph from a stereo-microscope of the colony morphology sample 1 (identified as *M. gallinaceum*) after 8 days of incubation. (**A**) The streak plate method was used. (**B**) Two different colony morphologies, obtained from what was believed to be a pure culture, can be seen i.e. (**r**) small, raised colonies, and (**f**) larger, flattened colonies.

Whole-cell PCR targeting the 16S-23S ITS region was performed on each of the colony morphologies. The sequences were aligned and found to be identical, confirming both isolates to be *M. gallinaceum*. Due to the small size of the colonies, it is possible that a pure culture was not achieved and two different strains of *M. gallinaceum* were present. Since the ITS-PCR would not be sensitive enough to distinguish between strains, more hyper-viable regions between strains would have to be targeted.

Another explanation for this occurrence could be phenotypic switching which has previously been described by Rosengarten and Wise (1990) in *Mycoplasma hyorhinis*. This isolate has been shown to undergo high-frequency phase transition in colony morphology and opacity and in the expression of diverse lipid-modified, cell-surface protein antigens. These proteins also spontaneously vary in size, contain highly repetitive structures, and are orientated with their carboxyl-terminal region outside of the membrane. This ability to rapidly alter the expression and structure of surface components reflects an important strategy for adaptation to changing environments.

2.4.2.4 Farm D

Of the 8 swab samples taken from Farm D, 4 samples showed positive colour change after overnight incubation at 37°C. The broths (100 µL) were plated out on to *Mycoplasma* agar, and within 5 hours of incubation the plates were overgrown with contamination. A gram stain showed gram negative rods, and the 16S rDNA-PCR confirmed the contamination to be *Klebsiella pneumoniae* and a *Proteus species*, both of which are known to cause respiratory problems. To minimize the fast growing contamination, the broth solution was passed through a 0.45 µm filter. After concentrating the filtrate by centrifugation, part was plated out on *Mycoplasma* agar and another part was used for DNA extraction. The 16S-23S ITS region PCR was carried out to detect any mycoplasmas (Figure 2.10). If multiple *Mycoplasma* species were present in one sample they would most likely appear as different sized bands. Of the four samples, 3 showed single bands (~700 bp); however, lower than that produced by the positive control *M. gallisepticum* strain A514 (~1000 bp).

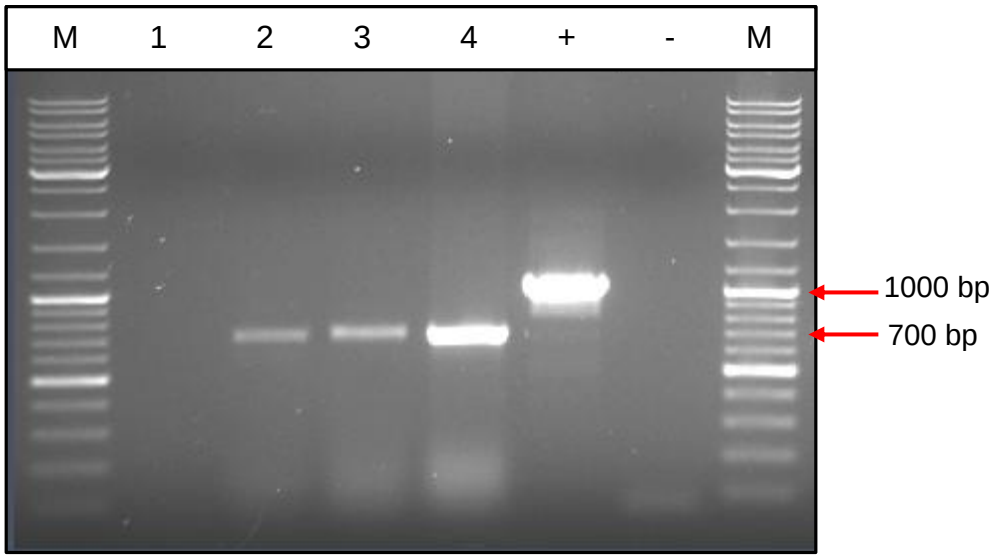


Figure 2.10: PCR amplified products using the primer pair IGSRG-F and IGSRG-R (Harasawa *et al.*, 2004) to amplify the 16S-23S ITS region, producing variable size products for different mycoplasmas. Lane M represents the molecular marker (O'GeneRuler™ Ladder mix). Both the MG A514 positive control (+) and the negative control (-) were present. Fragments were separated on a 1% (w/v) agarose gel, stained with ethidium bromide and visualised with UV illumination.

The samples 2, 3 and 4 depicted in Figure 2.10 were selected for sequencing, thereafter the data obtained was analyzed *in silico*. Nucleotide data of samples were aligned using ClustalW (Larkin *et al.,* 2007) and all shared identical sequences:

```
5′–TCATCATGCCTCTTACGAGTGGGGCAACACACGTGCTACAATGGACGGTACAAAGAGAAGCAAGA
CGGCGACGTGGAGCAAATCTCAAAAAACCGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATG
AAGTCGGAATCGCTAGTAATCGTAGATCAGCTACGCTACGGTGAATACGTTCTCGGGTCTTGTACACA
CCGCCCGTCAAACCATGGGAGCTGGTAATGCCCGAAGTCGGTTTTGTTAACTACGGAAACAACCGCCT
AAGGCAGGACTGGTGACTGGGGTTAAGTCGTAACAAGGTATCCCTACGAGAACGTGGGGATGGATTAC
CTCCTTTCTACGGAGTACATTACCTATATTAATAGGATACTTAAGACTATTTTTATTAAAATTATTTG
TTACAATGACACATGTACTATGTTAATAGTCCATAGATATATCTAGTTTTGAGAGAACTTTCTCTCTA
ATTGTTCTTTGAAAACTGAATAGTAAAGATATTACAACGACATCAAATAAAATAAATAAATTAAATTG
GTTAATTTGTTTTGATTGATACCGAGTAATTATTATTAAATATAATAATTTATTGAAATGTCTTTGAA
TATACATCAACAATAGGTCATATATTGTTACAACTTTTAAATAAGTAAGAGTTTGTGGTGGATGCCTT
TGGCAC- 3′
```

Nucleotide-nucleotide BLAST (Altschul *et al*., 1990) of the consensus sequence showed the isolate from this farm to most likely be *Mycoplasma glycophilum*. Although the identity is 100%, the query coverage is only 69% since the primers used to amplify the 16S-23S ITS region also amplify a large region of the flanking 16S rDNA, and isolate AY729932.1 only has its 16S-23S ITS region submitted in GenBank

Table 2.4: Indicating nucleotide-nucleotide BLAST results for the consensus sequence of samples from Farm D with isolates of highest percentage identity and their GenBank accession numbers.

ISOLATE / SPECIES	***ACCESSION NO.***	***QUERY COVERAGE***	***IDENTITY***
M. gallopavonis str. WR1	FJ226577.1	99%	95%
M. glycophilum	AY729932.1	69%	100%

Furthermore, sample 4 (Figure 2.10) was the only isolate to have grown on the agar plates. After which, the isolate failed to be passaged. In a study performed by Forrest and Bradbury (1984) regarding the characterization of *M. glycophilum*, they noted that the isolate would grow for a maximum of three passages.

2.4.2.5 Farm E

Of the 8 swab samples taken, a positive colour change was observed for 3 of the broths. After plating out 100 µL of each broth, 5 showed growth conforming to that typical of mycoplasmas. DNA was extracted from the original broths and screened for *M. gallisepticum* using the *mgc2*-PCR. This was done to ensure that if *M. gallisepticum* was present and being overgrown by other faster-growing mycoplasmas, the sensitive PCR would detect this minority species. All samples were however negative, suggesting the colonies were another species of *Mycoplasma*. Consequently, they were screened using the 16S-23S ITS region PCR (Ramírez *et al.,* 2008). The 5 samples previously showing growth all produced bands (~500 bp) slightly lower than that of the positive control of *MG* A514 (~800 bp). It is possible that the PCR did not detect *MG* because the high DNA load of other *Mycoplasma* species compared to that of *MG*.

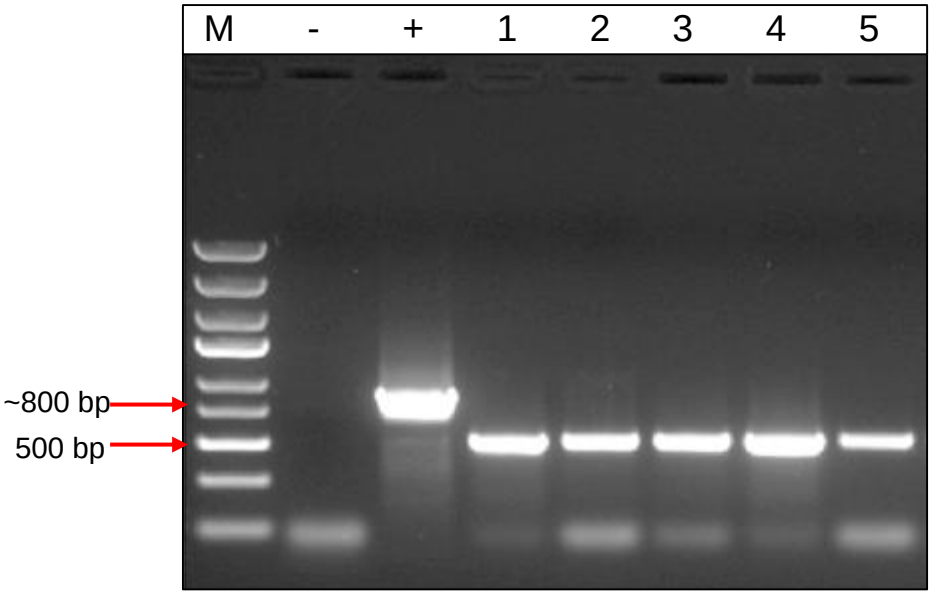


Figure 2.11: PCR amplified products using the primer pair ISR-F and ISR-R, previously described by Ramírez and co-workers (2008) to amplify the 16S-23S ITS region, producing variable size products for different mycoplasmas. Lane M represents the molecular marker (O'GeneRuler™ Ladder mix). Both the *MG* A514 positive control (+) and the negative control (-) were present. Fragments were separated on a 1% (w/v) agarose gel, stained with ethidium bromide and visualised with UV illumination.

The bands were excised and sequenced. All the isolates that showed a yellow colour change were identified as *M. gallinaceum*, owing to their ability to ferment glucose as does *MG*. The two isolates that grew on the agar plates, however with the broth remaining red,

were identified as *M. gallinarum*. The broth remained red since this species is incapable of fermenting glucose, but instead hydrolyzes arginine thus increasing the alkalinity. The sequences of each isolate identified as the same species were aligned using Clustal W (Larkin *et al.,* 2007) and showed to be identical.

2.5 CONCLUSIONS

Morphology may be markedly affected by the nutritional quality and osmotic strength of the medium and importantly by lipid content of the membrane (Rodwell & Mitchell, 1979; Razin, 1983b). Since the growth medium used was undefined, control isolates were used to test each batch of media and establish the cultivation techniques. By viewing the colony morphology using a stereo-microscope and SEM, they appeared to conform to the typical morphology of *Mycoplasma*. Once this was established, field isolates were collected.

Poultry farms were selected based on clinical signs of *MG* and some even on positive serological test results so as to increase *MG* detection, however *M. gallisepticum* was not detected via cultivation from any of the farms during this study. This is most likely due to faster-growing *Mycoplasma* species, such as *M. glycophilum*, *M. gallinaceum* and *M. gallinarum*, and also due to antibiotic resistant bacterial contaminants. The rapid growth of these isolates is thought to drop the pH of the media significantly, thereby killing *MG*, especially if it is present in low numbers (Bradbury *et al*., 2001). As recommended by Bradbury and co-workers (2001), the number of passages should be restricted in order to maximize the chances of *MG* detection and prevent the overgrowth of contaminating species. However, Ley (2008) states field isolates may take longer to grow and may require multiple passages. To prevent the growth of contaminating bacteria, excluding other mycoplasmas, the broth was passed through a 0.45 µm and the filtrate containing the *Mycoplasma* cells was concentrated by centrifugation. This showed to be effective, however would not be advised for *MG* present in very low numbers.

Mixed cultures of tiny *Mycoplasma* colonies pose a problem for isolation. Initially, DNA extract of multiple colonies of each sample was used as template for the 16S-23S ITS region PCR. This would determine if various *Mycoplasma* species were present in one sample,

resulting in different sized bands. If so, care should be taken to isolate each individual species. However, this would still exclude any *MG* isolates unable to be cultivated under the conditions previously mentioned. Immunofluorescence and immunoperoxidase techniques have been suggested in literature for use of identification of various mixed colonies, however these techniques tend to be costly (Del Giudice *et al*., 1967; Polak-Vogelzang *et al*., 1976).

Although we were unable to confirm the presence of *MG* in the field isolates, it remains a possibility that mycoplasmas such as *M. gallinaceum* and *M. gallinarum* may serve as a cofactor for pathogenic respiratory viruses or bacteria. Various research reports have observed this cofactor pathogenic link in *M. gallinarum* (Kleven *et al*., 1978; Bradbury, 1984; Shah-Majid, 1996) despite its apparent non-pathogenic nature. Although poultry farms were chosen based on clinical signs and previous positive serological test results for *MG*, the clinical signs are very broad-based and may have been a result of mycoplasmas such as *M. gallinarum* in combination with the opportunistic pathogens *K. pneumoniae* and *P. auruginosa*. Pathogens such as *M. synoviae* not selected for by culturing could also be responsible for the clinical signs. In the case of serology, it is well known that the closely related *M. imitans* may result in false positive results for *M. gallisepticum.*

The difficulty to cultivate *M. gallisepticum in vitro* has lead to the major impediment of its research and laboratory diagnosis. Despite many efforts through the years, there seems to be a general consensus that only a minority of the mycoplasmas existing *in vivo* have been cultivated so far, and even some cultivable mycoplasmas grow poorly on the best *Mycoplasma* media available (Razin, 1994). In order to overcome the strict nutritional requirements of mycoplasmas, complex media are used for their cultivation. However, the use of these complex undefined growth media have interfered with the molecular definition of mycoplasmal metabolic pathways, genetic analysis, preparation of mycoplasmal antigens free of serum components, etc (Razin, 1994).

For this reason, the next chapter will concentrate on screening *MG* on various poultry farms by extracting DNA directly from the swab sample. This will also facilitate the collection of samples, since the isolates need not remain viable. If a novel strain was detected, care could then be taken to isolate and cultivate this sample from the specific farm. In addition,

Bradbury and co-workers (2001) found that *M. gallisepticum* could be more readily isolated from specimens submitted as whole heads rather than swabs, due to significantly fewer “contaminating” species.

CHAPTER 3

MOLECULAR SCREENING OF *M. gallisepticum* FIELD ISOLATES

3.1 INTRODUCTION

The difficulty to cultivate *M. gallisepticum in vitro* has lead to the major impediment of *Mycoplasma* research and laboratory diagnosis. In addition, the use of complex undefined growth media has been shown to interfere with the molecular definition of mycoplasmal metabolic pathways, genetic analysis, preparation of mycoplasmal antigens free of serum components, etc (Razin, 1994). Mycoplasmas also occur as rapidly mutating populations and diversification may occur in very short periods (Miles, 1992). It is argued by Miles (1992) that sampling the host population by cultivation may select particular phenotypes. This then limits attempts to correlate phenotypes determined *in vitro* with properties of the organism *in vivo*.

Molecular techniques such as PCR have thus become a valuable tool in the diagnosis of mycoplasmas, not only for its sensitivity but for its increasing specificity (Garcia *et al*., 2005). PCR has allowed the study of microbial genes, directly amplified from samples, without the need for cultivation. The specificity of the method is highly dependent on the target chosen. Earlier methods primarily targeted the *16S rRNA* gene (Kempf *et al*., 1993), whereas more recent methods target the surface proteins and the 16S-23S intergenic spacer region (Liu *et al*., 2001; Garcia *et al*., 2005; Raviv *et al*., 2007).

The *16S rDNA* gene is highly conserved region with low levels of genetic variation, which reduces likelihood of excluding *MG* strains, however may not always be sufficient to differentiate closely related species such as *M. imitans* (Garcia *et al*., 2005). More recently the 16S-23S intergenic spacer region was shown by Garcia-Martinez and co-workers (1999) to be a better target for prokaryotic diversity, in that is it highly variable in both length and sequence, even within closely related species.

Primers may be designed to be species-specific as shown by Raviv and co-workers (2007), or genus-specific as shown by Harasawa and co-workers (2004) and Ramírez and co-workers (2008). Although capable of distinguishing isolates to the species level, extreme specificity is needed when live vaccine strains are used in the poultry industry. It is often critical that non-pathogenic strains such as vaccine strains can be distinguished from pathogenic strains. For this reason, membrane surface proteins such as cytadhesins are often targeted and offer more variability and therefore better discrimination between strains. A highly specific and sensitive PCR was developed by Lysnyansky and co-workers (2005) for the detection of *MG* by targeting a partial region of the *mgc2* cytadhesin gene. The PCR was tested by Garcia and co-workers (2005) amongst various other PCRs, including those targeting the *16S rRNA* gene and the 16S-23S ITS region, for the detection of *MG*. This *mgc2*-PCR was shown to be the most specific and sensitive of the PCRs.

3.2 OBJECTIVES

The aim of this chapter was to screen various poultry farms in southern Africa for *M. gallisepticum* using molecular methods. As an alternative to cultivation, PCRs targeting various genes were employed. A broad-based PCR targeting the 16S-23S ITS region for various avian mycoplasmas was used to observe the different populations of *Mycoplasma* species within a sample. More specific primer sets, targeting various genes, including *16S rRNA*, *mgc2* cytadhesin and the 16S-23S ITS region within *M. gallisepticum* were used.

3.3 MATERIALS AND METHODS

3.3.1 *REAGENTS AND CHEMICALS*

The reagents and chemicals used were of molecular biological and analytical grade, unless stated differently and were obtained from the following companies.

Amersham Biosciences: GFX™ PCR DNA and Gel Band Purification Kit; **Applied Biosystems**: BigDye terminator v3.1 Kit; 2720 Thermal Cycler; **Fermentas**; O'GeneRuler™ Express DNA Ladder, O'GeneRuler™, Orange Loading dye, Deoxynucleoside triphosphates (dNTPs); **Invitrogen**: TOP 10 *E.coli* competent cells; **New England Biolabs®**: Taq DNA Polymerase with ThermoPol Buffer; **Promega:** pGEM™TEasy vector; **Thermo Scientific**: NanoDrop™ 1000 Spectrophotometer v3.7; **Whitehead Scientific (PTY) LTD**; Agarose D1 LE, QIAamp® DNA mini kit, All synthesis of primers; **J. Bradbury**, **University of Liverpool, UK**: Freeze-dried samples of *M. gallisepticum* strains, PG31(18.11.99) and A514 (21.5.94).

3.3.2 *Mycoplasma ISOLATES*

3.3.2.1 Control isolates: Live freeze-dried reference *M. gallisepticum* strains, *MG* PG31 and *MG* A514, were kindly donated by Prof Janet Bradbury (University of Liverpool). DNA was extracted from these isolates (see chapter 3) and served as positive controls in the various PCRs.

3.3.2.2 Field isolates: Samples were taken from various poultry farms in southern Africa suspected of *M. gallisepticum* infection. Swabs were taken from the choanal cleft, oropharynx, oesophagus or trachea of the chickens. Since the samples were not going to be used for cultivation, a dry swab was used and the samples were transported on ice to preserve the DNA. Names of farms on which samples were collected have been omitted due to client confidentiality privileges.

Group A: Samples were collected from live birds on a poultry farm in Zimbabwe. The flock was treated with the antibiotics tylosin and tiamulin (Denagard), and also vaccinated prior to sampling. An inactive vaccine which was used, had been produced from the *MG* R low strain (manufactured by Lohmann Animal Health). Although the flock was shown to be serologically positive before vaccination, two sets of PCR tests done on FTA swabs from the same flock showed to be negative. These tests were performed by another testing laboratory.

Group B: Samples were taken from layers on two different poultry farms in South Africa during post mortem. Symptoms for Farm 1 included head shaking, cyanosis finally leading to death. Post mortem revealed plaque formation in trachea, moderate tracheitis and peritonitis. Farm 2 included symptoms such as sinusitis and conjunctivitis also leading to death. Post mortem revealed consolidated lungs, mild tracheitis and peritonitis. Neither of the farms had administered any antibiotics, however it was unknown whether the flocks had been vaccinated.

3.3.3 DNA EXTRACTION

DNA was extracted directly from dry swab samples taken from the various poultry farms using the QIAamp DNA mini kit following the manufacture's recommendations. The cotton swabs were cut off from the rod and suspended in a 1.5 mL microcentrifuge tube containing 400 µL PBS. The scissors was sterilized by flaming with 100% ethanol between each sample to prevent sample carry-over. The microcentrifuge tubes were incubated at 56°C for 10 min with Proteinase K (20 µL) and Lysis buffer (400 µL) in order to lyse the cells and digest proteins. The dielectric constant of the solution was decreased with the addition of 400 µL pure ethanol, thereby dehydrating the DNA molecules. The sample mixture (700 µL × 2) was transferred on to an assembled QIAamp Mini Spin Column and Collection tube and incubated for 1 min at RT. As the sample was passed through the column by centrifugation (6000 x *g* for 1 min), the DNA was absorbed on to the silica membrane. The binding of DNA to silica is driven by dehydration and hydrogen bond formation, which competes against weak electrostatic repulsion (Melzak *et al.,* 1996). Hence, a high concentration of salt will help drive DNA adsorption onto silica, and a low concentration will release the DNA. The flow-through from the column was discarded and the salts were removed by washing with 500 µL alcohol-based Buffer AW1 and Buffer AW2, respectively (× 2). The first wash step was carried out at 6000 x *g* for 1 min, while the second was carried out at 20 000 x *g* for 3 min to remove any excess ethanol that may interfere with downstream processes. The micro spin column was transferred to a sterile DNase-free 1.5 mL microcentrifuge tube, 200 µL of a low-ionic strength solution (TE buffer) was added to the centre of the membrane and incubated at RT for 1 min. The assembled column and microcentrifuge tube was centrifuged at 6000 x *g* for 1 min to elute the purified DNA. DNA was stored at -20°C.

3.3.4 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS

Each PCR reaction consisted of 2.5 mM MgCl; 0.2 mM dNTPs; 0.5 µM of each primer, 5 µL of template, 1.5 units of Taq Polymerase (5 U/µL) and sterile Milli-Q (MILLIPORE) water to the volume of 50 µL. Negative controls were performed which did not contain DNA template. In regards to whole-cell PCR, a single colony was picked and incubated at 96°C for 10 min in the Milli-Q water fraction. After which, the rest of the reaction mix was added. Reactions were thermocycled on a 2720 Thermal Cycler (Applied Biosystems). All PCRs were previously optimized during my Honours studies (Moretti, 2009, unpublished data). PCR

amplification product sizes were based on the *M. gallisepticum* R_{low} genome sequence (Genbank: *AE015450*).

Due to the limited amount of control isolates, primer sets were taken from literature, where their sensitivity and specificity had already been established against various other avian *Mycoplasma* species. This also ensured enough nucleotide data for the various regions of different *MG* strains was available for nucleotide-BLAST comparison (Altschul *et al*., 1990). The template DNA used was extracted directly from the swab sample, so as to detect *in vivo* conditions. The various PCRs are as follows:

Partial *16 rDNA* of *M. gallisepticum*

Primer pair MG 14F (5'- GAGCTAATCTGTAAAGTTGGTC -3') and MG 13R (5'- GCTTCCTTGCGGTTAGCAAC-3'), previously described by Lauerman (1998) were used to specifically target a 183 bp partial region of the 16 rDNA of *M. gallisepticum*. Initial denaturation was performed at 96°C for 5 minutes (min), followed by 35 cycles of denaturation at 96°C for 30 s; annealing carried out at 55°C for 30 s and extension at 72°C for 20 s. A final elongation step of 72°C for 7 min was performed to allow complete elongation of product.

Partial *mgc2* gene of *M. gallisepticum*

Primers mgc2-2F (5'- CGCAATTTGGTCCTAATCCCCAACA -3') and mgc2-2R (5'- TAAACCCACCTCCAGCTTTATTTCC -3') previously described by Hnatow and co-workers (1998) were used to amplify a 237-303 bp region of the *mgc2* gene specific for *M. gallisepticum*. The amplification reaction was performed as above, however with an annealing temperature of 58°C and an elongation time of 30 s. Garcia and co-workers (2005) showed this PCR to be both highly specific and sensitive, and so was used to screen for *M. gallisepticum*. It should be noted Garcia and co-workers (2005) published the primers *mgc2*-2R and MG13R in the incorrect orientation, however has been corrected in this study.

16S-23S Intergenic Spacer Region (ITS)

Various primer sets were used to amplify the 16S-23S ITS region, two sets were designed over different regions for the amplification of most avian mycoplasmas and another set used to specifically amplify *MG* only. The primer set, IGSRG-F (5'-GGGATGACGTCAAA TCATCATGCC-3') and IGSRG-R (5'- TAGTGCCAAGGCATCC ACC-3') described by Harasawa and co-workers (2004) were designed to amplify the ITS region of various avian *Mycoplasma* species including *M. imitans*. Based on the size of the PCR product, *M. gallisepticum* (~940 bp) can be distinguished from *M. imitans* (~2780 bp) and most other avian *Mycoplasma* (>800 bp). DNA amplification was achieved using the same PCR conditions as mentioned initially, however with an annealing temperature of 60°C and an elongation time of 30 s.

A second set of primers, ISR-F (5'-CGTTCTCGGGTCTTGTACAC-3') and ISR-R (5'-CGCAGGTTTGCACGTCCTTCATCG-3') was used to amplify the 16S-23 Intergenic spacer region. This primer set was first described by Ramírez and co-workers (2008) and designed to amplify most avian-associated mycoplasmas. As with the previous primer set, various sized amplicons (approximately 400-2700 bp) are produced for the different mycoplasmas, *M. gallisepticum* (~1000 bp) and most other *Mycoplasma* (approximately 400-600 bp). The amplification reaction was performed as initially mentioned, however with an annealing temperature of 60°C and an elongation time of 2 min.

A third set of primers, MG IGSR-F (5'-GTAGGGCCGGTGATTGGAGTTA -3') and MG IGSR-R (5'- CCCGTAGCATTTCGCAGGTTTG-3'), previously described by Raviv and co-workers (2007), were used to amplify an 812 bp product. The primers' annealing sites were sites were designed in the downstream region of the 16S rRNA gene and the upstream region of the 23S rRNA gene for the complete and specific amplification of the *M. gallisepticum* ITS segment. The amplification reaction was performed as initially mentioned, however with an annealing temperature of 55°C and an elongation time of 1 min.

Further methods such as analysis of PCR amplicons, purification of DNA from agarose gels, and sequencing of purified PCR products or plasmid DNA were performed as per mentioned in Chapter 2 (section 2.3.7- 2.3.9). It should be noted if chromatogram base peaks were seen to be overlapping, suggesting variable bases due to population diversification or non-specific amplification, the PCR amplification products were cloned and then re-sequenced.

3.3.5 CLONING OF PURIFIED PCR PRODUCTS INTO THE pGEM™TEasy VECTOR SYSTEM I

3.3.5.1 TRANSFORMATION INTO TOP 10 *E. coli* COMPETENT CELLS

Different sized PCR amplicons that were too close together to be cut out of the gel as separate bands were cloned in TOP 10 *E.coli* cells to be sequenced. Gel purified DNA was ligated into the pGEM™TEasy vector (Promega) in a 10 µL reaction. The amount of DNA insert (ng) used in the reaction was calculated using the following equation:

$$ng\ of\ insert = \frac{ng\ of\ vector \times bp\ size\ insert}{bp\ size\ vector} \times insert{:}\ vector\ molar\ ratio$$

$$ng\ of\ insert = \frac{50ng\ \times size\ insert}{3015\ bp} \times \frac{3}{1}$$

The reactions consisted of the appropriate amount of DNA, 1 µL of 50ng/µL vector, 2 µL 2x ligation buffer and 5 U T4 DNA ligase (Fermentas). The reactions were incubated for 2 hours at RT before transformation into TOP 10 *E. coli* cells.

The TOP 10 *E. coli* cells were made competent with an adjusted version of the rubidium chloride ($RbCl_2$) method as described by Hanahan (1983) and used in 50 µL aliquots.

For each ligation reaction, 50 µL of competent cells were thawed on ice after which 10 µL of ligated plasmid was added to it. A negative control was included which consisted of competent cells with no plasmid. The mixture was placed on ice for 30 min, followed by a heat-shock of 40s at 42°C to increase the permeability of the *E. coli* membranes to the plasmid, and placed on ice again for 2 min. A volume of 250 µL of pre-warmed (37°C) SOC medium (20 $g.L^{-1}$ Tryptone, 5 $g.L^{-1}$ Yeast Extract, 0.01 M NaCl, 0.0025 M KCl, 0.01 M MgCl, 0.01 M $MgSO_4$ and 0.02 M glucose) was added to the samples and shaken at 37°C for 1 hour. After which, 100 µL of the sample was plated out onto pre-warmed (37°C) Luria Bertani (LB [10 $g.L^{-1}$ Tryptone, 5 $g.L^{-1}$ Yeast Extract, 10 $g.L^{-1}$ NaCl and 12 $g.L^{-1}$ bacteriological agar]) plates that were supplemented with ampicillin (60 $mg.L^{-1}$), IPTG (9.6 $mg.L^{-1}$) and X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside [40 $mg.L^{-1}$]). The plates were incubated for 16 hours overnight (O/N) at 37°C after which they were stored at 4°C.

3.3.5.2 CONFIRMATION OF INSERT DNA IN pGEM^{TM}TEasy

Positive transformants (white colonies) were selected from the plates, inoculated into 5 mL LB media containing 50 µL of 10 mg.mL^{-1} ampicillin stock solution and incubated O/N for 16 hours at 37°C on a shaker. After which they were incubated at 4°C.

Screening for positive recombinants plasmids was performed using a whole-cell PCR with the primer set T7 (5'-TAATACGACTCACTATAGG-3') and SP6 (5'-ATTTAGGTGACACT ATAG-3'). Each PCR reaction consisted of 2.5 mM MgCl; 10 mM dNTPs; 0.2 µM of each primer, 0.2 µL of O/N samples in LB media as template, 1.5 units of Taq Polymerase (5 U/µL) and sterile Milli-Q (MILLIPORE) water to the volume of 10 µL. Reactions were thermocycled on a 2720 Thermal Cycler (Applied Biosystems). Initial denaturation was performed at 96°C for 5 minutes (min), followed by 35 cycles of denaturation at 96°C for 30 s; annealing carried out at 53°C for 30 s and extension at 72°C for 40 s. A final elongation step of 72°C for 7 min was performed to allow complete elongation of product. PCR amplicons were resolved on a 1% agarose gel stained with ethidium bromide. LB media samples containing the correct sized bands were selected for small-scale plasmid isolations using PureYield^{TM} Plasmid Miniprep System (Promega).

A volume of 1.5 mL of the O/N bacterial cultures were added to 1.5 mL microcentrifuge tubes and centrifuged at full speed for 30 s. The supernatant was discarded, and the process was repeated with another 1.5 mL of the same bacterial culture. The pellet was resuspended with 600 µL water and 100 µL Cell Lysis Buffer was added to the tubes and mixed by inversion 6 times, until the solution turned clear blue (indicating complete lysis). After not more than 2 min, 350 µL of cold Neutralization Solution was added and mixed thoroughly (complete neutralization was indicated by the solution turning yellow and the formation of a yellowish precipitate). The cells were incubated with Cell Lysis Buffer for no more than 2 minutes, otherwise the membrane would have completely lysed open and the genomic DNA would also have been released. The neutralized samples were centrifuged at full speed for 3 min. The supernatant (~900 µL) was transferred to the assembled PureYield^{TM} Minicolumn with a collection tube, whilst avoiding disturbing the cell pellet. The assembled columns were centrifuged at full speed for 15s, the flow-through was discarded, 200 µL of Endotoxin Removal Wash was added to the columns to removed proteins, RNA and endotoxin contaminants from the purified plasmid DNA, and centrifuged again at full speed for 15 s. Thereafter, 400 µL of an ethanol-based Column Wash Solution was added to the columns and the samples were centrifuged for 30 s at full speed. The columns were

transferred to clean 1.5 mL microcentrifuge tubes and 30 µL of pre-warmed (50°C) Elution Buffer was added directly to the column matrix and incubated at RT for 1 min. The samples were centrifuged for 15 s at full speed to elute the plasmid DNA which was stored at -20°C until required. The previously mentioned primer set, T7 and SP6 were used to sequence the plasmid insert.

3.4 RESULTS AND DISCUSSION

3.4.1 The 16S-23S Intergenic Spacer Region PCR (Harasawa *et al.*, 2004)

Primer set IGSRG-F and IGSRG-R (Harasawa *et al*., 2004) was designed to amplify the complete 16S-23S intergenic spacer region of various avian *Mycoplasma* species including *M. imitans*. Based on the size of the PCR product, *M. gallisepticum* (~940 bp) can be distinguished from the closely related *M. imitans* (~2780 bp) and most other avian *Mycoplasma* species (<800 bp).

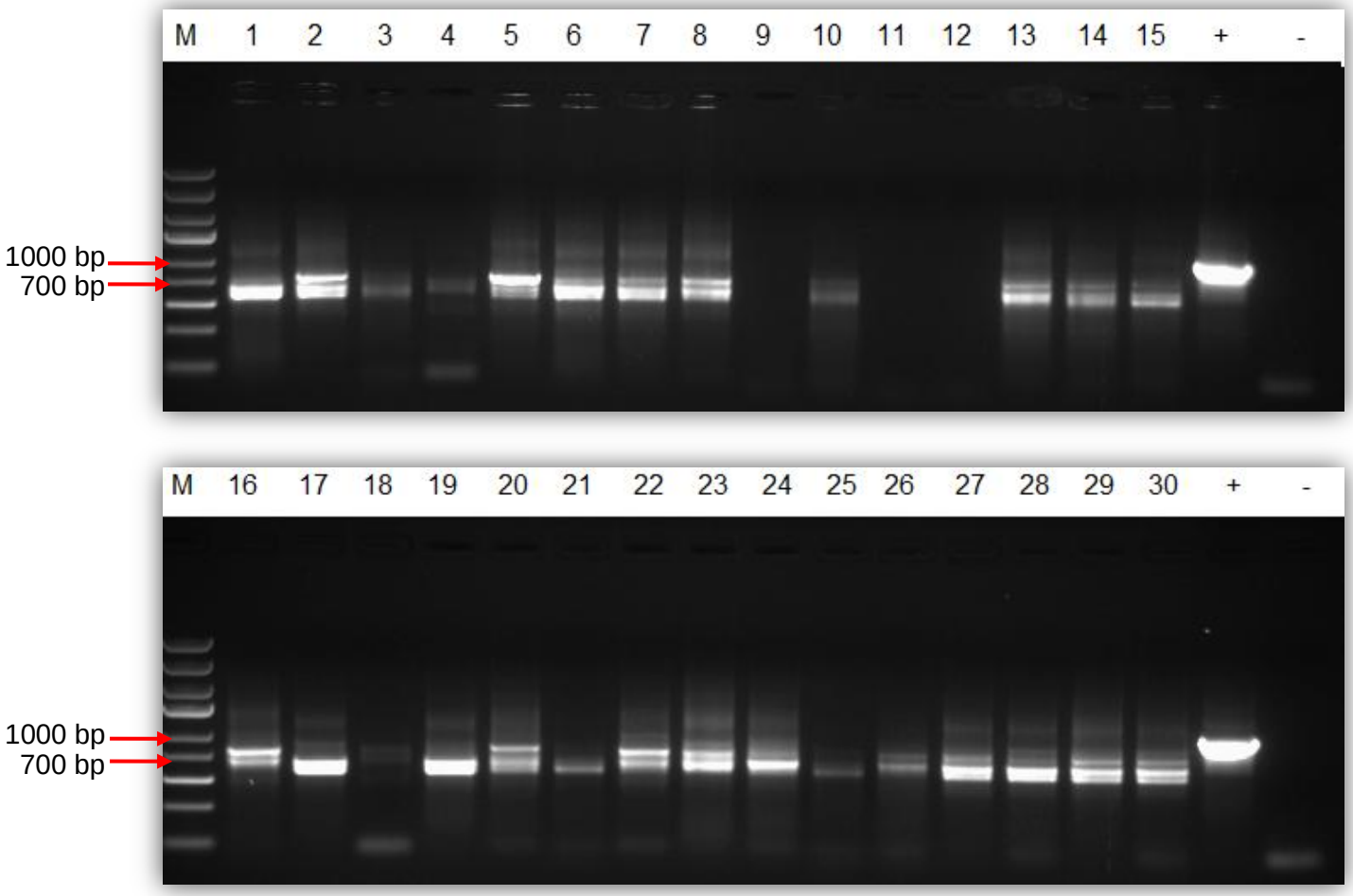


Figure 3.1: A 1% gel (w/v) of the PCR amplification products using primer pair IGSRG-F and IGSRG-R (Harasawa *et al.*, 2004) used to amplify the 16S-23S ITS region of various species of *Mycoplasma,* resulting in different size PCR amplicons. Samples 1-30 were isolated from a poultry farm in Zimbabwe believed to be positive for *M. gallisepticum*. Lane M represents the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* strain A514 of approximately 940 bp, while the last lanes of each gel represent the negative control (-).

Figure 3.1 indicates the amplified products obtained from this primer set, using DNA extracted directly from the swab sample as the template for Group A. The positive control, *MG* strain A514 produced the expected band of approximately 940 bp, while corresponding bands of this size could not be clearly seen from samples 1-30.

Most samples contained a band of approximately 600 bp, while multiple bands were also seen for many of the samples (e.g. samples 2, 8, 16, 20, 22). This may be a result of multiple *Mycoplasma* species present within a single sample or unintended amplification from isolates other than *Mycoplasma*. These primers had previously only been tested by the authors on DNA extracted from axenically cultured *Mycoplasma* species. Samples 9, 11 and 12 appeared to be negative, showing no amplicons. PCR amplicons from samples 1, 6, 17, and 29 were successfully sequenced and showed to be an *Enterococcus* species by nucleotide-nucleotide BLAST (Altschul *et al*., 1990). Both genera, *Enterococcus* and *Mycoplasma* belong to the phylum Firmicutes. An NCBI primer-BLAST (Rozen & Skaletsky, 2000) was performed and showed the primer set to be 100% identical to *Enterococcus faecalis* (CP002621.1) and also capable of amplifying various other *Enteroccocus* species. Three amplification products were predicted for *Enterococcus faecalis*, of 728 bp, 626 bp and 1314 bp. This may explain the multiple bands.

3.4.2 The 16S-23S Intergenic Spacer Region PCR (Ramírez *et al.*, 2008)

Another primer set ISR-F and ISR-R (Ramírez *et al*., 2008) was used to amplify the complete 16S-23S intergenic spacer region of various avian *Mycoplasma* species including *M. imitans*. Based on the size of the PCR product, *M. gallisepticum* (~900 bp) can be distinguished from the closely related *M. imitans* (~2700 bp) and most other avian *Mycoplasma* species (<600 bp). Figures 3.2 and 3.3 indicate the amplified products obtained from this primer set for Group A and Group B respectively. The positive control, *MG* strain A514 produced the expected bands of approximately 900 bp in both Group A and Group B, while the negative controls remained clear.

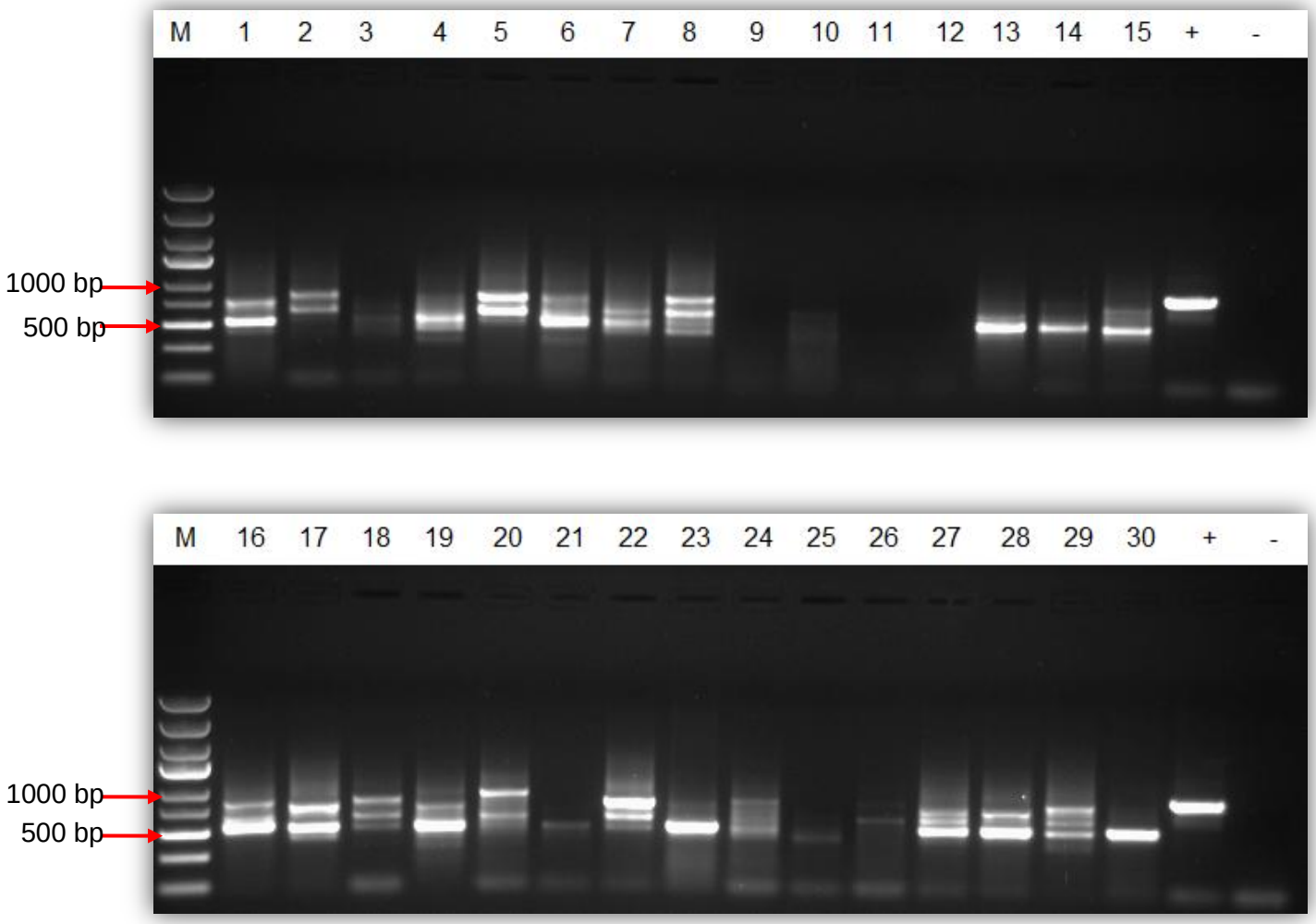


Figure 3.2: A 1% gel (w/v) of the PCR amplification products using primer pair ISR-F and ISR-R (Ramírez *et al.*, 2008) to amplify the 16S-23S ITS region of various species of *Mycoplasma,* resulting in different size PCR amplicons. Samples 1-30 were isolated from a poultry farm in Zimbabwe believed to be positive for *M. gallisepticum*. Lane M represents the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* (strain A514) of approximately 900 bp, while the last lanes of each gel represent the negative control (-).

Bands corresponding in size to the *MG* positive control from Group A could be seen in samples 2, 5, 6, 8, 18, 20, 22 and 24. Samples 9, 11 and 12 appeared to be negative, once again showing no amplicons (refer to section 3.4.1). Most samples from this group contained a band of approximately 550 bp. Samples 23 and 30, of this size range, were sequenced and identified as *M. synoviae*. As with the previous primer set (refer section 3.4.1) multiple bands were seen for many of the samples. As before, these primers had previously only been tested by the authors on DNA extracted from axenically cultured *Mycoplasma* species. Thus the effect of contaminants and unintentional PCR amplifications had not been taken in to account as for direct detection of field isolates.

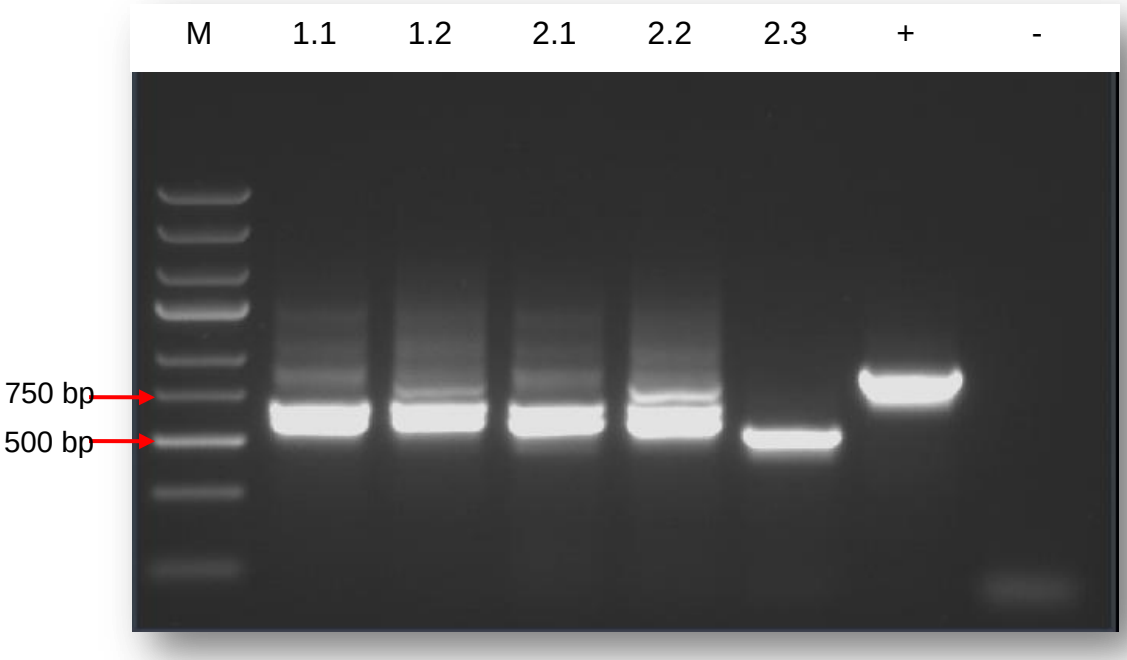
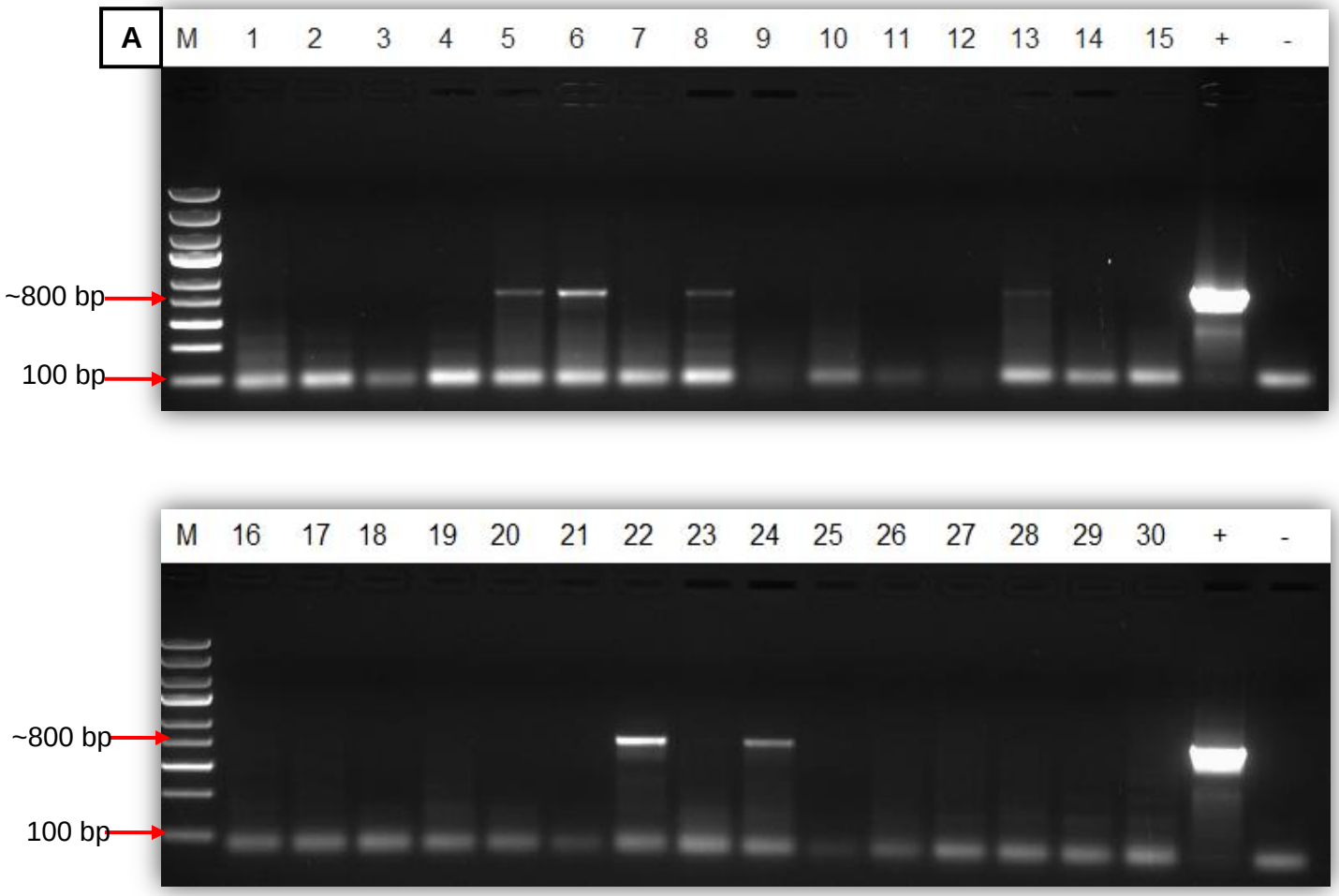


Figure 3.3: A 1% gel (w/v) of the PCR amplification products using primer pair ISR-F and ISR-R (Ramírez *et al.*, 2008) to amplify the 16S-23S ITS region of various species of *Mycoplasma,* resulting in different size PCR amplicons. Samples were isolated from poultry Farm 1 (samples 1.1 and 1.2) and Farm 2 (samples 2.1-2.3) in South Africa believed to be positive for *M. gallisepticum*. Lane M represents the molecular marker (O'GeneRuler^{TM} Express DNA ladder). The positive control (+) was *M. gallisepticum* (strain A514) of approximately 900 bp, while the last lane represents the negative control (-).

In Group B, samples from Farm 1 (1.1-1.2) and Farm 2 (2.1-2.3) isolated in South Africa produced smaller amplicons than that of the positive *MG* control as seen in Figure 3.3. Sample 2.3 produced a single amplicon of approximately 500 bp. This band was purified, sequenced and identified as *M. synoviae*. Samples 1.1-1.2 and 2.1-2.2 showed multiple bands (between approximately 550-750bp) which were identified as non-specific amplifications of *E. coli*. This is most likely a result of a high DNA load of *E. coli* ("burden" DNA) in ratio to the total isolated DNA.

3.4.3 The 16S-23S Intergenic Spacer Region PCR (Raviv *et al*., 2007)

Another primer set, IGSR-F and IGSR-R (Raviv *et al*., 2007) was used to amplify the complete 16S-23S intergenic spacer region of *M. gallisepticum* specifically, to produce an amplicon of approximately 800 bp. Figure 3.4 indicates the amplified products obtained from this primer set for Group A and Group B respectively, using DNA extracted directly from the swab sample as the template. The positive control, *MG* strain A514 produced the expected band of approximately 800 bp in both Group A and B.



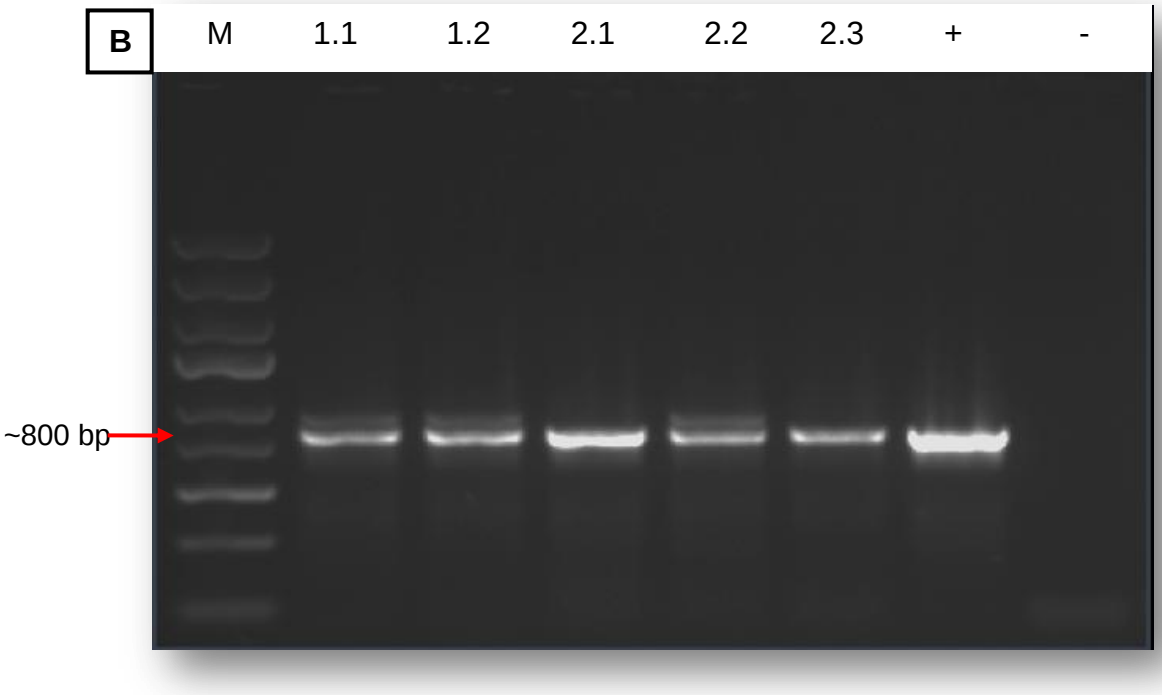


Figure 3.4: A 1% gel (w/v) of the PCR amplification products using primer pair MG IGSR-F and MG IGSR-R (Raviv *et al*., 2007) to amplify the 16S-23S ITS region of *M. gallisepticum* specifically. Lanes M represent the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* (strain A514) of approximately 800 bp, while the last lanes of each gel represent the negative (-) control. **A**) Samples 1-30 were isolated from a poultry farm in Zimbabwe believed to be positive for *M. gallisepticum*. **B**) Samples were isolated from poultry Farm 1 (samples 1.1 and 1.2) and Farm 2 (samples 2.1-2.3) in South.

In Group A and B, bands corresponding to the size range of the *MG* positive controls were seen, however with less intensity. Samples 5, 6, 8, 13, 22 and 24 of Group A showed positive amplification. Bands observed just below 100 bp in Figure 3.4A were most likely primer-dimers formed during the PCR reaction. In Group B, despite *MG* being absent in the previous 16S-23S ITSR PCR in section 3.4.2 targeting various mycoplasmas (which was performed twice, but gave the same results), it is detected in all the samples using this more specific primer set, as seen in Figure 3.4B.

Samples showing positive amplification from Group A and Group B were excised from the gel and purified to be sequenced. Positive amplicons of samples 6, 20 and 22 from Group A and all the samples from Group B were sequenced successfully. The data was analysed using Geneious Pro v5.4.4 (Drummond *et al*., 2011), and showed samples isolated from the same farms to be identical in sequence. Thus samples from Group A were all identical, while

samples from Group B were divided in to Farm 1 and Farm 2. Subsequently Farms 1 and 2 were aligned using ClustalW (Larkin *et al.,* 2007) and showed to be 100% identical to each other, however did show minor nucleotide differences to Group A as seen in Figure 3.5.

```
Farm 1          AACTTATGTATTTGATCCATTAGCTATTTTTATTGAATCTATA-TTATATGAAATTTGAT 59
Farm 2          AACTTATGTATTTGATCCATTAGCTATTTTTATTGAATCTATA-TTATATGAAATTTGAT 59
Group A         AACTTATGTATTTGATCCATTAGCTATTTTTATTGAATCTATAATTATATGAAATTTGAT 60
                ************************************** ***************

Farm 1          TTGATACTTTAATCAAATTTCGGACTAGAAAGATTTGTCGTATTCAGTTTTCAAAGAACA 119
Farm 2          TTGATACTTTAATCAAATTTCGGACTAGAAAGATTTGTCGTATTCAGTTTTCAAAGAACA 119
Group A         TTGATACTTTAATCAAATTTCGGACTAGAAAGATTTGTCGTATTCAGTTTTCAAAGAACA 120
                ************************************************************

Farm 1          ATGTAGCTATATAAGCCTTAAAGATTATAGCACAATAAATCAGTTTGCACAACACTTTTC 179
Farm 2          ATGTAGCTATATAAGCCTTAAAGATTATAGCACAATAAATCAGTTTGCACAACACTTTTC 179
Group A         ATGTAGCTATATAAGCCTTAAAGATTATAGCACAACAAATCAGTTTGCACAACACTTTTT 180
                ********************************** ***********************

Farm 1          TAAAAAAACTTTTATCATTGGATTTTTTTATAAGCATTAAAGCTAAAATCACAAGTAAAA 239
Farm 2          TAAAAAAACTTTTATCATTGGATTTTTTTATAAGCATTAAAGCTAAAATCACAAGTAAAA 239
Group A         TAAAAAA-CTTTTATCATTAAATTTTTT-ATAAGATTTAAAGCTAAAATCACAAGTAAAA 238
                ******* **********  ******* *****  ************************

Farm 1          AA-TAACCAATGATTAATAATAAAAAAAAGCCTTTTTAAATCATTGGTTTGAACAGATAA 298
Farm 2          AA-TAACCAATGATTAATAATAAAAAAAAGCCTTTTTAAATCATTGGTTTGAACAGATAA 298
Group A         AAATAACGAATGATTAATAATAAAAAAA-GCCTTTTTAAATAATTGGTTTGAACAGATGA 297
                ** **** ****************** ************ *************** *

Farm 1          ATCTGCATTTTTTGCAGTAAAAATAATAAAAAAA 332
Farm 2          ATCTGCATTTTTTGCAGTAAAAATAATAAAAAAA 332
Group A         ATCTGCATTTTTTGCGGTAAAAATAACAAAAAAA 331
                *************** ********** *******
```

Figure 3.5: ClustalW sequence alignment of the consensus sequences from Group A and Group B, namely Farm 1 and Farm 2, of the 16S-23S ITS region. Nucleotides with a star below (*) are indicative of identical nucleotides for the sequences, while differences are highlighted in yellow.

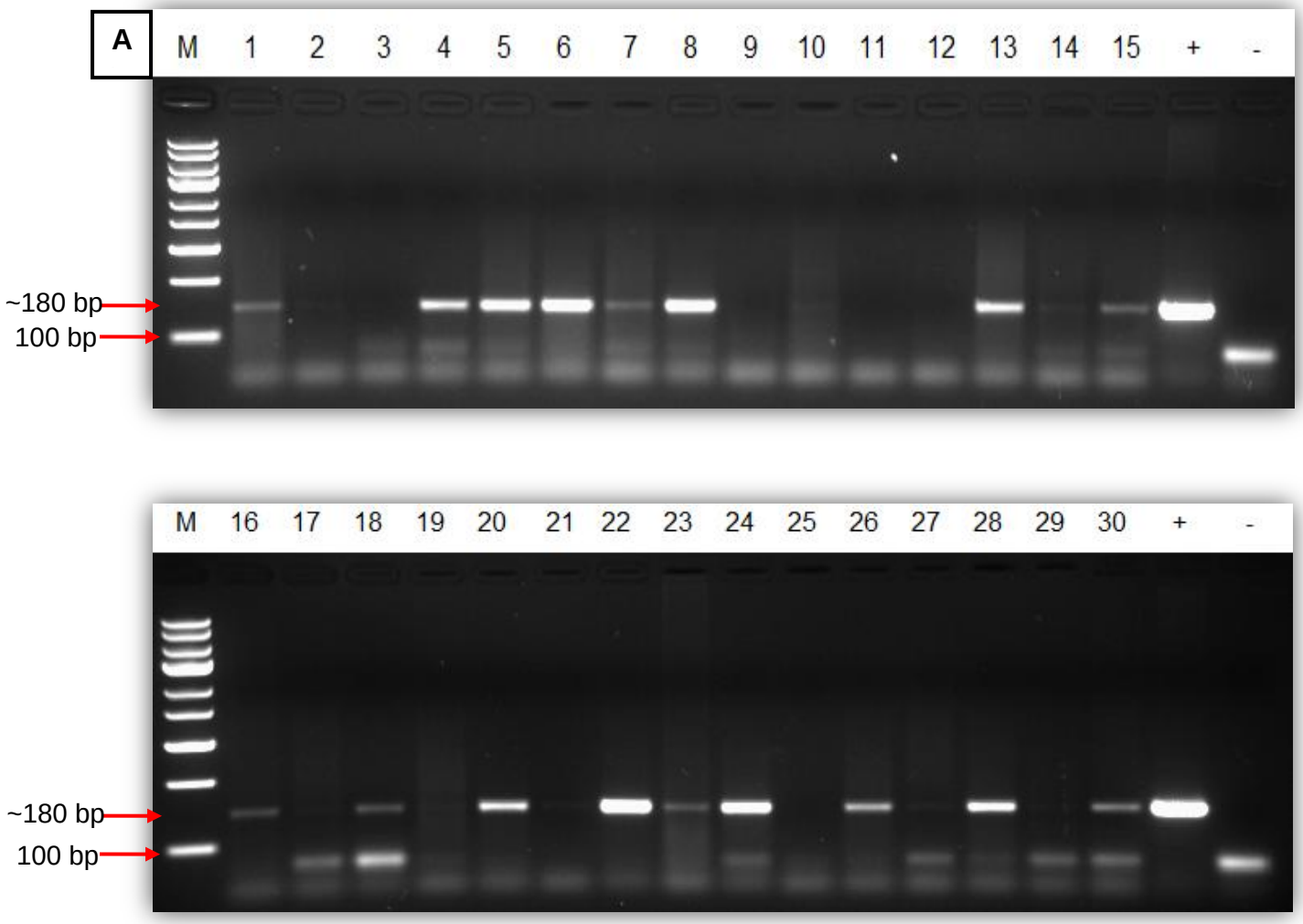
Nucleotide-nucleotide BLAST analysis (Altschul *et al*., 1990) was performed and the results displayed in Table 3.1. In Group A, the isolates showed 99% identity to *M. gallisepticum* strains A5969, R (high and low passage) and F. In Group B, the isolates showed 100% identity to *M. gallisepticum* strains F and R (low). It should be noted the *MG* RV-2 strain 16S-23S ITS region was not available on GenBank, and the percentage identity could therefore not be determined. From these results, it can be seen that the 16S-23S intergenic spacer region is not specific enough to distinguish between strains.

Table 3.1: Indicating nucleotide-nucleotide BLAST (Altschul *et al*., 1990) results for the 16S-23S ITS region consensus sequences from Group A and Group B field isolates, with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
Group A	*MG* str. A5969	L08897.1	100%	99%
	MG str. R(high)	CP001872.1	100%	99%
	MG str. R(low)	AE015450.2	100%	99%
	MG str. F	CP001873.1	100%	99%
	MG str. S6	FJ468422.1	90%	95%
Group B	*MG* str. R (low)	AE015450.2	99%	100%
	MG str. F	CP001873.1	100%	100%
	MG str. S6	FJ468422.1	100%	97%
	MG str. A5969	L08897.1	99%	95%

3.4.4 Partial 16S rRNA gene specific for M. gallisepticum (Lauerman, 1998)

Primer set MG 14F and MG 13R (Lauerman, 1998) was designed to specifically amplify a 183 bp partial region of the 16 rDNA gene of *M. gallisepticum.* Figure 3.6 indicates the amplified products obtained from this primer set for Group A and Group B respectively. The positive control, *MG* strain A514 produced the expected band of approximately 180 bp, while the negative control remained clear of a corresponding amplicon.



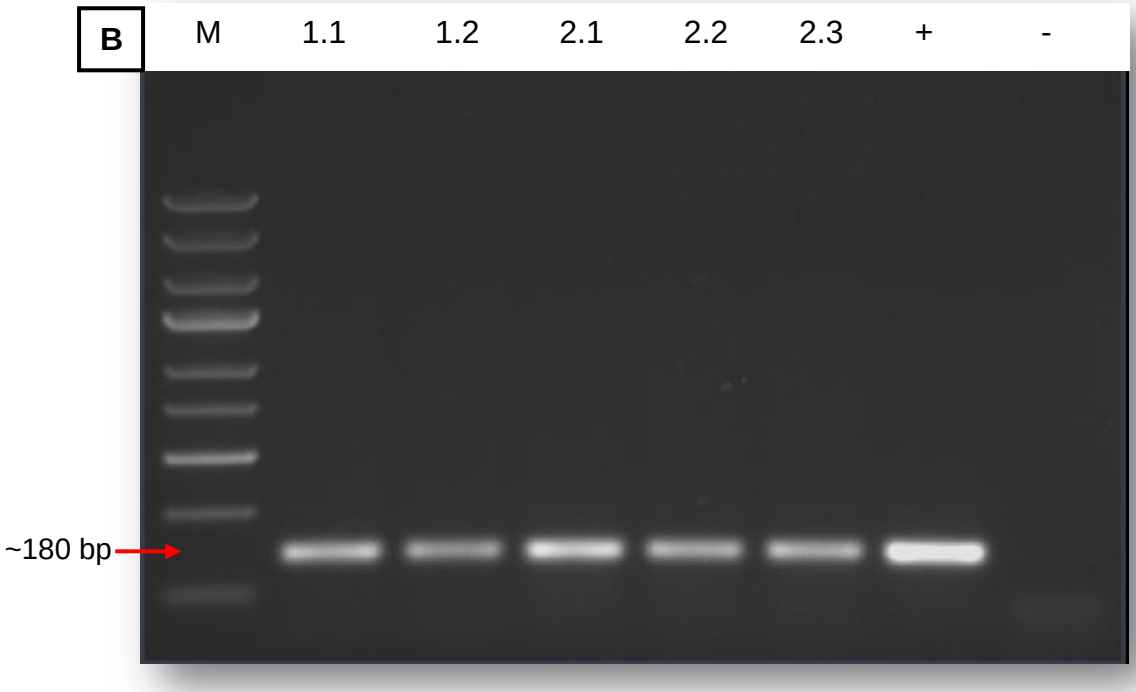


Figure 3.6: A 2% gel (w/v) of the PCR products stained with ethidium bromide and visualized with UV illumination. PCR amplification products using primer pair MG14F and MG13R (Lauerman, 1998) to amplify the partial *16s rRNA* gene specific for *M. gallisepticum*. **A**) Samples 1-30 were isolated from a poultry farm in Zimbabwe. **B**) Samples were isolated from poultry Farm 1 (samples 1.1 and 1.2) and Farm 2 (samples 2.1-2.3) in South Africa. Lane M represents the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* (strain A514) of approximately 186 bp, while the last lane represents the negative control (-).

Considerably more positive samples could be seen for Group A using this primer set compared to the primer sets targeting the 16S-23S ITS region. In Figure 3.6A, clear positive amplicons were seen in samples 4, 5, 6, 8, 13, 20, 22, 24, 26 and 28, while faint amplicons could be seen for samples 1, 7, 14, 15, 16, 18, 23 and 30. The bands observed just below 100 bp were most likely primer-dimers formed during the PCR reaction. Figure 3.6B shows positive amplification for all the samples from Farm 1 and Farm 2 isolated in South Africa. Once again sample 2.3 produced a positive band for *MG*, despite being negative for *MG* with the 16S-23S ITS region PCR (Ramírez *et al.*, 2008) capable of amplifying various mycoplasmas.

Samples showing positive amplification from Group A and B were excised from the gel and purified to be sequenced. Samples 4, 5, 6, 8, 13, 20, 22, 24 and 28 from Group A and all samples from Group B were sequenced successfully. The data was analysed using Geneious Pro v5.4.4 (Drummond *et al*., 2011), and showed all samples from Group A to be identical to each other, additionally Farm 1 and 2 from Group B were also 100% identical to each other. The consensus sequence of Group A was compared to that of Group B using ClustalW (Larkin *et al.,* 2007), as shown in Figure 3.7. The consensus sequences differed by only one base pair.

```
Group A         GAGCTAATCTGTAAAGTTGGTCTCAGTTCGGATTGAGGGCTGCAATTCGTCCTCATGAAG 60
Group B         GAGCTAATCTGTAAAGTTGGTCTCAGTTCGGATTGAGGGCTGCAATTCGCCCTCATGAAG 60
                ************************************************ **********

Group A         TCGGAATCACTAGTAATCGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGT 120
Group B         TCGGAATCACTAGTAATCGCGAATCAGCCATGTCGCGGTGAATACGTTCTCGGGTCTTGT 120
                ************************************************************

Group A         ACACACCGCCCGTCAAACTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAA 180
Group B         ACACACCGCCCGTCAAACTATGAGAGCTGGTAATATCTAAAACCGTGTTGCTAACCGCAA 180
                ************************************************************

Group A         GGAAGC 186
Group B         GGAAGC 186
                ******
```

Figure 3.7: ClustalW sequence alignment of the consensus sequences from Group A and Group B of a partial region of the 16S rDNA. Nucleotides with a star below (*) are indicative of identical nucleotides for the sequences, while differences are highlighted in yellow.

Nucleotide-nucleotide BLAST analysis (Altschul *et al*., 1990) was performed on the sequenced samples and the results displayed in Table 3.2. The isolates in Group A showed 99% identity to *M. gallisepticum* strains A5969, R (high and low passage) and F, and also *M. imitans* (str. 4229) differing by only one bp. The South African isolates (Group B) showed 100% identity to *M. gallisepticum* strains, however also shared this identity with *M. imitans*. Thus targeting the *16S rRNA* gene for PCR does not have a high enough sensitivity for distinguishing closely related *Mycoplasma* species. It should be noted the *MG* RV-2 strain 16S rDNA was not available on GenBank, and the percentage identity could therefore not be determined.

Table 3.2: Indicating nucleotide-nucleotide BLAST results for the consensus sequence of the partial *16S rDNA* gene of the field samples for Group A and B, with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
GROUP A	*MG* str. A5969	L35043.3	100%	99%
	MG str. R(high)	CP001872.1	100%	99%
	MG str. R(low)	AE015450.2	100%	99%
	MG str. F	CP001873.1	100%	99%
	M. imitans str. 4229	NR 025912.1	100%	99%
	M. pirum	NR 029165.1	100%	97%
GROUP B	*MG* str. R (low)	AE015450.2	100%	100%
	MG str. F	CP001873.1	100%	100%
	MG str. A5969	L08897.1	100%	100%
	M. imitans str. 4229	NR025912.1	100%	100%

3.4.5 The partial mgc2 gene (Hnatow *et al*., 1998)

Primer set mgc2-2F and mgc2-2R (Hnatow *et al*., 1998) was designed to amplify a 237-303 bp region of the *mgc2* gene specific for *M. gallisepticum.* Figure 3.8 depicts the amplified products obtained from this primer set for Group A and B respectively. The positive *MG* control strain A514 produced the expected band of approximately 270 bp; however, other *MG* strains may vary from 237-303 bp.

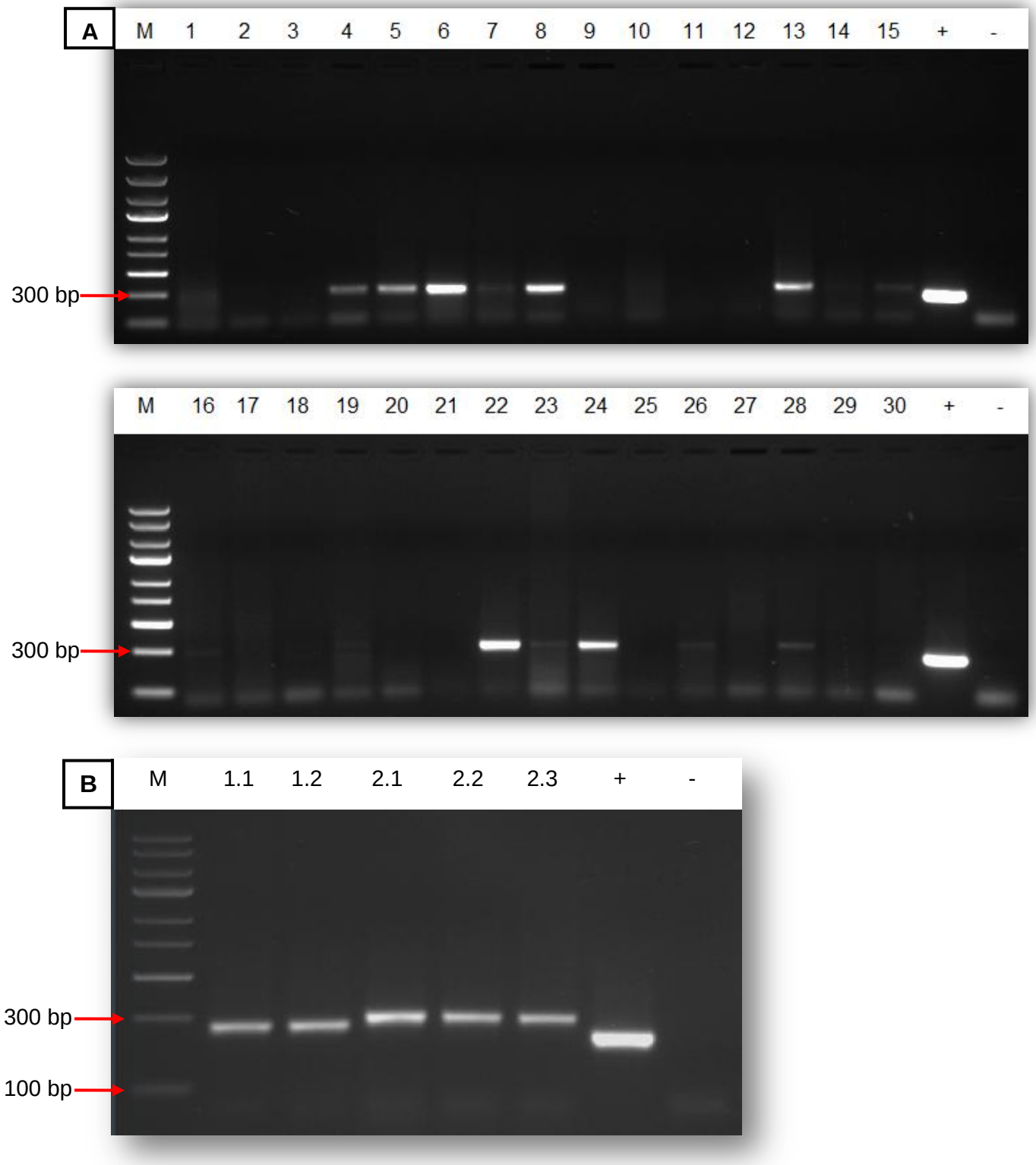


Figure 3.8: A 2% gel (w/v) of the PCR amplification products using primer pair mgc2-2F and mgc2-2R (Hnatow *et al*., 1998) to amplify the partial *mgc2* gene specific for *M. gallisepticum*. **A**) Samples 1-30 were isolated from a poultry farm in Zimbabwe. **B**) Samples were isolated from poultry Farm 1 (samples 1.1 and 1.2) and Farm 2 (samples 2.1-2.3) in South Africa. Lane M represents the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* (strain A514) of approximately 270 bp, while the last lanes of each gel represent the negative control (-).

The Zimbabwean isolates of Group A showing amplicons in this size range were clearly seen from samples 4, 5, 6, 8, 13, 20, 22 and 24, while faint bands were observed for samples 7, 15, 23, 26 and 28. All positive sample amplicons in Group A appeared to be of the same size, approximately 300 bp. Opposed to this, Group B showed amplicons of different sizes for Farm 1 and Farm 2, suggesting the presence of different strains between the farms.

Samples from Group A and B showing strong positive amplification were excised from the gel and purified to be sequenced. Samples 5, 6, 8, 13, 22 and 24 from Group A and all samples from Group B were sequenced successfully. The data was analysed using Geneious Pro v5.4.4 (Drummond *et al*., 2011), and showed Group A samples to be identical, while Group B was divided into Farm 1 and Farm 2. Nucleotide-nucleotide BLAST (Altschul *et al*., 1990) was performed on the consensus sequences and the results displayed in Table 3.3.

Table 3.3: Indicating nucleotide-nucleotide BLAST (Altschul *et al*., 1990) results for the sequenced partial *mgc2* gene of the field samples with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCES	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
GROUP A	*MG* str. RV-2	EU939449.1	100%	100%
	MG str. S6	AY556229.1	99%	90%
	MG str. A5969	AY556227.1	99%	90%
	MG str. R(low)	AE015450.2	99%	90%
	MG str. F	AY5562300.1	98%	83%
FARM 1	*MG* str. RV-2	EU939449.1	100%	100%
	MG str. S6	AY556229.1	100%	90%
	MG str. A5969	AY556227.1	100%	90%
	MG str. R low	AE015450.2	100%	90%
	MG str. Ts-11	AY556232.1	100%	86%
	MG str. F	CP001873.1	100%	84%
FARM 2	*MG* str. Ts-11	AY556232.1	100%	100%
	MG str. S6	AY556229.1	100%	97%
	MG str. A5969	AY556227.1	100%	97%
	MG str. R low	AE015450.2	100%	97%
	MG str. F	CP001873.1	100%	92%

The Zimbabwean isolates, illustrated as Group A showed only 90% identity to the *M. gallisepticum* strains A5969, R (high and low passage) and F, while showing 100% identity to *MG* RV-2 strain. Despite this high identity to the *MG* RV-2 strain, gaps were present in the sequence. For this reason an alignment was performed using ClustalW (Larkin *et al.,* 2007) for the *MG* RV-2 strain, the sample isolate from Zimbabwe and the *MG* R (low) strain as seen in Figure 3.9. The *MG* R low passage strain was included as the inactive vaccine used on this farm was produced from this strain, as mentioned in section 3.3.2.2.

In Group B, the isolates collected from Farm 1 showed 100% identity to the *MG* RV-2 strain, with the next closest matches only showing 90% identity. The isolates from Farm 2 showed 100% identity to the Ts-11 vaccine strain; however other *MG* strains showing little difference at 97% identity. As mentioned previously in section 3.3.2.2, it was unknown whether Farms 1 or 2 had been vaccinated. These results could suggest that Farm 2 had been vaccinated with the *MG* Ts-11 strain, and the clinical signs that were evident in the flock were a result of *M. synoviae* infection (as identified in sample 2.3 in section 3.4.2). The consensus sequence from Farm 1 and Farm 2 where aligned to their closest matches, namely the *MG* RV-2 strain and the *MG* Ts-11 vaccine strain respectively, as seen in Figure 3.9.

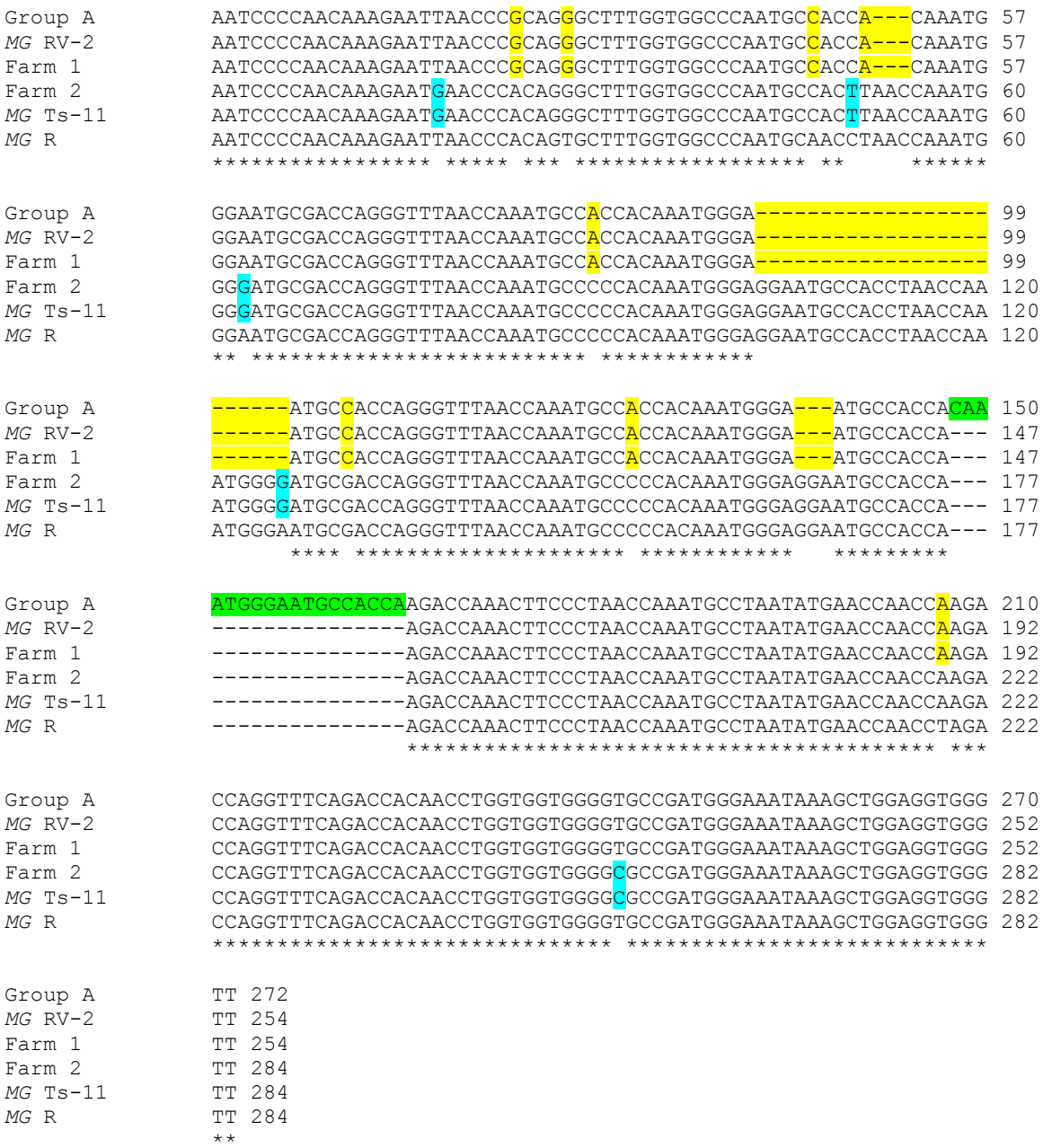


Figure 3.9: Multiple sequence alignment of the partial *mgc2* cytadhesin gene from the field isolates and *MG* strains showing highest identity. Field samples were divided into those isolated from Zimbabwe (Group A) and South Africa (Group B), while the latter was further divided into Farm 1 and Farm 2 within South Africa. Both Group A and Farm 1 isolates showed closest percentage identity to the *MG* RV-2 strain (EU939449.1), the unique nucleotide regions highlighted in yellow. Group A isolate however contains a unique nucleotide insert highlighted in green. Farm 2 showed closest percentage identity to the *MG* Ts-11 vaccine strain (AY556232.1) with mutual unique nucleotides shown in blue.

The alignment of the sequences showed many areas of identity between the *MG* strains, however there were significant differences. It is important to note that all insertions and deletions occurred in-frame, thereby not resulting in any frame shifts which could render the protein non-functional. The partial *mgc2* region of the isolate from Farm 2 was found to be identical to that of the *MG* Ts-11 vaccine strain (AY556232.1), sharing unique nucleotides as highlighted in blue in Figure 3.9. Both Group A and Farm 1 isolates showed highest percentage identity to the *MG* RV-2 strain (EU939449.1), however the Group A isolate showed a unique nucleotide insert. Together with the nucleotide differences between Group A and the inactivated vaccine *MG* R (low) strain, these changes may lead to antigenic variation. Since the *mgc2* gene is a cytadhesin and is exposed to the host immune system, changes in this region could aid the pathogen in evading the host immune system. This hypothesis will be further addressed in the following chapter.

3.5 CONCLUSIONS

Poultry farms from two geographically different regions, namely Zimbabwe (Group A) and South Africa (Group B) were screened for *MG*. Group B was further divided into Farm 1 and Farm 2. As an alternative to cultivation, PCRs targeting various genes were employed.

Two broad-based PCRs (primers designed by Harasawa *et al.*, 2004 and Ramírez *et al.*, 2008) targeting the 16S-23S intergenic spacer region for various avian mycoplasmas were used to observe the different populations of *Mycoplasma* species within a sample. These two “general” primer sets were unable to detect *M. gallisepticum* in Group B, however were capable of detecting *M. synoviae* and even *Enterococcus* species. Even though *MG* was detected from the samples using the more specific primer sets, it is possible they were not detected in the general 16S-23S ITS region PCRs since the *Enterococcus*, *E. coil* and *M. synoviae* species were more dominant in the sample. The primers were previously only tested on axenically cultured *Mycoplasma* species. These general primer sets therefore proved to be limited in detecting *MG* from field isolates due to higher DNA load of other mycoplasmas or unintentional amplicons such as those of *Enterococcus* species or *E.coli*. They did however show to be useful in getting a general overview of other *Mycoplasma* present in field samples with DNA extracted directly from swab samples.

Due to the population of *Mycoplasma* and other "contaminating" species present simultaneously, specific primer sets were needed for the direct detection of *MG* from field samples. Specific primers for the 16S-23S intergenic spacer region were useful, however produced faint amplicons which suggests it will not be sensitive in detecting low levels of *MG*. Sequencing of this 16S-23S ITS region was able to distinguish *M. gallisepticum* from other species, however was not able to distinguish between various strains. The 16S rDNA-PCR was found to be the most sensitive of the PCRs, however it must be kept in mind that closely related species such as *M. imitans* may result in false positives for the detection of *MG,* even after sequencing the PCR amplicons. Thus despite the sensitivity of the 16S rDNA, it is not always specific enough to distinguish *Mycoplasma* species. As stated in literature (Garcia *et al*., 2005) the *mgc2*-PCR was the most specific and the most sensitive PCR for the detection of *MG*. It was not only specific for *MG* and sensitive in producing bright PCR amplicons, but also produced amplicons of various sizes as seen from the samples of Group B.

From analysis of a partial region of the *mgc2* gene, the Zimbabwean isolates (Group A) appeared to be most similar to the *MG* RV-2 strain, however with an additional insert of significant length. Farm 1 from the South African isolates however were 100% identical to the partial *mgc2* region of *MG* RV-2 strain, while Farm 2 was very similar to the *MG* Ts-11 vaccine strain. Clinical signs from Farm 2 could be explained by the presence of *M. synoviae* which was identified from the 26S-23S ITS-PCR. The *MG* RV-2 strain was originally isolated from two outbreaks in broiler-breeders in 2007 in Israel (Lysnyansky *et al*., 2008). The strain was noted as being distinct from the "Israeli" type strain previously described by Ferguson and co-workers (2005), and suggested as being a new *MG* strain introduced to Israel. The strain also showed susceptibility to enrofloxacin (0.05 $\mu g.\mu L^{-1}$), unlike other strains isolated in Israel at the time (Gerchman *et al*., 2008). Thus a link between the atypical strain found in Israel and those prevalent in southern Africa has been established.

The following chapter will concentrate on amplifying more *MG* specific, but divergent genes so as to further identify the strains present from these two geographical areas. A larger region of the *mgc2* gene would also be amplified so as to view any significant changes on protein level. Various cytadhesin genes and membrane associated proteins would be targeted due to their use in gene-targeted sequencing (GTS) and their interactions with the host cells. The possibility of the unique insert observed in the *mgc2* gene of isolates found in Zimbabwe would be addressed as being responsible for the noted failure in vaccination of flocks, thus aiding the pathogen in evading the host immune system.

CHAPTER 4

MOLECULAR CHARACTERIZATION OF *M. gallisepticum* FIELD ISOLATES AND *in silico* ANTIGENIC DETERMINATION

4.1 INTRODUCTION

With the increased use of vaccinations, and for purposes of global epidemiological studies, more powerful tools are needed for the differentiation of various *MG* strains and for their subsequent characterization. Unlike other widely used *MG* typing methods (e.g. RAPD), the sequencing methods do not require the isolation of the test organism in a pure culture, thus providing a significant advantage.

The sequencing method denoted gene-targeting sequencing (GTS) and described by Ferguson and co-workers (2005) was employed and targeted four surface protein genes, one of which was only predicted as a surface protein. The discriminatory power hierarchy, from lowest to highest, for GTS analysis of individual genes was ranked as: *gapA*, MGA_0319*, mgc2* and *pvpA*. The *gapA* gene is considered the primary cytadhesin (Goh *et al*., 1998), while the *mgc2* gene has also been shown to encode a second cytadhesin protein known to play a role in the attachment process (Hnatow *et al*., 1998). The gene designated MGA_0319 is a putative, conserved surface lipoprotein (Nascimento *et al.*, 1991). PvpA is an integral membrane surface protein with a free C-terminus, predicted to function as an accessory cytadhesin that exhibits size variation among *MG* strains (Boguslavsky *et al*., 2000; Liu *et al*., 2001). Boguslavsky and co-workers (2000) showed it to possess a proline-rich carboxy-terminal region (28%) containing two identical directly repeated sequences and a tetrapeptide motif [Pro-Arg-Pro-X (X being Met, Gln or Asn)] repeated numerous times. The authors noted truncations often seen within this region that result in size variations of the protein between strains. Due to the high divergence rate of *MG*, these targeted regions were tested for their stability within a strain and showed to be stable after sequencing different *in vitro* passages of *MG* reference strains.

These cytadhesin proteins play a vital role in establishing a specific and firm attachment to sialic acid residues along the respiratory epithelial host cells, in order to avoid rapid clearance by innate host defense mechanisms (Papazisi *et al.*, 2000). Variation within these regions may not only contribute to altered host binding, but may contribute in the organism altering its antigenic profile and thereby evading the host's immune system. This antigenic variation may be accomplished in two distinct ways. Microbial pathogens may use signal transduction pathways to sense signals in the host environment and respond accordingly by expressing virulence-gene products necessary for survival in the host (Robertson & Meyer, 1992); or alternatively, the microbial population as a whole may spontaneously and randomly generate distinct cell populations with varied antigenic phenotypes that will survive the specific host response capable of eliminating the predominant "homotypes" (Razin *et al.*, 1998). With the lack of a both dark and light DNA repair in *MG* (Ghosh *et al.,* 1977), the frequency of occurrence of such genetic variants is strikingly high.

4.2 OBJECTIVES

The aim of this chapter was to further characterize isolates identified as *M. gallisepticum* in Chapter 3, collected from poultry farms in Zimbabwe (Group A) and South Africa (Group B). These isolates would be characterized using GTS described by Ferguson and co-workers (2005). Sequences showing significant nucleotide differences would be translated to further examine if and how these changes affected the amino acid composition of the gene.

For Group A, the aim was not only to characterize the *MG* strain, but to determine if adequate differences were present to result in a varied antigenic profile. By doing this, the *MG* strain may evade antibodies produced from the inactivated *MG* R_{low} vaccine administered to the poultry farm of collection, thus explaining the breaks in vaccination. Antigenicity of the proteins was studied *in silico* by constructing hydrophilicity plots and using BepiPred linear epitope prediction to calculate the location of linear B-cell epitopes.

4.3 MATERIALS AND METHODS

4.3.1 Reagents and Chemicals

The reagents and chemicals used were of molecular biological and analytical grade, unless stated differently and were obtained from the following companies.

Amersham Biosciences: GFX^{TM} PCR DNA and Gel Band Purification Kit, **Applied Biosystems**: BigDye terminator v3.1 Kit, 2720 Thermal Cycler; **Fermentas**; O'GeneRuler^{TM} Express DNA Ladder, O'GeneRuler^{TM} , Orange Loading dye, Deoxynucleoside triphosphates (dNTPs): **Merck**; Ethanol (99.5%); EDTA: Tris; NaCl: NaOH pellets; **Merial South Africa**: Live attenuated *Mycoplasma gallisepticum* Ts11 vaccine; **New England Biolabs**: Taq DNA Polymerase with ThermoPol Buffer; **Thermo Scientific**: NanoDrop^{TM} 1000 Spectrophotometer v3.7; **Whitehead Scientific (PTY) LTD**; Agarose D1 LE, QIAamp$^{®}$ DNA mini kit, All synthesis of primers; **J. Bradbury**, **University of Liverpool, UK**: Freeze-dried samples of *M. gallisepticum* strains, PG31(18.11.99) and A514 (21.5.94)

4.3.2 *Mycoplasma ISOLATES*

4.3.2.1 Control isolate: Refer to *MG* strain A514 used in chapter 3 (section 3.3.2.1).

4.3.2.2 Field isolates: DNA extracted from swab samples in Chapter 4 was used for further characterization of the isolates. Of the various PCRs performed in Chapter 4, the *mgc2*-PCR (Hnatow *et al*., 1998) showed the highest variation in sequence. ClustalW alignment (Larkin *et al.,* 2007) of *mgc2* gene in sample sequences suggested *MG* isolates taken from the same farm were of the same *MG* strain. Thus there were a total of three representative *MG* isolates: one from Group A in Zimbabwe and two from Farm 1 and Farm 2 isolated in South Africa. To ensure good quality and quantity of template DNA, samples showing strong positive results for the various PCRs from Chapter 4 were selected for further characterization. From Group A these included samples 6, 8, 22 and 24, while all samples from Group B were further analyzed due to the small sample size from the farms.

4.3.3 POLYMERASE CHAIN REACTION (PCR) AMPLIFICATIONS

PCR reactions were set up as previously mentioned in chapter 3 (section 3.3.4). PCR amplification product sizes were based on the *M. gallisepticum* R_{low} genome sequence (Genbank: *AE015450*) as seen in Table 4.1.

Amplifications were performed in a 2720 Thermal Cycler (Applied Biosystems) for an initial denaturation of 94°C for 3 min, followed by 35 cycles of denaturation of 94°C for 30 sec; annealing carried out at 55 to 60°C for 40 sec and extention at 72°C for 1 min. A final elongation of 72°C for 5 min was performed to allow complete elongation of product. The optimal annealing temperature utilized to amplify the MGA_0319 and *pvpA* genes was 55°C, to amplify the *mgc2* gene an annealing temperature of 58°C was utilized, and 55°C was utilized to amplify the parital *gapA* gene. The annealing temperature for *gapA* PCR was originally 60°C; however, this was lowered to 55°C when the field samples failed to produce PCR amplification. Primers were described by Ferguson and co-workers (2005) and PCR product sizes given in Table 4.1 are based on the *M. gallisepticum* R_{low} genome sequence (AE015450).

Table 4.1: Indicating nucleotide sequence of the primers and the predicted PCR product sized based on the *M. gallisepticum* R_{low} genome sequence (AE15450).

PRIMER	SEQUENCE(5'→ 3')	PCR PRODUCT SIZE (bp)
lp 1F	CCAGGCATTTAAAAATCCCAAAGACC	590
lp 1R	GGATCCCATCTCGACCACGAGAAAA	
gapA 3F	TTCTAGCGCTTTAGCCCTAAACCC	332
gapA 4R	CTTGTGGAACAGCAACGTATTCGC	
pvpA 3F	GCCAMTCCAACTCAACAAGCTGA	702
pvpA 4R	GGACGTSGTCCTGGCTGGTTAGC	
mgc2 1F	GCTTTGTGTTCTCGGGTGCTA	824
mgc2 1R	CGGTGGAAAACCAGCTCTTG	

Further methods such as analysis of PCR amplicons, purification of DNA from agarose gels and sequencing of the PCR products were performed as mentioned in Chapter 2 (section 2.3).

4.3.4 HYDROPHILICITY PREDICTION

Hydrophilicity analysis of the protein was predicted *in silico* with DN*Assist* 3.0 (Patterton & Graves, 2000) and carried out using the following method: Each amino acid in the protein sequence is assigned a hydrophilicity value; these values are then repetitively averaged down the length of the polypeptide chain, which then generates a series of local hydrophilicity values (Hopp & Woods, 1981). The graph is then generated as hydrophilicity verses the residue number of the amino acid sequence.

4.3.5 ANTIGENIC DETERMINATION in silico

The antigenicity of the partial Mgc2 and PvpA membrane proteins were predicted *in silico* using BepiPred linear epitope prediction (Larsen *et al*., 2006) via the Immune Epitope Database (http://tools.immuneepitope.org). It predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method. B-cell epitopes are the sites of molecules that are recognized by antibodies of the immune system.

All prediction calculations are based on propensity scales for each of the 20 amino acids. Each scale consists of 20 values assigned to each of the amino acid residues on the basis of their relative propensity to possess the property described by the scale. Parameters such as hydrophilicity, flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptides chains have been correlated with the location of continuous epitopes.

4.4. RESULTS AND DISCUSSION

4.4.1 The gapA partial gene

Primer set gapA (nucleotide sequence seen in Table 4.1) described by Ferguson and co-workers (2005) was used to amplify a partial region of a *gapA* gene of *M. gallisepticum*. The gene encodes a protein shown to be involved in the cytadhesin process (Goh *et al*., 1998). All samples yielded PCR amplicons of the same size (Figure 4.1), thus showing no gene size polymorphorism as previously observed by Ferguson and co-workers (2005). The authors assigned a discrimination index of 0.713 for the individual *gapA* gene, being the lowest of the targeted surface protein genes and thus the most conserved.

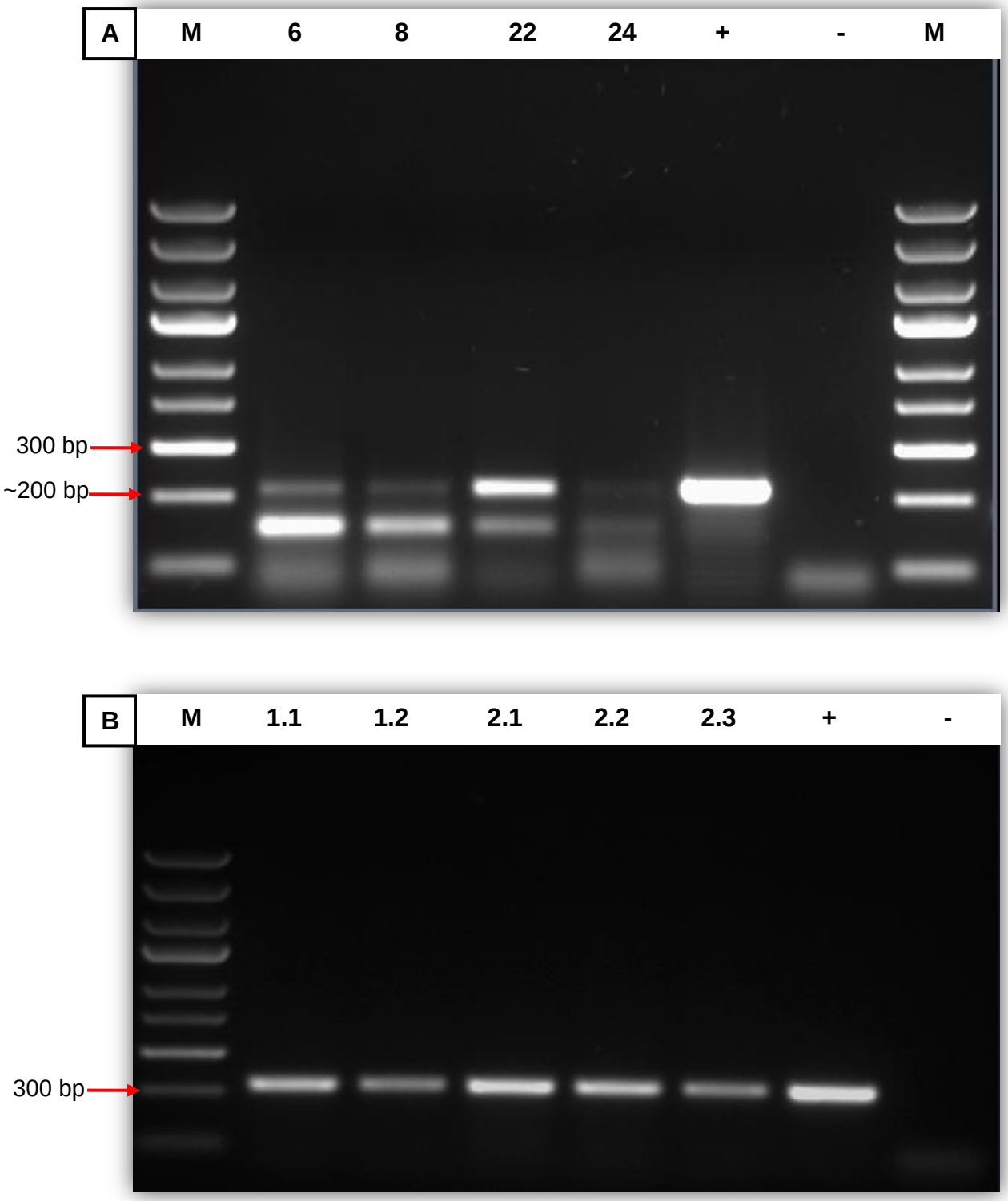


Figure 4.1: A 1% gel (w/v) of the PCR amplification products using the gapA primer pair (Ferguson *et al.*, 2005) to amplify a partial region of the *gapA* surface protein encoded gene of *M. gallisepticum,* resulting in a constant PCR amplicon of 332 bp. **A**) Zimbabwean isolates (Group A) and (**B**) South Africa isolates from Farm 1 (1.1-1.2) and Farm 2 (2.1-2.3) selected for further characterization. Lane M represents the molecular marker (O'GeneRuler™ Express DNA ladder). The positive control (+) was *M. gallisepticum* strain A514, while the last lanes of each gel represent the negative control (-).

In Figure 4.1A, non-specific amplification products of approximately 200 bp were seen with varying intensity throughout the samples. These bands were excised from the gel and successfully sequenced. BLAST results showed 100% identity to an uncultured organism (AY765219.1) resulting as an occasional non-specific amplification when using genomic DNA from tracheal swabs (Garcia *et al*., 2005).

These non-specific amplifications were not however seen from samples in Group B, Figure 4.1B. This may be explained by Group B samples being isolated post-mortem, as opposed to from live birds, and thus contained fewer contaminants; a phenomenon also noted by Bradbury and co-workers (2001). Amplicons of appropriate size from samples 6, 8 and 22 from Group A and all samples from Group B were cut out of the gel and successfully sequenced. Farms 1 and 2 were shown to be identical upon ClustalW alignment (Larkin *et al.,* 2007), no doubt due to the low discrimination index of the *gapA* gene. The consensus sequence for Farms 1 and 2 was therefore represented as Group B in Figure 4.2, showing minor nucleotide differences to that of Group A, as highlighted in yellow.

```
Group A         CGTATTCGCCATCAACTAATACTTGAGGTAATAACTTAATGAAGTCATTAGTTGCTCTAG 60
Group B         CGTATTCGCCATCAACTAATACTTGAGGTAATAACTTAATGAAGTCATTAGTTGCTCTAG 60
                ************************************************************

Group A         AACGATCAATTACTAATAATCTTAATCTAATATCAGGATTGTTTTGAGGTGGAGTATATT 120
Group B         AACGATCAATTACTAATAATCTTAATCTAATATCAGGATTGTTTTGAGGTGGAGTATATC 120
                ************************************************************

Group A         TATAGATTGAATTAATGTATTTACTATCTAATACTTCTGTACCATCAGCTTCTGTAGTTA 180
Group B         TATAGATTGAATTAATGTATTTACTATCTAATACTTCTTTACCATCAGCTTCTGTAGTTA 180
                ************************************ ********************

Group A         CAATATCATTAATTTTGTTTTCAAAGAATGATGATGGAATATCATTTCTACTAATGAAAG 240
Group B         CAATATCATTAATTTTGTTTTCAAAGAATGATGATGGAATATCATTTCTACTAATGAACG 240
                ************************************************************ *

Group A         TACCTTGACCGATTACGTTATTTCTATTCATCAATGGGTTAGTAATTCGGTTAGGGTTTA 300
Group B         CACCTTGACCGATTACGTTATCTCTATTCATTAATGGGTTAGTAATTCGGTTAGGGTTTA 300
                 ******************** ********* ****************************

Group A         GGGCTAA 307
Group B         GGGCTAA 307
                *******
```

Figure 4.2: ClustalW sequence alignment of the consensus sequences from Group A and Group B of the partial *gapA* gene. Nucleotides with a star below (*) are indicative of identical nucleotides shared for the sequences, while nucleotides highlighted in yellow show differences between Group A and B.

Nucleotide-nucleotide BLAST (Altschul *et al*., 1990) was performed on the consensus sequences for Groups A and B, with isolates of highest percentage identity seen in Table 4.2. For the Zimbabwean samples represented as Group A, no 100% identity was observed for the *gapA* gene. The highest identity of 97% was for the reference *MG* R_{low} strain and the *MG* TLS-2 strain isolated from Israel in 2010 from broiler breeders (Gerchman *et al*., 2011). In Chapter 4, the 300 bp *mgc2* amplicon of Group A showed closest identity to that of the *MG* RV-2 isolate, also previously isolated in Israel. Demonstrating the discrimination index of the gene, the P1 analog of *M. synoviae* shares 92% to that of the *gapA* amplicon belonging to *MG*.

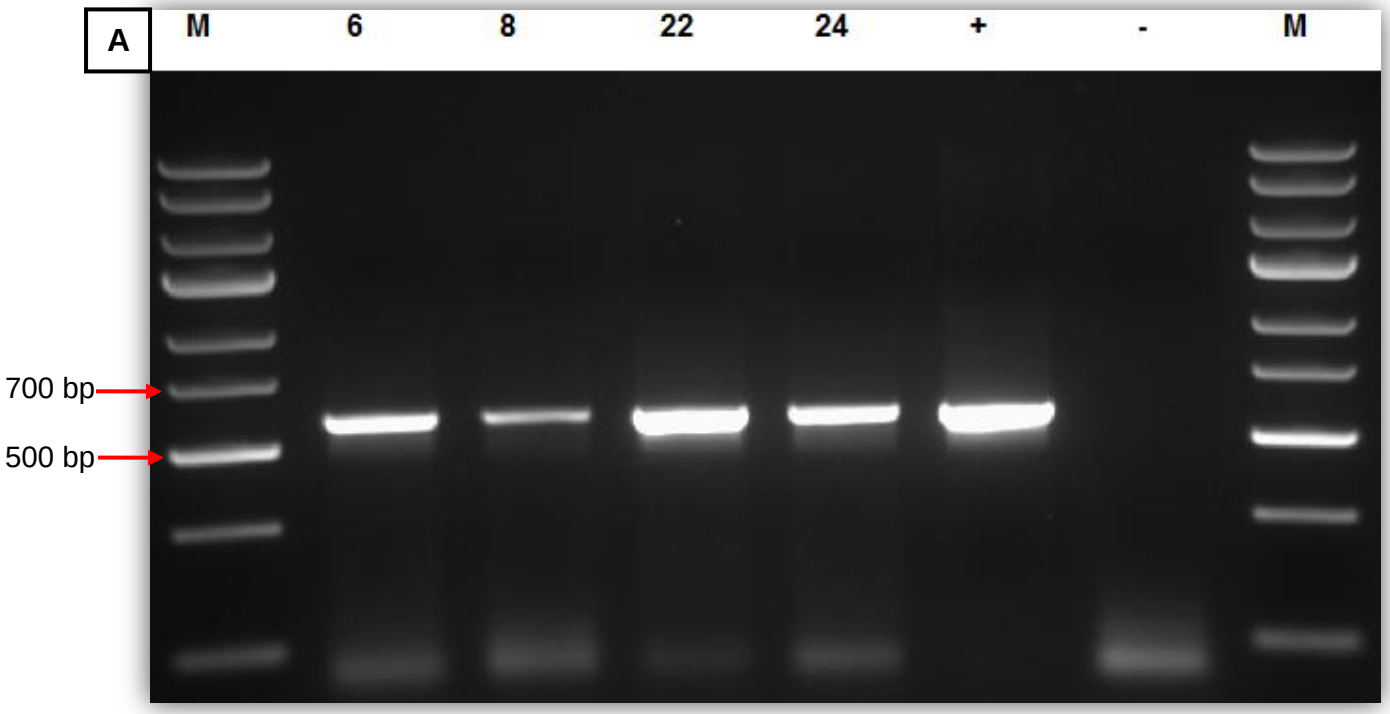
The consensus sequence for the two isolates in Group B showed a high percentage identity to various *MG* strains as seen in Table 4.2. The vaccine *MG* strains, Ts-11 and 685 showed 100% identity, while the *MG* RV-2 strain showed an identity of 97%. Therefore, the individual *gapA* gene did not show enough nucleotide variation to distinguish the isolates from Farms 1 and 2 apart.

Table 4.2: Indicating nucleotide-nucleotide BLAST results for the targeted *gapA* region consensus sequences from Group A and Group B field isolates, with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
Group A	*MG* str. R_{low}	AE015450.2	99%	97%
	MG str. RV-2	EU939451.1	99%	96%
	MG str. S6	U44804.1	99%	96%
	MG str. F	CP001873.1	99%	96%
	MG str. TLS-2	JN102618.1	93%	97%
	MG str. A5969	AY556149.1	95%	96%
	M. synoviae WVU1859	S70129.1	99%	92%
Group B	*MG* str. R_{low}	AE015450.2	99%	99%
	MG str. F	CP001873.1	99%	96%
	MG str. S6	U44804.1	99%	97%
	MG str. Ts11	AY556154.1	92%	100%
	MG str. 685	AY556153.1	92%	100%
	MG str. RV-2	EU939451.1	99%	97%

4.4.2 The lp partial gene sequence (MGA_0319)

Primer set lp (nucleotide sequence seen in Table 4.1) as described by Ferguson and co-workers (2005) was used to amplify a 590 bp partial region of a hypothetical conserved surface lipoprotein (lp) of *M. gallisepticum* designated MGA_0319. All samples yielded PCR amplicons of the same size (Figure 4.3), thus showing no gene size polymorphorism as with the *gapA* partial gene. The authors assigned a discrimination index of 0.874 for the individual MGA_0319 gene, being the second lowest of the targeted surface protein genes.



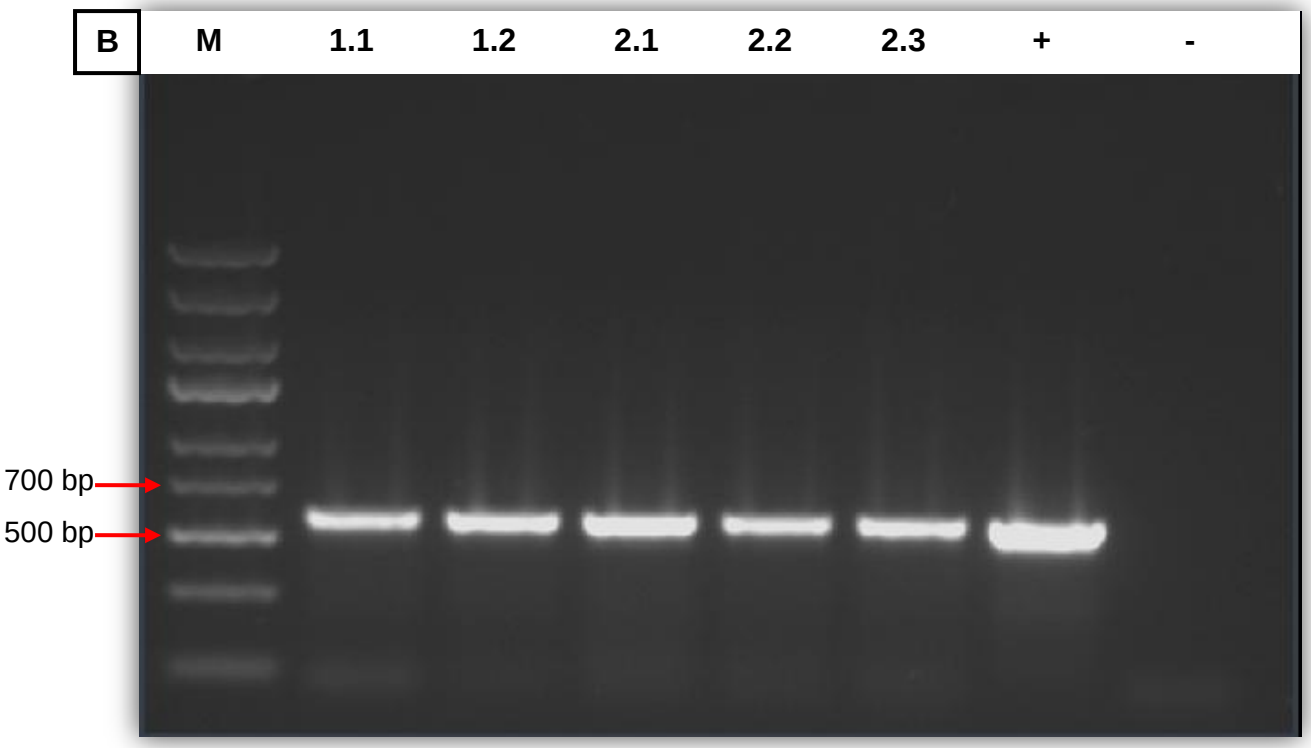


Figure 4.3: A 1% gel (w/v) of the PCR amplification products using primer pair Ip (Ferguson *et al.*, 2005) to amplify the putative surface lipoprotein of *M. gallisepticum,* resulting in a constant PCR amplicon of 590 bp. **A**) Zimbabwean isolates (Group A) and (**B**) South Africa isolates from Farm 1 (1.1-1.2) and Farm 2 (2.1-2.3) selected for further characterization. Lane M represents the molecular marker (O'GeneRuler^{TM} Express DNA ladder). The positive control (+) was *M. gallisepticum* strain A514, while the last lanes of each gel represent the negative control (-).

All amplicons from Groups A and B were successfully sequenced and upon ClustalW alignment (Larkin *et al.,* 2007) it was confirmed that isolates collected from the same farm were identical in sequence. Similarly to the *gapA* gene, aligned consensus sequences of Farm 1 and 2 showed to be identical to each other (Figure 4.4). Various nucleotide differences could however be seen between Group A and Group B (Farms 1 and 2) as highlighted in yellow.

```
Farm 1          GACCACGAGAAAATAAGAACTTACTAGCGTTCATATTTTTACTCATGACATTCGTTGATG 60
Farm 2          GACCACGAGAAAATAAGAACTTACTAGCGTTCATATTTTTACTCATGACATTCGTTGATG 60
Group A         GACCACGAGAAAATAAGAACTTACTAGCGTTCGTATTTTTACTCATTACGTTTGTTGATG 60
                ****************************** ************* ** ** *******

Farm 1          AATTATCTGATTTCGAAGAATCAACTGTCTTCATCCCATTTCATTCAAAGATTTCGTGGA 120
Farm 2          AATTATCTGATTTCGAAGAATCAACTGTCTTCATCCCATTTCATTCAAAGATTTCGTGGA 120
Group A         AATTATCTGATTTCGAAGAATCAACTGTCTTCATCCCATTTCATTCAAAGATTTCGTGGA 120
                ************************************************************

Farm 1          TATCTTTAGTTCCAGCTGCTAAATCTGCATAAGCATTGTAATATGGATCAGTAGTTCGAT 180
Farm 2          TATCTTTAGTTCCAGCTGCTAAATCTGCATAAGCATTGTAATATGGATCAGTAGTTCGAT 180
Group A         TATCTTTAGTTCCAGCTGCTAAATCTGCATAAGCATTGTAATATGGATCAGTAGTTCGAT 180
                ************************************************************

Farm 1          TCGTTTCACCTGTTTTTAATAATGAATTATTAGTAATCTTTTGTGAGTAGGTGTTGGCTG 240
Farm 2          TCGTTTCACCTGTTTTTAATAATGAATTATTAGTAATCTTTTGTGAGTAGGTGTTGGCTG 240
Group A         TCGTTTCACCTGTTTTTAATAATGAATTATTAGTAATCTGTTGTGAGTAGGTGTTGCTTG 240
                ************************************ ***************  **

Farm 1          TGTTACCATTTCCCTTAGCTCTAATGAAATTATTCATCAGATTGATCTCATCAATATCTG 300
Farm 2          TGTTACCATTTCCCTTAGCTCTAATGAAATTATTCATCAGATTGATCTCATCAATATCTG 300
Group A         TGTTATCATTTCCCTTAGCTCTAATGAAATTATTCATCAGATTGATCTCATCAATATCTG 300
                ***** ********************************************************

Farm 1          ATTGAATTACACCAACGTTATCACGGTTAGTATTATTAGTTTGTTTTAAATATTGTTGAA 360
Farm 2          ATTGAATTACACCAACGTTATCACGGTTAGTATTATTAGTTTGTTTTAAATATTGTTGAA 360
Group A         ATTGAACTACACCAACGTTATCGCGGTTAGTATTATTAGTTTGTTTTAAATATTGTTGAA 360
                ****** *************** ************************************

Farm 1          CATAAGCTGCACTAAATGATACGTCAAAACTATTAAACATATCAGAAGCAGTCATTAACA 420
Farm 2          CATAAGCTGCACTAAATGATACGTCAAAACTATTAAACATATCAGAAGCAGTCATTAACA 420
Group A         CATAAGCTGCACTAAATGATACGTCAAAACTATTGAACATATCAGAAGCAGTCATTAACA 420
                ********************************* ************************

Farm 1          ACTTACCACCAGAATCTGATGATAAGTTGTTGGGAATATTAATCCCTTTAGTTGTACTAT 480
Farm 2          ACTTACCACCAGAATCTGATGATAAGTTGTTGGGAATATTAATCCCTTTAGTTGTACTAT 480
Group A         ACTTACCACCAGAATCTGATGATAAGTTGTTGGGAATATCAATCCCTTTAGTTGTACTAT 480
                ************************************ ********************

Farm 1          TTACATAACTGTCCAACCCTCTAGTAATGAATTGGTTATAAGCTCTCATCCCCTTGTTTT 540
Farm 2          TTACATAACTGTCCAACCCTCTAGTAATGAATTGGTTATAAGCTCTCATCCCCTTGTTTT 540
Group A         TTACATAACTGTCCAACCCTCTAGTAATGAATTGGTTATAAGCTCTCATCCCCTTGTTTT 540
                ************************************************************

Farm 1          CATCAAAAGGTTGGTCTTTGGGATTTTT 568
Farm 2          CATCAAAAGGTTGGTCTTTGGGATTTTT 568
Group A         CATCAAACGGTTGGTCTTTGGGATTTTT 568
                ******* ********************
```

Figure 4.4: ClustalW sequence alignment of the consensus sequences from Group A and Group B, namely Farm 1 and Farm 2, of partial region of the *lp* (lipoprotein) gene. Nucleotides with a star below (*) are indicative of identical nucleotides for the sequences, while nucleotides highlighted in yellow are identical for Farm 1 and 2 however differ from that of Group A.

Nucleotide-nucleotide BLAST (Altschul *et al*., 1990) was performed using the consensus sequences for Group A and B. Yet again, there were no 100% identities for Group A, only a variety of *MG* strains (including *MG* R_{low}) of 97% identity and the *MG* TLS-2 strain showing the highest identity of 99% (Table 4.3). Group B also showed high identity (>97%) to various *MG* strains including the TS-11 vaccine strain and the TLS-2 Israeli strain. The RV-2 strain showed an identity of 99%. Unfortunately, the lp-PCR was unable to discriminate between the isolates collected from Farms 1 and 2 of the South African isolates.

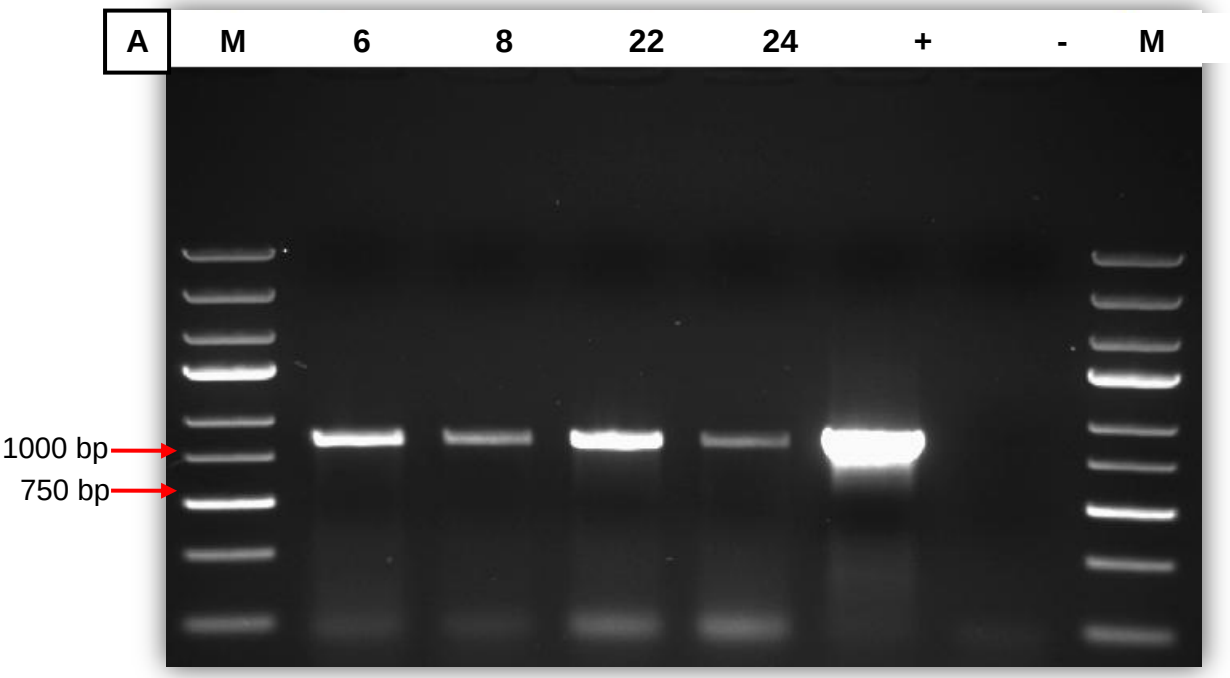
Table 4.3: Indicating nucleotide-nucleotide BLAST results for the partial *lp* gene consensus sequences from Group A and Group B field isolates, with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
Group A	*MG* str. TLS-2	JN102669.1	94%	99%
	MG str. R_{low}	AE015450.2	100%	97%
	MG str. F	CP001873.1	99%	97%
	MG str. RV-2	JN102659.2	94%	97%
	MG str. A5969	AY556071.1	84%	97%
	MG str. S6	AY556073.1	84%	97%
Group B	*MG* str. R (low)	AE015450.2	100%	98%
	MG str. F	CP001873.1	100%	97%
	MG str. S6	AY556073.1	87%	100%
	MG str. Ts11	AY556076.1	87%	97%
	MG str. 685	AY556075.1	87%	98%
	MG str. RV-2	JN102659.2	96%	99%
	MG str. TLS-2	JN102669.1	96%	97%

4.4.3 The carboxy-terminus of the mgc2 gene

The mgc2 primer set (Table 4.10) described by Ferguson and co-workers (2005) was used to amplify a large region of the *mgc2* gene of *MG*, showing a size variation of 761-857 bp between various strains. The genetic variability of this gene has yet to be addressed; however, it is known that the gene encodes a cytadhesin protein important in the process of attachment (Hnatow *et al*., 1998). This *mgc2* region was assigned a discrimination index of 0.915 by Ferguson and co-workers (2005), being the second highest of the targeted surface proteins.

All samples showed positive amplification. Those from Group A produced amplicons of the same size while those from Group B produced slightly dissimilar sized-amplicons for Farms 1 and 2. This corresponds to the results from Chapter 4 (section 4.4.5), amplifying a shorter region of the *mgc2* gene with isolates from Farm 2 producing larger amplicons. All amplicons were successfully sequenced, and the shorter region of the *mgc2* region from Chapter 4 was aligned to the corresponding samples to check for contamination. All sequences showed 100% sequence correlation.



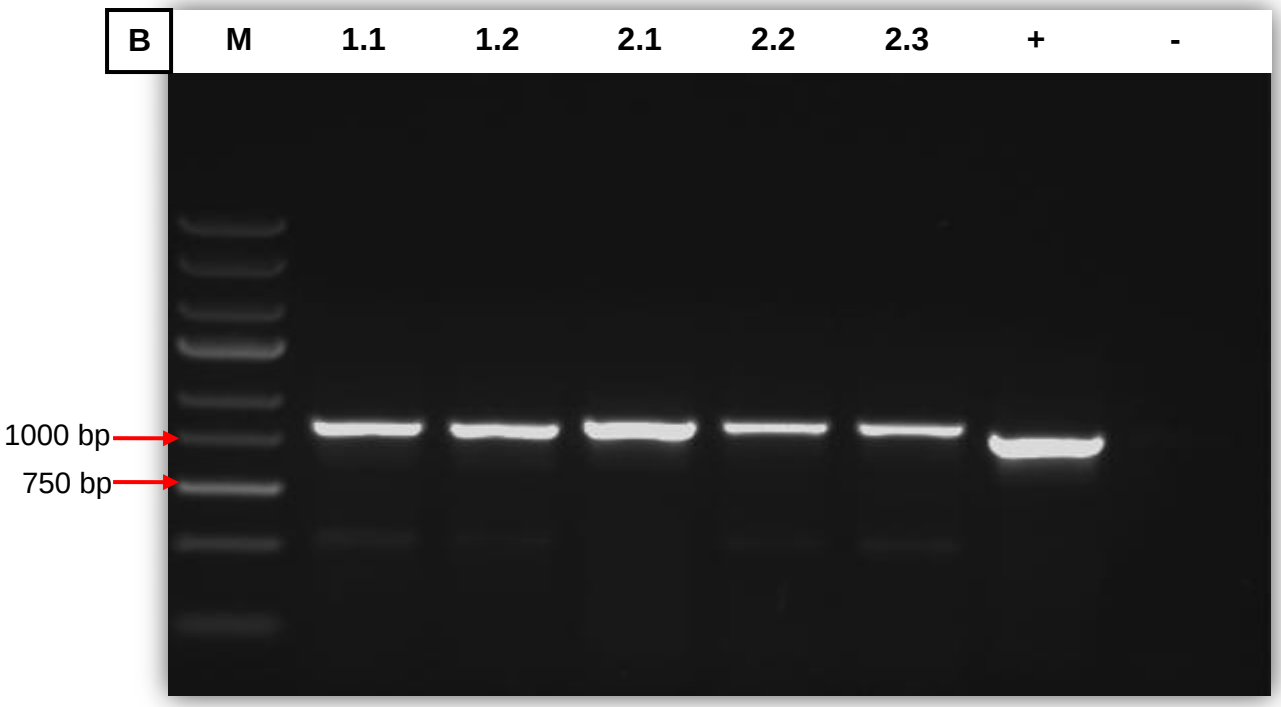


Figure 4.5: A 1% gel (w/v) of the PCR amplification products using primer pair mgc2 (Ferguson *et al.*, 2005) were used to amplify the surface mgc2 protein of *M. gallisepticum,* resulting in variable PCR amplicons of approximately 820 bp. **A**) Zimbabwean isolates (Group A) and (**B**) South Africa isolates from Farm 1 (1.1-1.2) and Farm 2 (2.1-2.3) were selected for further characterization. Lane M represents the molecular marker (O'GeneRuler^{TM} Express DNA ladder). The positive control (+) was *M. gallisepticum* strain A514, while the last lanes of each gel represent the negative control (-).

Nucleotide-nucleotide BLAST analysis (Altschul *et al*., 1990) showed the Zimbabwean isolate (Group A) to be most similar to the *MG* S6 strain in terms of percentage identity (93%) and available query coverage (100%). The Israeli isolates, RV-2 and TLS-2 did show highest identities of 100% and 96% respectively. However the query coverage was very low as the entire sequence was not available on the NCBI database. More importantly, the *MG* R_{low} strain used to vaccinate the poultry on this farm showed only 90% identity to the strain isolated from the farm. These differences could result in an altered antigenic profile, which could aid the pathogen in evading the host immune system.

From Group B, Farm 1 showed closest identity to the RV-2 strain (99%), however with very low query coverage. The *mgc2* gene region of isolates from Farm 2 showed 100% identity to that of the Ts-11 vaccine strain, indications consistent with BLAST results from the *gapA* and *lp* gene regions. Important to note is that the *mgc2* gene region was able to discriminate between the isolates collected from Farms 1 and 2 that were otherwise identical in sequence for the targeted *gapA*, lp, 16S rDNA and 16S-23S IGS regions.

Table 4.4: Indicating nucleotide-nucleotide BLAST results for the partial *mgc2* gene consensus sequences from Group A and Group B (Farms 1 and 2) field isolates, with various isolates of highest percentage identity and their GenBank accession numbers

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
Group A	*MG* str. S6	U34842.1	100%	93%
	MG str. F	CP001873.1	100%	90%
	MG str. R (low)	AE015450.2	100%	90%
	MG str. A5969	AY556227.1	81%	93%
	MG str. RV-2	EU939449.1	38%	100%
	MG str. TLS-2	JN113387.1	18%	96%
Farm 1	*MG* str. R (low)	AE015450.2	100%	90%
	MG str. F	CP001873.1	100%	91%
	MG str. S6	AY556229.1	83%	94%
	MG str. Ts11	AY556232	83%	93%
	MG str. 685	AY556231.1	83%	96%
	MG str. RV-2	EU939449.1	38%	99%
Farm 2	*MG* str. R (low)	AE015450.2	100%	95%
	MG str. F	CP001873.1	100%	94%
	MG str. S6	AY556229.1	84%	97%
	MG str. Ts11	AY556232	84%	100%
	MG str. 685	AY556231.1	84%	99%

The nucleotide sequences for the sample isolates were aligned with the strains showing highest identity in the BLAST results, including the *MG* R_{low} strain used for vaccination in Group A (Figure 4.6). Farm 2 showed highest sequence identity to Ts-11 with 100% identity, while that of Group A and Farm 1 shared highest identity with the RV-2 strain, despite its limited nucleotide sequence available on the NCBI database. The Farm 1 South African isolate differed by only one base pair from that of the nucleotide sequence available of the RV-2 strain (highlighted in blue), while the Group A isolate showed an insert region (highlighted in green) unique to any of the represented strains. The Group A isolate also showed significant differences (highlighted in yellow) to the *mgc2* gene region of the *MG* R_{low} strain used to vaccinate the flock.

```
Farm 2          TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
Ts-11           TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
MG R_low        TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
MG S6           TTTTACCCAGTAGTGGGCGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
Group A         TTTTATCCAGTAGTGGGTGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
Farm 1          TTTTACCCAGTAGTGGGCGCAGGTGCTGGGTTGATTGTTGTTTCTTTACTCTTGGGTTTA 60
MG RV2          ------------------------------------------------------------
                ***** ********** ****************************************

Farm 2          GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
Ts-11           GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
MG R_low        GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
MG S6           GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
Group A         GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
Farm 1          GGGATTGGGATTCCGATCGCTAAGAAAAAAGAAAGAATGATGATCCAAGAACGTGAAGAA 120
MG RV2          ------------------------------------------------------------
                ************************************************************

Farm 2          CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
Ts-11           CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
MG R_low        CACCAAAAGATGGTTGAATCCCTTGGAATAATTGAAGAACAAAATAAAACAGAAGCGATT 180
MG S6           CACCAAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
Group A         CACCAAAAGATGGTTGAATCCCTTGGAATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
Farm 1          CACCAAAAGATGGTTGAATCCCTTGGTATAATCGAAGAACAAAATAAAACAGAAGCGATT 180
MG RV2          ------------------------------------------------------------
                ************************* ***** ****************************

Farm 2          GAGCCAACTACATCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
Ts-11           GAGCCAACTACATCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
MG R_low        GAGCCAACT---------------GAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 225
MG S6           GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
Group A         GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
Farm 1          GAGCCAACTGCAGCAGTGCCAACTGAAGAAGTTAATACTCAAGAACCAACTCAACCAGCT 240
MG RV2          ------------------------------------------------------------
                *********               ************************************

Farm 2          GGTGTTAATGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
Ts-11           GGTGTTAATGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
MG R_low        GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCA---------------ACCT 270
MG S6           GGTGTTAATGTAGCTAATAACCCTCAGATGGGGATCAATCAACCAGGATTTAATCAACCT 300
Group A         GGTGTTAATGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
Farm 1          GGTGTTAATGTAGATAATAACCCTCAGATGGGGATCAATCAGCCAGGATTTAATCAACCT 300
MG RV2          ------------------------------------------------------------
                ************* ***************************              ****

Farm 2          CAGATTAATCCGCAATTTATTCCTAATCCCCAACAAAGAATGAACCCACAGGGCTTTGGT 360
Ts-11           CAGATTAATCCGCAATTTATTCCTAATCCCCAACAAAGAATGAACCCACAGGGCTTTGGT 360
MG R_low        CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGTGCTTTGGT 330
MG S6           CAGATTAATCCGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCACAGGGCTTTGGT 360
Group A         CAGATTAATCAGCAATTTGGTCCTCATCCCCAACAAAGAATTAACCCGCAGGGCTTTGGT 360
Farm 1          CAGATTAATCCGCAATTTGGTCCTCATCCCCAACAAAGAATTAACCCGCAGGGCTTTGGT 360
MG RV2          ----------CGCAATTTGGTCCTAATCCCCAACAAAGAATTAACCCGCAGGGCTTTGGT 51
                ********** *******  **** ***************** ***** *** ********

Farm 2          GGCCCAATGCCACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
Ts-11           GGCCCAATGCCACTTAACCAAATGGGGATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
MG R_low        GGCCCAATGCAACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 390
MG S6           GGCCCAATGCCACCTAACCAAATGGGAATGCGACCAGGGTTTAACCAAATGCCCCCACAA 420
Group A         GGCCCAATGCCACCA---CAAATGGGAATGCGACCAGGGTTTAACCAAATGCCACCACAA 417
Farm 1          GGCCCAATGCCACCA---CAAATGGGAATGCGACCAGGGTTTAACCAAATGCCACCACAA 417
MG RV2          GGCCCAATGCCACCA---CAAATGGGAATGCGACCAGGGTTTAACCAAATGCCACCACAA 108
                ********** **     ******** ************************** ******
```

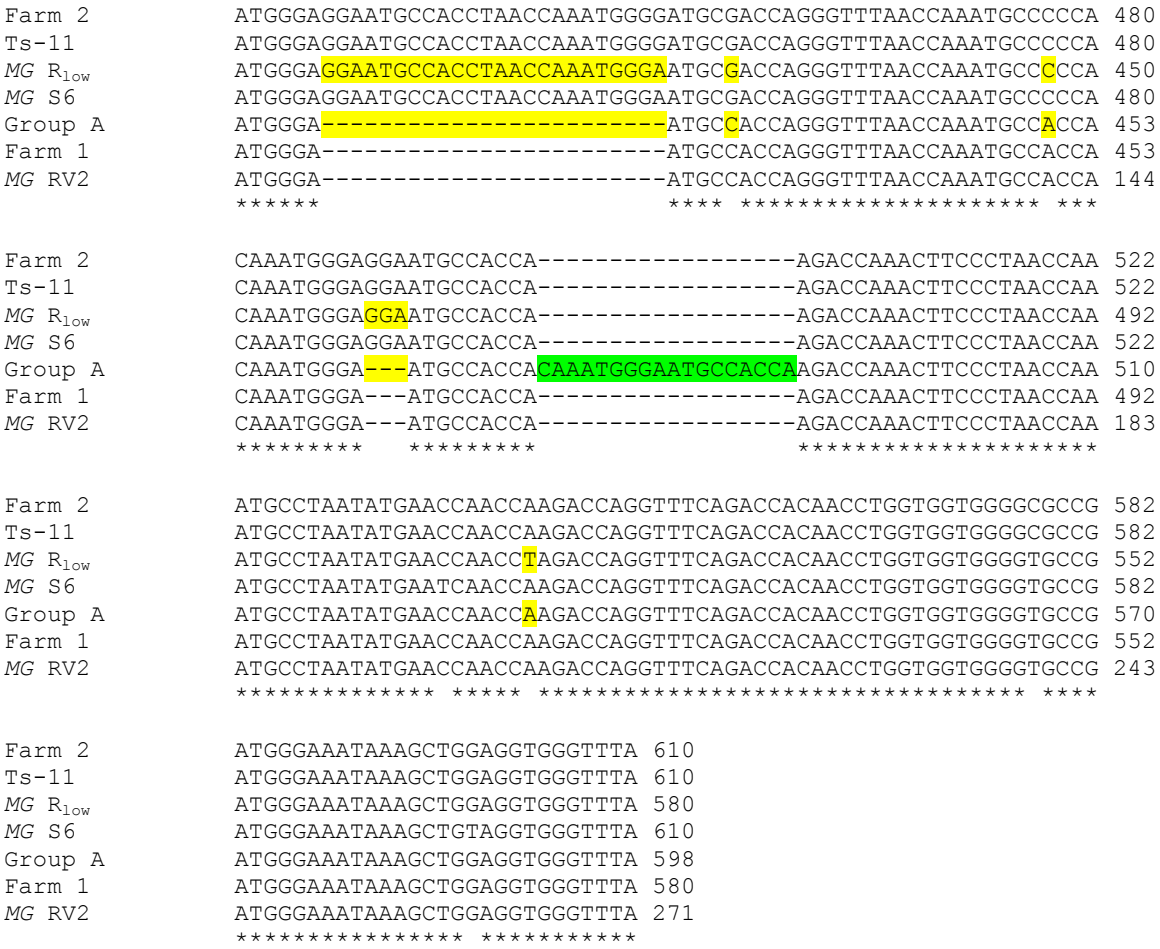


Figure 4.6: ClustalW sequence alignment of the consensus sequences from Group A and Group B, namely Farm 1 and Farm 2, of the variable *mgc2* region compared to that of isolates showing highest identity. These isolates include the *MG* S6 strain (AY556229.1), the Ts-11 vaccine strain (AY556232), the *MG* RV-2 strain (EU939449.1) and the reference *MG* R_{low} strain (AE015450.2). Nucleotides with a star below (*) are indicative of identical nucleotides for the sequences. Nucleotides highlighted in yellow indicate differences between the Group A sequence to that of the *MG* Rl_{ow} strain, while those highlighted in green show unique regions of the Group A isolate. Nucleotides highlighted in blue mark the difference between Farm 2 and the Ts-11 vaccine strain.

Samples sequences were translated to observe the amino acid composition and to subject the *mgc2* gene sequences to further analysis in order to hypothesize whether or not differences in genetic composition are significant in the antigenicity of the protein. During translation of the sequences with Geneious Pro v5.4.4 (Drummond *et al*., 2011), the "Mycoplasma" option was selected since the UGA codon encodes tryptophan as opposed to a stop codon in other Prokaryotes.

In Figure 4.7, the whole *mgc2* gene of the *MG* R_{low} and the *MG* S6 strains are illustrated in comparison to the partial sequences of the Zimbabwean isolate (Group A) and the highly similar RV-2 strain. As highlighted in green, the Zimbabwean isolate contained a significant insert region and considerable amino acid differences are seen in comparison to the R_{low} strain (highlighted in yellow). Whether or not these changes occur in an antigenic region would illustrate the significance of these amino acid changes with regard to aiding the pathogen in resisting the host immune system and promoting their survival in an otherwise inhospitable environment. Additionally, BLASTp (Altschul *et al*., 1990) was performed so as not to exclude any other strains at protein alignment level. The S6 strain (AAB02986.1) showed the highest identity of 93%, however the Zimbabwean sample isolate still showed the unique insert region as seen in Figure 4.7.

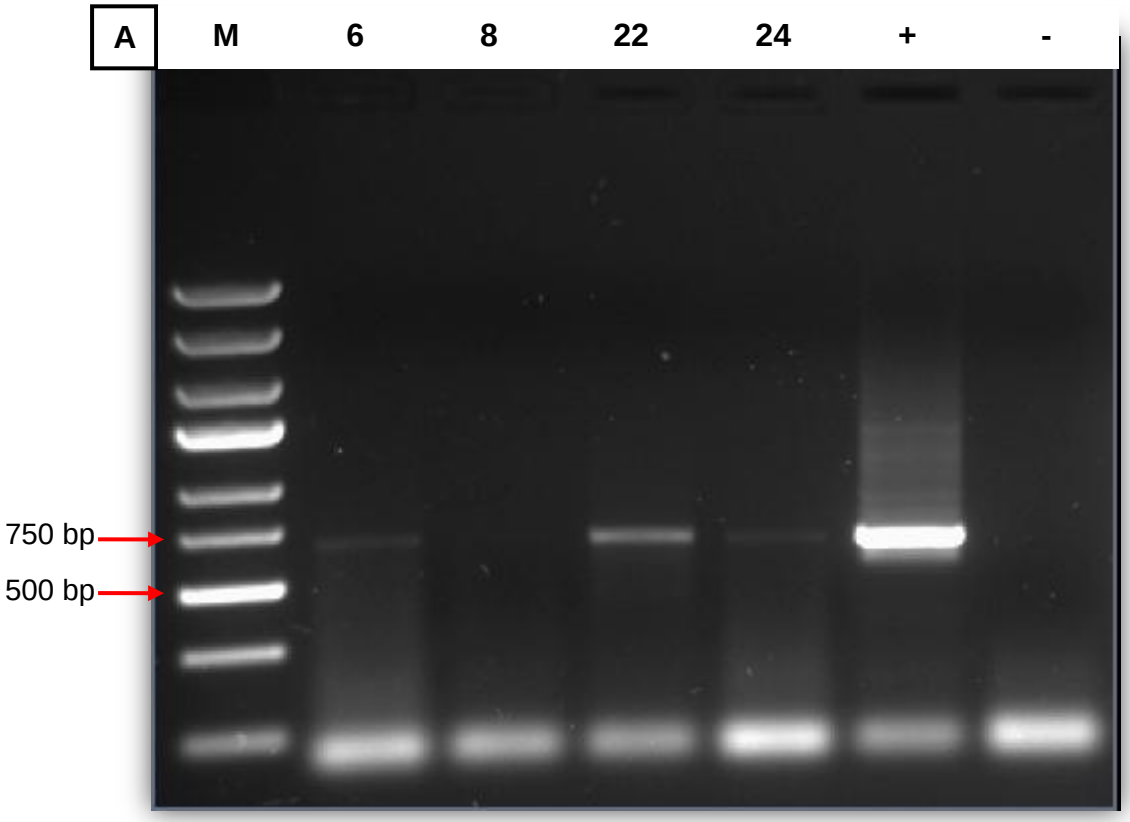
```
RV-2            ------------------------------------------------------------
Group A         -------------------------GVGLTSEHKYEHSPTLVLHEGETNSVGPRKITSE 34
MG Rlow         MFSLKKLKSKLVGVSFVFSGVIALGTGVGLTSEHKYEHSPTLVLHEGETNSVGPRKITSE 60
S6              MFSLKKLKSKLVGVSFVFSGAIALGTGVGLTSEHKYEHSPTLVLHEGETNSVGPRKITSE 60

RV-2            ------------------------------------------------------------
Group A         PWFYPVVGAGAGLIVVSLLLGLGIGIPIAKKKERMMIQEREEHQKMVESLGIIEEQNKTE 94
MG Rlow         PWFYPVVGAGAGLIVVSLLLGLGIGIPIAKKKERMMIQEREEHQKMVESLGIIEEQNKTE 120
S6              PWFYPVVGAGAGLIVVSLLLGLGIGIPIAKKKERMMIQEREEHQKMVESLGIIEEQNKTE 120

RV-2            ----------------------------------------------SQFGPNPQQRINPQG 15
Group A         AIEPTAAVPTEEVNTQEPTQPAGVNVDNNPQMGINQPGFNQPQINQQFGPHPQQRINPQG 154
MG Rlow         AIEP-----TEEVNTQEPTQPAGVNVANNPQMGINQP-----QINPQFGPNPQQRINPQC 170
S6              AIEPTAAVPTEEVNTQEPTQPAGVNVANNPQMGINQPGFNQPQINPQFGPNPQQRINPQG 180
                                                  ****:********

RV-2            FGGPMPP-QMGMRPGFNQMPPQMG--------MPPGFNQMPPQMG-MP------PRPNFP 59
Group A         FGGPMPP-QMGMRPGFNQMPPQMG--------MPPGFNQMPPQMG-MPPQMGMPPRPNFP 204
MG Rlow         FGGPMQPNQMGMRPGFNQMPPQMGGMPPNQMGMRPGFNQMPPQMGGMP------PRPNFP 224
S6              FGGPMPPNQMGMRPGFNQMPPQMGGMPPNQMGMRPGFNQMPPQMGGMP------PRPNFP 234
                ***** * ***************        * *********** **      ******

RV-2            NQMPNMNQPRPGFRPQPGGGVPMGNKAGGGF---------------------------- 91
Group A         NQMPNMNQPRPGFRPQPGGGVPMGNKAGGGFNHPGAPMGPNRM----------------- 248
MG Rlow         NQMPNMNQPRPGFRPQPGGGVPMGNKAGGGFNHPGTPMGPNRMNFPNQGMNQPPHMAGPR 284
S6              NQMPNMNQPRPGFRPQPGGGVPMGNKAVGGFNHPGAPMGPNRMNFPNQGMNQPPYMAGPR 294
                ************************** ***

RV-2            ----------
Group A         ----------
MG Rlow         AGFPPQNGPR 294
S6              AGFPPQNGPR 304
```

Figure 4.7: Protein sequence alignment of the partial *mgc2* region of the Zimbabwean isolate (Group A) compared to that of the *MG* R_{low} strain used to vaccinate the flock, and the high identity strains RV-2 and S6. Regions highlighted in yellow show differences between the R_{low} strain and the Zimbabwean isolate. The regions highlighted in green are unique for the Zimbabwean isolate.

4.4.4. The Carboxy-teminus of the pvpA gene

The primer set pvpA (Table 4.1) described by Ferguson and co-workers (2005) was used to amplify the C-terminal of the *pvpA* gene of *MG,* where truncations have been reported to be located within the proline-rich direct repeat (DR) region. This gene has been shown to encode a putative accessory cytadhesin that exhibits size variation among *MG* strains (Liu *et al*., 2001), producing amplicons of 437, 578, 606 or 665 bp. Furthermore, it is also subject to spontaneous high-frequency variation in expression (Boguslavsky *et al*., 2000). The individual *pvpA* gene was shown by Ferguson and co-workers (2005) to have the highest discrimination index of 0.920.

In Figure 4.8A, the Zimbabwean isolates 6, 22 and 24 produced a faint band of approximately 700 bp, of which only sample 22 was successfully sequenced. Of Group B (Figure 4.8B), all samples of Farm 1 and 2 produced a band of approximately 700 bp that were successfully sequenced.



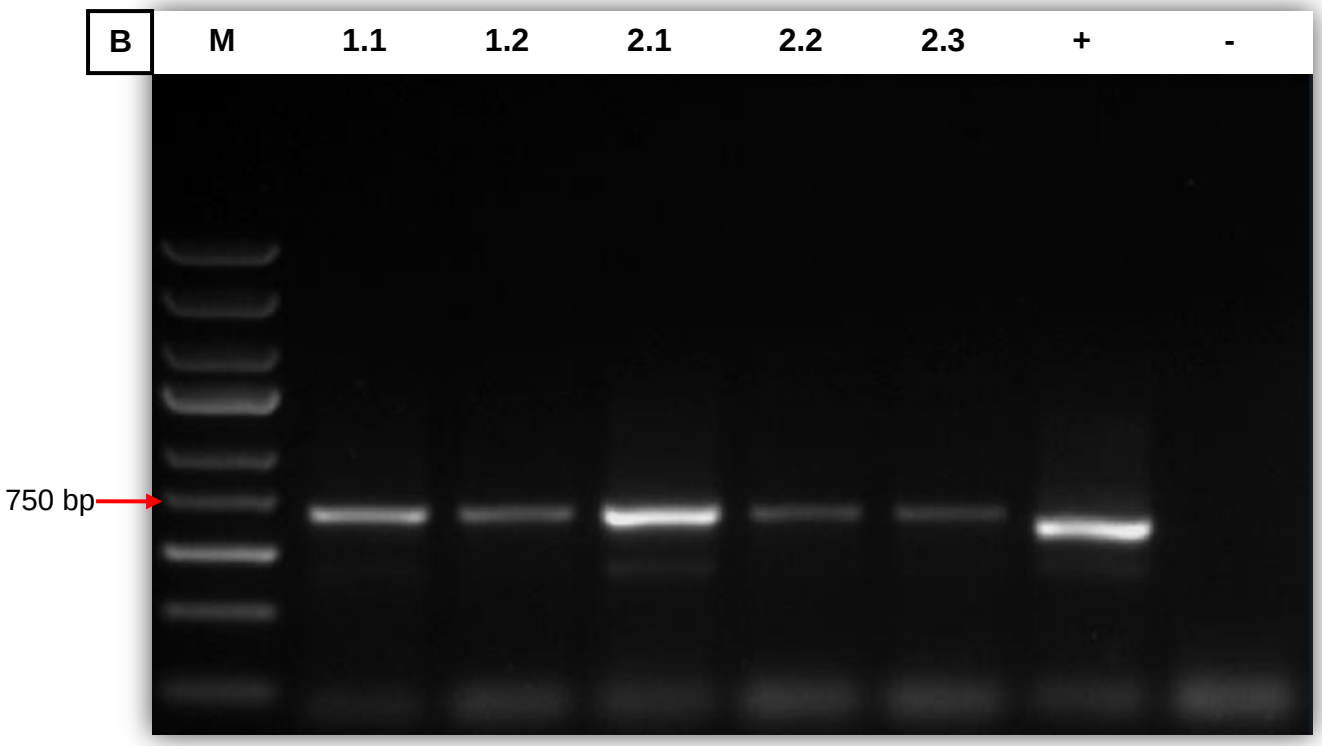


Figure 4.8: A 1% gel (w/v) of the PCR amplification products using primer pair pvpA (Ferguson *et al.*, 2005) to amplify the integral membrane surface protein PvpA of *M. gallisepticum,* resulting in PCR amplicons of approximately 700 bp. **A**) Zimbabwean isolates (Group A) and (**B**) South Africa isolates from Farm 1 (1.1-1.2) and Farm 2 (2.1-2.3). Lane M represents the molecular marker (O'GeneRuler^{TM} Express DNA ladder). The positive control (+) was *M. gallisepticum* strain A514, while the last lanes of each gel represent the negative control (-).

The consensus sequences of the samples were aligned with isolates previously showing high percentage identity, namely the Ts-11 vaccine strain, the R_{low} reference strain used as an inactivated vaccine for Group A, and lastly the Israeli RV-2 strain. As seen from the alignment in Figure 4.9, the *pvpA* nucleotide sequences of Farms 1 and 2 were identical and differed by only one base pair from that of the Ts-11 vaccine strain as highlighted in yellow. A few nucleotide differences (highlighted in green) were seen between the Zimbabwean isolate from Group A and the R_{low} strain used for vaccination, however these lead to significant differences when translated (Figure 4.10) to amino acids. The antigenicity of this PvpA region was determined in section 4.4.5 to hypothesize whether or not differences in this region would be antigenically significant. Isolates from Group A and Group B were also seen to share some nucleotide differences. Although with the *mgc2*-PCR Group A and Farm 1 were found to be most similar to the RV-2 strain, these isolates lack the large deletion in the *pvpA* gene as seen in Figure 4.9 for the RV-2 strain.

```
Farm 1          TTAGTAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGCTCAAGGT 60
Farm 2          TTAGTAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGCTCAAGGT 60
MG Ts-11        TTAGTAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGCTCAAGGT 60
MG R_low        TTAGTAACAACTGATGTAGCTAGCAC---TCAAGCTGTAGGTACTGAAGAAGTTCAAGGT 57
MG TLS-2        TTAGTAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGCTCAAGGT 60
MG RV-2         TTAGTAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGCTCAAGGT 60
Group A         TTAGCAACAACTGATGTAGCTAGCACTACTCAAGCTGCAGGTACTGAAGAAGTTCAAGGT 60
                **** ********************   ******** ************** *******

Farm 1          GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTCACCAATGGGT 120
Farm 2          GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTCACCAATGGGT 120
MG Ts-11        GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTCACCAATGGGT 120
MG R_low        GTTTTATTACCTCCTAGTCAACAACCAACGGAAATGCGTCCAGCTCCTTCACCAATGGGT 117
MG TLS-2        GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTTACCAATGGGT 120
MG RV-2         GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTTACCAATGGGT 120
Group A         GATTTATTACCTCCTAGTCAACAACCAACGGGAATGCGTCCAGCTCCTTTACCAATGAGT 120
                * ***************************** ***************** ******* **

Farm 1          AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATCCACAACACGGACCACGTCCGATG 180
Farm 2          AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATCCACAACACGGACCACGTCCGATG 180
MG Ts-11        AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATCCACAACACGGACCACGTCCGATG 180
MG R_low        AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATCCACAACACGGACCACGTCCGATG 177
MG TLS-2        AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATTCACAACACGGACCACGTCCGATG 180
MG RV-2         AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATTCACAACACGGACCACGTCCGATG 180
Group A         AGTCCTAAGTTATTAGGTCCAAACCAAGCTGGTCATTCACAACACGGACCGCGTCCGATG 180
                ************************************ ************* *********

Farm 1          AATGCTCATCCAGGTCAACCACGCCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 240
Farm 2          AATGCTCATCCAGGTCAACCACGCCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 240
MG Ts-11        AATGCTCATCCAGGTCAACCACGCCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 240
MG R_low        AATGCTCATCCAGGTCAACCACGTCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 237
MG TLS-2        AATGCTCATCCAGGTCAACTACGTCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 240
MG RV-2         AATGCTCATCCAGGTCAACCACGTCCTCAACAAGCTGGCCCACGTCCAATGGGAGCTGGT 240
Group A         AATGCTCATCCAGGTCAACCACGTCCTCAACCAGCTGGCCCACGTCCAATGGGAGCTGGT 240
                ******************* *** ******* ****************************

Farm 1          GGATCTAACCAACCAAGACCAATGCCAAATGGTCTACAAAACCCACAAGGTCCACGACCA 300
Farm 2          GGATCTAACCAACCAAGACCAATGCCAAATGGTCTACAAAACCCACAAGGTCCACGACCA 300
MG Ts-11        GGATCTAACCAACCAAGACCAATGCCAAATGGTCTACAAAACCCACAAGGTCCACGACCA 300
MG R_low        GGATCTAACCAACCAAGACCAATGCCAAATGGTCTACAAAACCCACAAGGTCCACGACCA 297
MG TLS-2        GGATCTAACCAACCAAGACCCATGCCAAATGGTCCACAAAACCAACAAGGTCCAAGACCA 300
MG RV-2         GGATCTAACCAACCAAGACCCATGCCAAATGGTCCACAAAACCAACAAGGTCCAAGACCA 300
Group A         GGATCTAACCAACCAAGACCAATGCCAAATAGTCCACAAAACCCACAAGGTCCACGACCA 300
                ******************* ********* *** ******** ********** *****

Farm 1          ATGAACCCTCAAGGCGATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAA 360
Farm 2          ATGAACCCTCAAGGCGATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAA 360
MG Ts-11        ATGAACCCTCAAGGCGATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAA 360
MG R_low        ATGAACCCTCAAGGCGATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAA 357
MG TLS-2        ATGAACCCTCAAGGCAATCCTCGTCCTGAACCAGCTGGCCCACGACCTAACGGCCCACAA 360
MG RV-2         ATGAACCCTCAAGGCAATCCTCGTCCTGG-------------------------------- 329
Group A         ATGAACCCTCAAGGTAATCCTCGTCCTCAACCAGCTGGTGTCAGACCTAACAGCCCACAA 360
                ************** ***********

Farm 1          AATTCTCAACCACGCCCAATGCCAAATAAACCACAAGGTCCACGACCAATGGGTGCTCCA 420
Farm 2          AATTCTCAACCACGCCCAATGCCAAATAAACCACAAGGTCCACGACCAATGGGTGCTCCA 420
MG Ts-11        AATTCTCAACCACGCCCAATGCCAAATAAACCACAAGGTCCACGACCAATGGGTGCTCCA 420
MG R_low        AATTCTCAACCACGCCCAATGCCAAATAACCACAAGGTCCACGACCAATGGGTGCTCCA 417
MG TLS-2        AATTCTCAACCACG---------------------------------------------- 376
MG RV-2         ------------------------------------------------------------ 329
Group A         AATTCTCAACCACGCCCAACGCCAAATAACCCACAAGGTCCACTACCAATGGGTGCTCCA 420
```

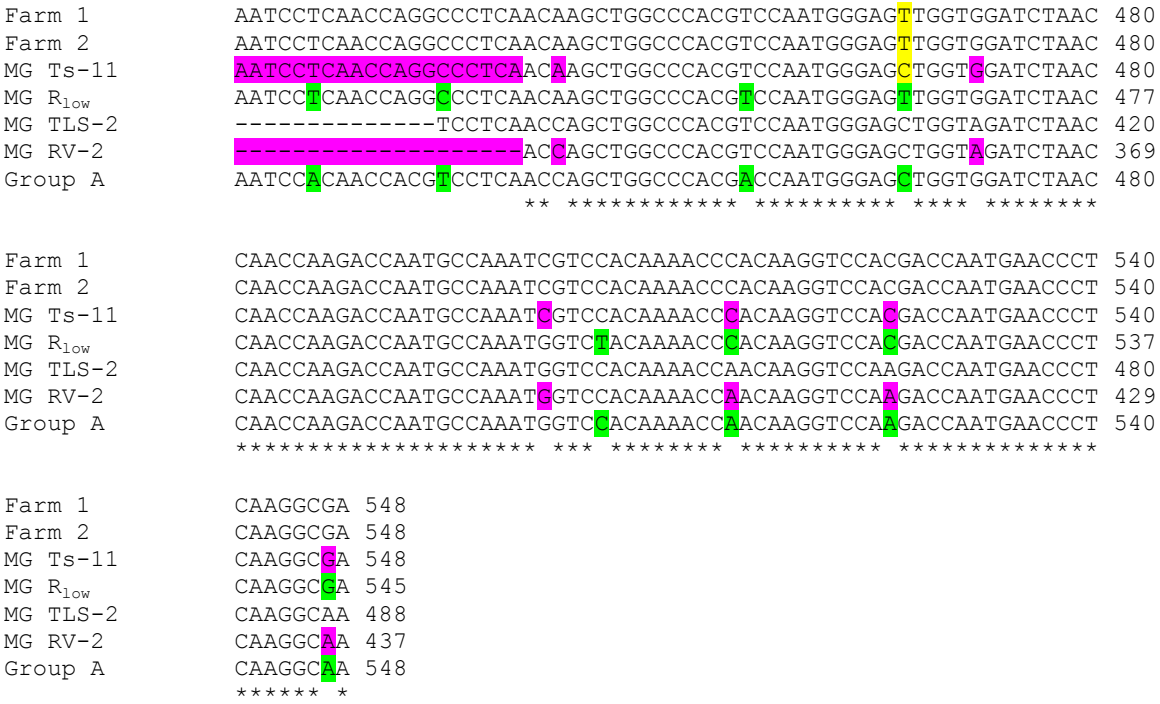


Figure 4.9: ClustalW sequence alignment of the consensus sequences from Group A and Group B, namely Farm 1 and Farm 2, of the variable *pvpA* region compared to that of isolates showing highest identity. Nucleotides with a star below (*) are indicative of identical nucleotides for the sequences. These isolates include the the Ts-11 vaccine strain (JN001167.1), the Israeli *MG* RV-2 (EU939450.1) and TLS-2 strains (JN113336.1), and the reference *MG* R_{low} strain (AE015450.2). Nucleotides highlighted in green indicate the differences between the Group A sequence to that of the *MG* RI_{ow} strain, while those highlighted in yellow mark the difference between Group B isolates from that of the Ts-11 vaccine strain. Differences between the *MG* RV-2 strain and the Ts-11 vaccine strain are highlighted in purple.

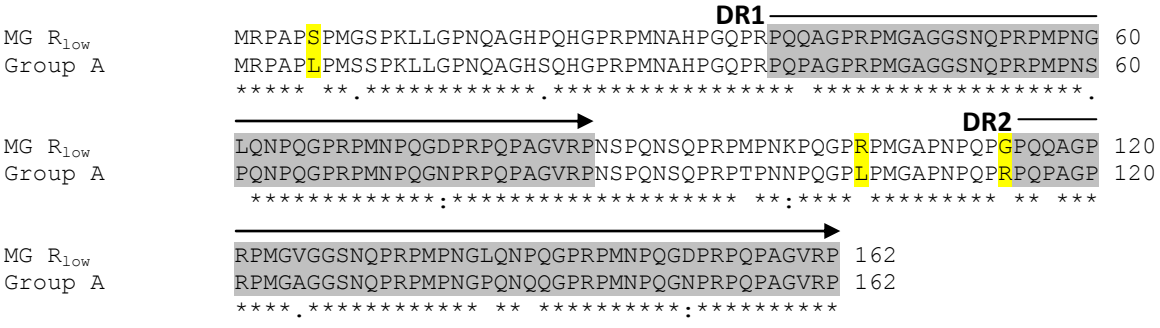


Figure 4.10: Protein sequence alignment of the partial PvpA region of the Zimbabwean isolate (Group A) compared to that of the *MG* R_{low} strain used to vaccinate the flock. Labelled arrows show the position and direction of two directly repeated amino acid sequences (DR-1 and DR-2). The asterisk (*) are indicative of identical residues, the colon (:) for conservation between groups of residues with strongly similar properties, while the period (.) indicates conservation between groups of weakly similar properties.

Nucleotide-nucleotide BLAST (Altschul *et al*., 1990) results showed there were no 100% identity matches for Groups A or B. The *MG* S6 strain showed the closest identity to the Group A isolate taking into account a lower query coverage is often indicative of gaps within the sequences as with the RV-2 strain in Figure 4.9. The *pvpA* region of the isolates from Farms 1 and 2 in Group B were indistinguishable from each other and showed closest identity to the Ts-11 vaccine strain and the *MG* R reference strain.

Table 4.5: Indicating nucleotide-nucleotide BLAST results for the partial *pvpA* gene consensus sequences from Group A and Group B field isolates, with various isolates of highest percentage identity and their GenBank accession numbers.

SEQUENCE	***ISOLATE / SPECIES***	***ACCESSION NO.***	***QUERY COVERAGE***	***% IDENTITY***
Group A	*MG* str. R (low)	AE015450.2	98%	95%
	MG str. A5969	AY556305.1	75%	95%
	MG str. F	CP001873.1	98%	94%
	MG str. TLS-2	JN113336.1	92%	96%
	MG str. RV-2	EU939450.1	95%	96%
	MG str. S6	EU847585.1	99%	96%
Group B	*MG* str. R (low)	AE015450.2	100%	98%
	MG str. F	CP001873.1	97%	98%
	MG str. S6	JN001170.1	91%	96%
	MG str. Ts11	JN001167.1	100%	99%
	MG str. 685	JN001168.1	74%	97%
	MG str. RV-2	JN113326.1	87%	97%

4.4.5. ANTIGENICITY PREDICTION *in silico*

4.4.5.1 Hydrophilicity Prediction

Studies have demonstrated that antigenic determinants are surface features of a protein and they are mostly found on regions of a molecule with a high degree of exposure to solvent, thus projecting into the medium (Hopp & Woods, 1981). To predict possible antigenicity, each amino acid was assigned a hydrophilicity value (Table 4.6). Hydrophilicity profiles of hydrophilicity verses residue number were predicted for the Mgc2 and PvpA amino acid sequences with DN*Assist* 3.0 (Patterton & Graves, 2000) using the default programme settings (Figure 4.11).

Table 4.6: Indicating the solvent parameter values (s, hydrophilicity values) assigned to each amino acid by Levitt in 1976 (as cited by Hopp & Woods, 1981).

AMINO ACID	s. kcal/mol (Hydrophilicity value)	AMINO ACID	s. kcal/mol (Hydrophilicity value)
Arginine	3.0	Alanine	-0.0
Aspartic acid	2.5	Histidine	-0.5
Glutamic acid	2.5	Cysteine	-1.0
Lysine	3.0	Methionine	-1.3
Serine	0.3	Valine	-1.5
Asparagine	0.2	Isoleucine	-1.8
Glutamine	0.2	Leucine	-1.8
Glycine	0.0	Tyrosine	-2.3
Proline	-1.4	Phenylalanine	-2.5
Threonine	-0.4	Tryptophan	-3.4

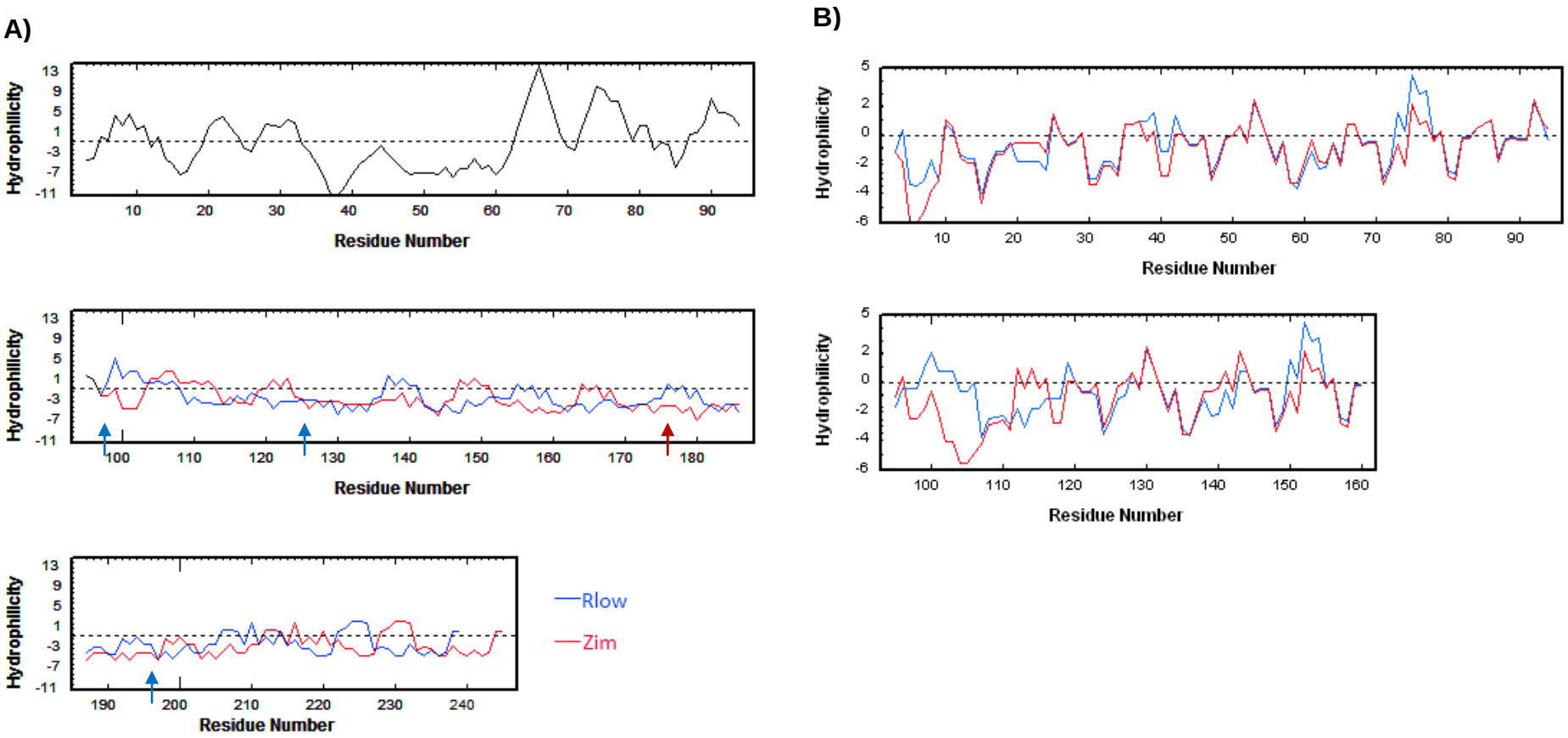


Figure 4.11: Hydrophilicity profiles for (**A**) the partial *mgc2* gene and (**B**) the partial *pvpA* gene for the Zimbabwean (Zim) isolate in comparison to the *MG* R_{low} strain used to vaccinate the flock. The blue arrows indicate the amino acid position where gaps are seen in the R_{low} strain, whereas the red arrow indicates the amino acid position of gaps present in Zimbabwean sequence.

From Figure 4.11 it is evident that the differences in Mgc2 and PvpA amino acid sequences between the R_{low} strain and the Zimbabwean isolate influence the hydrophilicity of the protein *in silico*. In Figure 4.11A, the first 96 residues remained the same (highlighted in black) since there were no differences in the sequences; however at residues 99, 127 and 190 the R_{low} strain showed gap regions compared to the Zimbabwean (Zim) isolate and so the plot of the Zim isolate shifted to the right. Baring this in mind, the peak formed at residue 107 for the Zim isolate is lower than that of the R_{low} strain at the corresponding peak of residue 109. The gap in the R_{low} strain caused by the unique insert of the Zim isolate at residue 177 resulted in lowering of the hydrophilicity of the region. This may result in the absence of certain antigens recognized by the host immune system. Likewise, the hydrophilicity is largely decreased in the Zimbabwean strain at residue 6 with the substitution of a polar serine residue to a hydrophobic leucine residue. The hydrophilicity of the Zimbabwean isolate is however increased between residues 96-99 with the substitution of various hydrophobic residues for polar residues. The substitution of the arginine residue for a leucine at position 104 disturbed the conserved Pro-Arg-Pro-X motif, however this motif is re-established at position 114 where a glycine residue is substituted for an arginine residue in the Zim strain.

```
MG R_low          LQNPQGPRPMNPQGDPRPQPAGVRPNSPQNSQPRPMPNKPQGPRPMGAPNPQPGPQQAGP 120
Group A           PQNPQGPRPMNPQGNPRPQPAGVRPNSPQNSQPRPTPNNPQGPLPMGAPNPQPRPQPAGP 120
                   ************:********************** **:**** ********* ** ***
```

Figure 4.12: Extract from the protein sequence alignment of the partial PvpA region of the Zimbabwean isolate (Group A) compared to that of the *MG* R_{low} strain used to vaccinate the flock. Residues highlighted in yellow indicate the amino acid changes disturbing or re-establishing the P-R-P-X motif.

*4.4.5.*2 *BepiPred Linear Epitope Prediction*

Antigenicity is however not only based on hydrophilicity, although it is an important contributer. Other factors such as flexibility, accessibility, turns, exposed surface, polarity and antigenic propensity of polypeptide chains correlated with the location of continuous epitopes need to be considered. The antigenicity of the Mgc2 and PvpA membrane surface proteins was predicted *in silico* using BepiPred linear epitope prediction via the Immune Epitope Database (http://tools.immuneepitope.org). More specifically, it was used to predict the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method (Larsen *et al*., 2006). B-cell epitopes are the sites of molecules that are recognized by antibodies of the immune system. Each scale consists of 20 values assigned to each of the amino acid residues on the basis of their relative propensity to possess the property described by the scale.

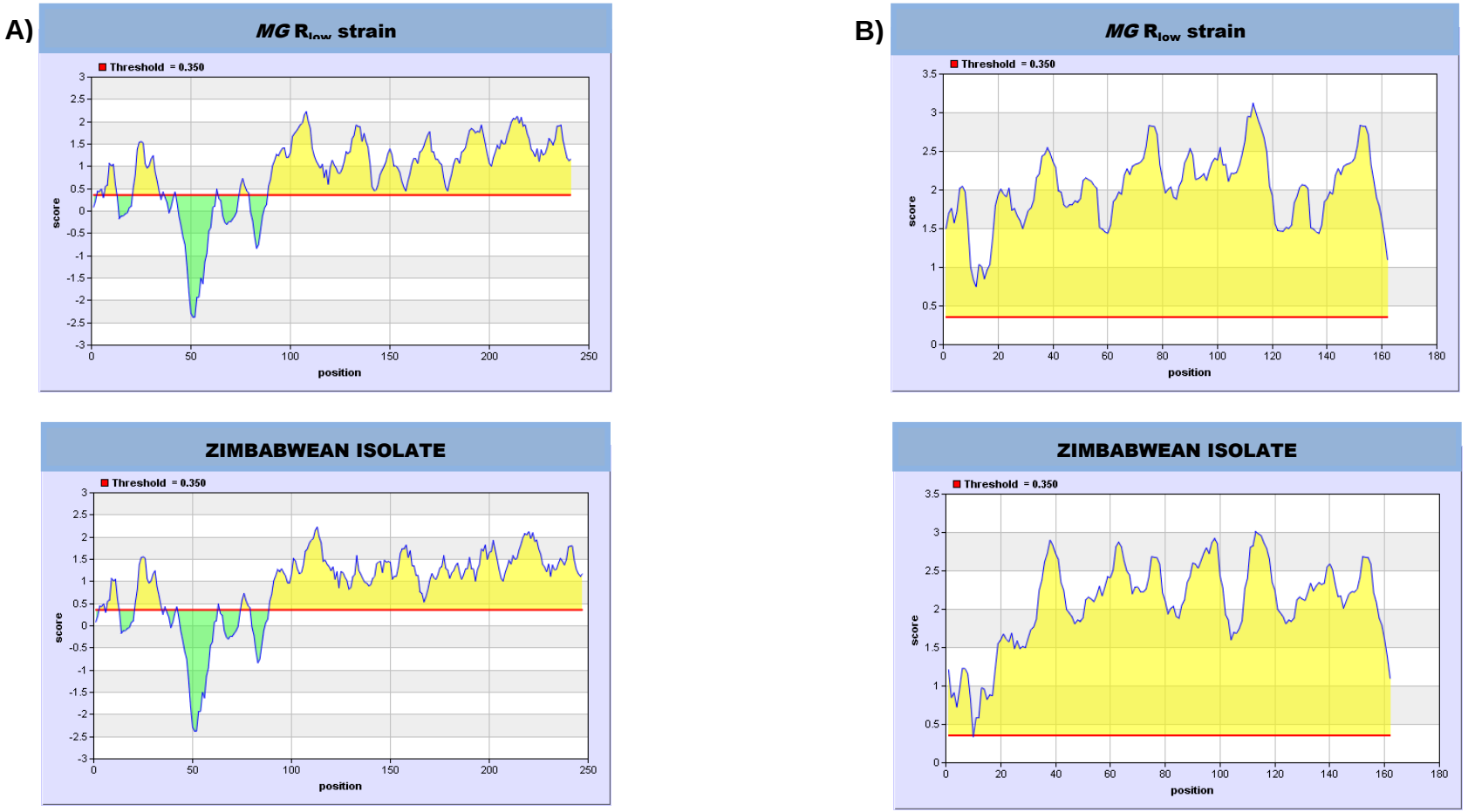


Figure 4.13: Antigenicity profiles for (**A**) the partial *mgc2* gene and (**B**) the partial *pvpA* gene for the Zimbabwean (Zim) isolate in comparison to the *MG* R_{low} strain used to vaccinate the flock. Peaks highlighted in yellow above the 0.35 threshold represent regions predicted for linear B-cell epitopes.

From Figure 4.13 it is clear that the predicted antigenicity profile between the *MG* R_{low} strain used to vaccinate the flock differed from that of the Zimbabwean strain isolated from the farm. In Figure 4.12A, the *mgc2* region amplified was predicted to be mostly antigenic approximately after residue 90. It was from this residue onwards where nucleotide differences were observed. Thus, the Zimbabwean isolate showed higher genetic variation in a region predicted to be antigenic.

In Figure 4.12B, the amplified *pvpA* region is predicted to be highly antigenic, although the *pvpA* gene had already been shown to be recognised by the chicken immune system by Levisohn and co-workers (1995). A steep decrease in antigenicity is seen between residues 1-10. This is simply because of the substitution of a single polar serine residue for a hydrophobic leucine residue at position 5. Owing to the use of a propensity scale, this single residue substitution influences the score for the neighbouring amino acids, thus decreasing the antigenicity of the region. Another decrease in antigenicity is seen at residue 104, with the substitution of a positively charged arginine residue for another leucine residue (disturbing the Pro-Arg-Pro-X motif). These predicted antigenic changes may all contribute to helping the pathogen evade the host immune system and survive in an otherwise inhospitable environment.

4.5 CONCLUSIONS

GTS, previously described by Ferguson and co-workers (2005) was employed to further differentiate and characterize the *MG* field strains isolated from poultry farms in southern Africa. Four surface protein genes were targeted, one of which was only predicted as a surface protein. This method seemed highly plausible since much sequence data of various strains with these regions were available for comparison on the NCBI database. Due to the vital role these proteins play during attachment to the host epithelial cells and with being exposed to the host environment, they served as a good platform to test for antigenicity. Ferguson and co-workers (2005) calculated various discrimination (D) indexes for the individual genes, ranking them from highest to lowest in the following order: the *gapA, Ip, mgc2* and *pvpA*. Ferguson and co-workers (2005) used a total of 67 *MG* field isolates from the USA, Israel and Australia, as well as 10 reference strains to validate the study. The work presented in this study is the first study using these techniques on strains from southern Africa.

Sequencing results of the various target genes showed the isolates collected from the same poultry farm to be identical, thus resulting in three representative field isolates: one from Group A (Zimbabwe) and two from Group B (South Africa), namely Farm 1 and Farm 2. The South African isolates collected from Farms 1 and 2 were, however indistinguishable from each other based on the sequences of the *gapA*, *Ip* and *pvpA* targeted regions. These regions suggested Group B isolates to be similar in sequence (>97% identity) to various *MG* strains, including the Ts-11 vaccine strain and the reference *MG* R_{low} strain.

The *mgc2* region, on the other hand showed considerable difference between these two isolates. BLAST and alignment results for the *mgc2* gene region showed Farm 1 to have closest identity (99%) to the Israeli *MG* RV-2 strain, albeit the shorter sequence length available on the NCBI database. Contradictory to the *mgc2* gene region for the Farm 1 isolate, the targeted *pvpA* gene region showed to be dissimilar to the *MG* RV-2 strain by lacking the large deletion present in the latter strain. Thus showing a unique clustering of the four genes, perhaps suggesting the Farm 1 isolate to be an ancestor of the *MG* RV-2 strain or have evolved independently.

On the other hand, the Farm 2 isolate showed 100% identity to the Ts-11 vaccine strain. Since vaccination history was unavailable for Farm 2, it can be speculated from the sequence data recovered that the strain isolated from this farm is Ts-11-derived vaccine subpopulations or isolates closely related to the vaccine that evolved independently in the field. To precisely determine the relation of this vaccine-like isolate to the commercially available vaccine, complete vaccine genome sequences and further analysis of the genetic stability of live *MG* vaccines in the field are needed. Despite this, clinical signs were present on Farm 2 where this Ts-11-like strain was isolated. Whether the slight nucleotide modifications observed in the targeted regions resulted in a diverged pathogenic strain, from the otherwise non-pathogenic vaccine strain remains unknown. El Gazzar and co-workers (2011) have however been the first to report of a field case of the apparent reversion to virulence and vertical transmission of the Ts-11 vaccine. It is however, more probable the clinical signs may be better explained by the presence of *M. synoviae* detected from this farm in Chapter 4 (section 4.4.2) while screening the 16S-23S ITS region.

For the Group A strain isolated from Zimbabwe, the aim was not only to characterize the strain but also to look for any significant genetic changes that could result in a different antigenic profile from that of the *MG* R_{low} strain used to vaccinate (inactive vaccine) the flock. By doing so, this could explain the break in vaccination experienced by this poultry farm. From the nucleotide data of the four targeted genes, the Group A isolate did not show 100% identity to any *MG* strains available in the NCBI database; apart from the *mgc2* gene region showing 100% identity to the partial sequence of the *MG* RV-2 strain.

The *gapA* region showed highest identity (97%) to the Israeli *MG* TLS-2 strain and the *MG* R_{low} vaccine strain, while the *MG* RV-2 strain shared 96% identity with various other strains. The *Ip* gene region also showed highest percentage identity (99%) to the *MG* TLS-2 strain, while the *MG* R_{low} and RV-2 strains shared an identity of 97% with various other strains. BLAST results of the *mgc2* gene region from the Zimbabwean isolate showed 100% identity to that of the *MG* RV-2 strain, however with very low query coverage (38%). Despite the low query coverage, the *MG* RV-2 sequence data did cover the nucleotide area showing the most genetic variation within the targeted gene. Upon alignment, the Zimbabwean isolate showed the same unique deletions as the *MG* RV-2 strain, but contained an insert which was not detected in any of the *MG* strains available on the NCBI database. With the *pvpA* gene region, the *MG* S6 strain showed highest identity (96%) with a corresponding high query coverage. The query coverage was important since low query coverage was indicative of nucleotide deletions present within the sequence, as with the *MG* TLS-2 and RV-2 strains, despite the high percentage identities (96%). Significant nucleotide differences were also observed between the Zimbabwean isolate and the *MG* R_{low} strain used to vaccinate the flock. Otherwise, due to all the differences seen within these four targeted regions, the Zimbabwean isolate is very likely to be a new *MG* strain.

The *mgc2* and *pvpA* gene regions were selected for further analysis since they showed the most genetic variation between the Zimbabwean isolate and the *MG* R_{low} vaccine strain. These nucleotide sequences were translated so as to observe any changes in the amino acid composition. The protein sequences were also further analysed in order to hypothesize whether or not differences in genetic composition were significant in the antigenicity of the protein. The Mgc2 and PvpA regions showed significant residue differences where amino acid substitutions were not conserved in terms of their properties.

In the case of the *pvpA* gene region, a hydrophobic leucine residue replaced the positively-charged, polar arginine residue (position 104) of the *MG* R_{low} strain; thereby not only decreasing the hydrophilicity of the region, but also disturbing one of the otherwise conserved Pro-Arg-Pro-X motifs. This motif is however restored with the substitution of an arginine residue in the Zimbabwean strain at position 114. Various insertions and deletions could also be seen within the *mgc2* gene region. All these changes contributed to a varied antigenic profile *in silico* for the Zimbabwean isolate compared to the *MG* R_{low} strain. These predicted antigenic changes are postulated to contribute in helping the pathogen to evade the host immune system and survive in an otherwise unfavourable environment. However, these are just prediction models and future work should involve testing the antigenicity *in vitro* either by expressing the Mgc2 and PvpA proteins through a vector, or isolation of the same *MG* strain from the poultry farm in Zimbabwe.

The occurrence of multiple *MG* infection from the same poultry flock or even sample has been previously reported (Mallinson & Rosenstein, 1976). This could pose as a foreseeable predicament for GTS when analysing various target regions of a sample. Although cloning of the PCR fragments would eliminate mixed sequencing data, the various targeted regions would not be able to be correlated to a specific isolate, especially when dealing with novel *MG* strains. With this in mind, pooling of samples from the same poultry farms should be avoided as a precautionary measure.

Otherwise, GTS worked well in this study with the resources available for identifying and characterizing the southern African *MG* field isolates. No mixed *MG* infections were detected from the various poultry farms, as evident in the sequencing chromatogram. By combining sequence data from the various target genes, a more accurate identification and characterization could be made. Ferguson and co-workers (2005) reported an improved discrimination index of 0.965 when doing so, as compared to the RAPD method offering only 0.958. Despite the hierarchy order of the targeted regions, the *mgc2* gene region was able to discriminate better between the isolates collected from Farms 1 and 2 in South Africa. The genetic variability of the *pvpA* gene has been previously well documented (Boguslavsky *et al*., 2000; Liu *et al*., 2001; Pillai *et al*., 2003); however, the genetic variability of the *mgc2* gene requires further study.

CHAPTER 5

FINAL CONCLUSIONS

Cultivation of *Mycoplasma* is considered the gold standard test for confirmation of diagnosis, and through which many other techniques may be carried out for further characterization. For this reason, the difficulty to cultivate *M. gallisepticum in vitro* has lead to the major impediment of its research and laboratory diagnosis. In this study the major setback was with the isolation of *M. gallisepticum* colonies from contaminating isolates, including other *Mycoplasma* species and most likely with the overgrowth of *MG* by faster growing organisms. The isolation of *Mycoplasma* species is therefore usually restricted to more specialized laboratories that have specific conjugated antibodies used for the identification of *MG* colonies.

Despite the advantages which cultivation offers, molecular techniques such as PCR are readily becoming favoured and may be performed in a wider scope of laboratories worldwide. This consequently allows for more inter-laboratory comparisons between *MG* strains and contributes more significantly towards the epidemiology of the species. PCR techniques allowed for the direct detection of the isolates nucleic acid with the identification of *in vivo* conditions. It also widens the possibility of *MG* isolates identified as opposed to those that are only culturable.

For the screening of poultry farms in southern Africa, DNA was extracted directly from the swab samples. The 16S-23S ITS region PCR, capable of amplifying a wide variety of avian *Mycoplasma* species (described by Harasawa *et al.*, 2004 and Ramírez *et al.*, 2008) gave a good indication of the dominant *Mycoplasma* species present. Unfortunately, the primers were also capable of strongly amplifying the phylogenetically-related *Enterococcus* genus and *E. coli*, resulting in multiple bands and masking or preventing the amplification of *MG*. To resolve this, primers designed by Raviv and co-workers (2007) were employed and were found to be specific for the 16S-23S ITS region of *MG* isolates. These primers were however

lacking in sensitivity. Thus this PCR might not detect low levels of *MG* within a sample. The PCR is however useful in distinguishing *MG* from the closely related *M. imitans*. It was found that the *mgc2* gene target (Hnatow *et al*., 1998) was the most sensitive and specific for screening of *MG* isolates from DNA extracted directly from swab samples.

The work presented in this study was the first to report the use of gene-targeted sequencing on strains from southern Africa. From which, three *MG* strains were identified: one from Zimbabwe (Group B) and two from South Africa (Farms 1 and 2). The *MG* strain isolated from Farm 1 showed the four targeted genes to belong to numerous possible strains. The *mgc2* gene region showed closest identity (99%) to the Israeli *MG* RV-2 strain, however was lacking a large deletion in the *pvpA* region for this strain. Nevertheless, there seems to be a link between the atypical *MG* RV-2 strain found in Israel and those prevalent in South Africa. The Farm 2 isolate from South Africa was found to be a Ts-11-like strain, but whether or not it is pathogenic or not remains unknown. Clinical signs observed in these birds could have been a result of *M. synoviae* infection which was also detected from this farm during this study.

Lastly, an *MG* strain was isolated from a poultry farm in Zimbabwe that was previously vaccinated with an inactivated *MG* R_{low} strain. The four targeted surface proteins of the isolated strain did not show 100% identity to any *MG* strains available in the NCBI database. This suggested that it is likely to be a novel *MG* strain. A significantly large nucleotide insert was found to be present in the *mgc2* gene, unique only to this isolate. Additionally, this unique insert was located within a surface-exposed region of the protein and was shown *in silico* to be antigenically significant. Together with this and various other significant amino acid substitutions in antigenically-relevant regions between the strain isolated and the *MG* R_{low} strain used to vaccinate the flock; these changes are believed to help the pathogen in evading the host immune system, thus explaining the reports of failed vaccinations within southern Africa.

Further work would however be needed to conclusively prove this speculation. From this study it is evident that there may be diverse and unique *MG* strains present within southern Africa. Epidemiology studies of *MG* strains within southern Africa would also lead to better biosecurity and perhaps the use of a unique vaccine strain for this region.

SUMMARY

Mycoplasma gallisepticum (*MG*) is an economically important pathogen of poultry worldwide, causing chronic respiratory disease in both chickens and turkeys. Little research has been done to characterize the *MG* field strains present in southern Africa. Field evidence however, suggested breaks in the vaccinations against *MG*, despite the proper cold chain and correct administration. Molecular methods were used to screen various poultry farms in South Africa and Zimbabwe for *MG*. Isolates were further characterized by gene-targeted sequencing (GTS). Portions of the cytadhesin *pvpA, gapA, mgc2* genes and the uncharacterized surface lipoprotein gene designated MGA_0319 were sequenced and analysed.

Three *MG* strains were identified in this study: a Ts-11-like strain; a strain showing closest percentage identity to the atypical *MG* RV-2 strain found in Israel, however with the lack of a large deletion within the *pvpA* region; and lastly an isolate from Zimbabwe, likely to be a novel *MG* strain. The latter contained a unique, large, in-frame nucleotide insertion in the *mgc2* gene. Antigenically-significant variation (determined *in silico*) within the membrane surface proteins of this isolate was found compared to the *MG* R_{low} strain used as an inactive vaccine on the poultry farm. It is thus postulated that by *MG* altering its antigenic profile, it allows effective avoidance of immune recognition and antibodies produced as a result of vaccination with inactivated and possibly live *MG* vaccines. Further research is however needed to substantiate this claim.

Keywords: *Mycoplasma gallisepticum*, Gene-targeted sequencing (GTS), RV-2 strain, Epidemiology, Antigenicity, 16S-23S intergenic spacer region, *mgc2*, *pvpA*.

OPSOMMING

Mycoplasma gallisepticum (MG) is 'n ekonomies belangrike patogeen van pluimvee wêreldwyd, wat chroniese respiratoriese siekte in beide hoenders en kalkoene veroorsaak. Min navorsing is al gedoen om die *MG* veld stamme wat teenwoordig is in Suider-Afrika te karakteriseer. Ten spyte van die behoorlike koue ketting en korrekte administrasie was daar in die veld bewys dat breke in inentings teen *MG* wel voorkom. Molekulêre metodes was gebruik om verskeie pluimvee plase in Suid-Afrika en Zimbabwe te ondersoek vir die teenwoordigheid van *MG*. Isolate was verder gekarakteriseer deur geen geteikende volgordebepalings (GTS). Die volgorde van gedeeltes van die cytadhesin *pvpA*, *gapA*, *mgc2* gene en die nie gekarakteriseerde oppervlak-lipoproteïen geen genoem MGA_0319 was bepaal en ontleed.

Drie *MG* stamme was in hierdie studie geïdentifiseer: 'n Ts-11-agtige stam, 'n stam met die naaste persentasie identiteit aan die a-tipiese *MG* RV-2 stam gevind in Israel, maar met 'n gebrek aan die groot delesie in die *pvpA* streek, en laastens 'n isolaat van Zimbabwe, waarskynlik 'n novel *MG* stam. Laasgenoemde het 'n unieke, groot inraam nukleotied invoeging in die *mgc2* geen. Antigenies-beduidende variasie (soos bepaal *in silico*) binne die membraan proteïene oppervlak van hierdie isolaat was gevind in vergelyking met die *MG* R_{low} stam wat gebruik was as 'n onaktiewe entstof op die pluimvee plaas. Dit is dus gepostuleer dat die veranderinge wat *MG* aan sy antigeniese profiel ondergaan het dit effektief van immuun erkenning vermy asook van die teenliggaampies wat as gevolg van inenting met geïnaktiveerde en moontlik lewendige *MG* entstowwe geproduseer word. Verdere navorsing is egter nodig om hierdie stelling te staaf.

Sleutelwoorde: *Mycoplasma gallisepticum*, Gene-teiken volgordebepaling (GTS), RV-2 stam, Epidemiologie, Antigenesiteit, 16S-23S intergeniese spasies, *mgc2*, *pvpA*.

REFERENCES

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

Altschul S.F., Gish W., Miller W., Myers E.W. & Lipman D.J. (1990) Basic local alignment search tool. *J. Mol. Biol*., Vol. 215: 403-410.

Beumer A. & Robinson J.B. (2005) A broad-host-range, generalized transducing phage (SN-T) acquires 16S rRNA genes from different genera of bacteria. *Appl. and Environ. Microbiol.,* Vol. 71 (12): 8301-8304.

Bhandari S. & Asnani P.J. (1989) Characterization of phospholipase A_2 of *Mycoplasma* species. *Folia Microbiol*.,Vol. 34: 294-301.

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

Bradbury J.M. (1984) Avian *Mycoplasma* infections: prototype of mixed infections with mycoplasmas, bacteria and viruses. *Ann. Microbiol. (Paris)*, Vol. 135A: 83-89.

Bradbury J.M., Abdul-Wahab O.M.S., 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.,* Vol. 43(4): 721-728.

Bradbury J.M., Yavari C.A. & Dare C.M. (2001) Mycoplasmas and respiratory disease in pheasants and partridges. *Avian Pathol.,* Vol. 30(4): 391-396.

Bragg R.R. (2010) Personal communication.

Bram R.J., Young R.A. & Steitz J.A. (1980) The ribonuclease III site flanking 23S sequences in the 30S ribosomal precursor RNA of *Escherichia coli. Cell.*,Vol. 19: 393-401.

Chen T.R. (1977) *In situ* detection of *Mycoplasma* contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res*., Vol. 104(2): 255-262.

Cole B.C., Ward J.R. & Martin C.H. (1968) Hemolysin and peroxide activity of *Mycoplasma* species. *J. Bacteriol*., Vol. 95(6): 2022-2033.

Cooper G.N. (1957) The prolonged survival of upper respiratory tract and intestinal pathogens on swabs. *J. Clin. Pathol*., Vol. 10: 226-230.

Dallo S.F., Lazzell A.L., Chavoya A., Reddy S.P. & Baseman J.B. (1996) Biofunctional domains of the *Mycoplasma pneumoniae* P30 adhesins. *Infect. Immun*., Vol. 64: 2595–2601.

Del Giudice R.A., Robillard N.F. & Carski T.R. (1967) Immunofluorescence identification of *Mycoplasma* on agar by use of incident illumination. *J. Bacteriol*., Vol. 93: 1205-1209.

Domanska-Blicharz K., Tomczyk G. & Minta Z. (2008) Comparison of different molecular methods for detection of *Mycoplasma gallisepticum*. *Bull. Vet. Inst. Pulawy.,* Vol. 52: 529-532.

Dramsi S., Dehoux P. & Cossart P. (1993) Common features of gram-positive bacterial proteins involved in cell recognition. *Mol. Microbiol*., Vol. 9(5): 1119-1121.

Drummond A.J., Ashton B., Buxton S., Cheung M., Cooper A., Duran C., Field M., Heled J., Kearse M., Markowitz S., Moir R., Stones-Havas S., Sturrock S., Thierer T. & Wilson A. (2011) Geneious v5.4, Available from http://www.geneious.com/

Dybvig K., Simecka J.W., Watson H.L. & Cassell G.H. (1989) High-frequency variation in *Mycoplasma pulmonis* colony size. *J. Bacteriol*., Vol. 171: 5165-5168.

El Gazzar M., Laibinis V.A. & Ferguson-Noel N. (2011) Characterization of a ts-11-like *Mycoplasma gallisepticum* isolate from commercial broiler chickens. *Avian Dis*., Vol. 55(4): 569-574.

Evans J.D., Leigh S.A., Branton S.L., Collier S.D., Pharr G.T. & Bearson S.M.D. (2005) *Mycoplasma gallisepticum*: Current and developing means to control the avian pathogen. *J. Appl. Poult. Res*., Vol.14: 757-763.

Fales W.H., McCune E.L. & Berg J.N. (1978) The isolation of gram negative nonfermentative bacteria from turkeys with respiratory distress. *Proc. Am. Assoc. Vet. Lab. Diag.,* Vol. 21: 227-242.

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

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

Forrest M. & Bradbury J.M. (1984) *Mycoplasma glycophilum*, a new species of avian origin. *J. Gen. Microbiol*., Vol. 130(3): 597-603.

Frey M.L., Hanson R.P. & Anderson D.P. (1968) A medium for the isolation of avian Mycoplasmas. *Am. J. Vet. Res*., Vol. 29: 2163-2171.

Ganapathy K. & Bradbury J.M. (1998) Pathogenicity of *Mycoplasma gallisepticum* and *Mycoplasma imitans* in red-legged partridges (*Alectroris rufa*). *Avian Pathol.,* Vol. 27, 455-463.

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.,* Vol. 49: 125-132.

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

Garcia-Martinez J., Acinas S.G., Anton A.I. & Rodreguez-Valera F. (1999) Use of the 16S-23S ribosomal genes spacer region in studies of prokaryotic diversity. *J. Microbiol. Methods,* Vol. 36: 55-64.

Geary S.J., Forsyth M.H., Saoud S.A., Wang G., Berg D.E. & Berg C.M. (1994) *Mycoplasma gallisepticum* strain differentiation by arbitrary primer PCR (RAPD) fingerprinting. *Mol. Cell Probes,* Vol. 8: 311-316.

Gerchman I., Levisohn S., Mikula I., Manso-Silván L. & Lysnyansky I. (2011) Characterization of *in vivo*-aquired resistance to macrolides of *Mycoplasma gallisepticum* strain isolated from poultry. *Vet. Res.,* Vol. 42: 90. http://www.veterinaryresearch.org/content/42/1/90.

Gerchman I., Lysnyansky I., Perk S. & Levisohn S. (2008) *In vitro* susceptibilities to fluoroquinolones in current and archieved *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates from meat-type turkeys. *Vet. Microbiol*., Vol. 131: 266-276.

Ghosh A., Das J. & Maniloff J. (1977) Lack of repair of UV light damage in *Mycoplasma gallisepticum*. *J. Mol. Biol.,* Vol. 116: 337-344.

Glasgow L.R. & Hill R.L. (1980) Interaction of *Mycoplasma gallisepticum* with sialyl glycoproteins. *Infect. Immun*., Vol. 30(2): 353-361.

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,* Vol. 144: 2971-2978.

Hanahan D. (1983) Studies on transformation of *Escherichia coli* with plasmids*. J. Mol. Biol.,* Vol. 166: 557-580.

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

Hnatow L.L., Keeler C.L, Tessmer L.L., Czymmek K. & Dohms J.E. (1998) Characterization of MGC2, a *Mycoplasma gallisepticum* cytadhesin with homology to the *Mycoplsma pneumonia* 30-kilodalton protein P30 and *Mycoplasma genitalium* P32. *Infect. Immun.,* Vol. 66(7): 3426-3442.

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

Hopp T.P. & Woods K.R. (1981) Prediction of protein antigenic determinant from amino acid sequences. *Proc. Natl. Acad. Sci. USA*, Vol. 78(6): 3824-3828.

Immune Epitope Database. Available at: http://tools.immuneepitope.org

Jordan F.T.W., Erno H., Cottew G.S., Hinz K.H. & Stipkovits L. (1982) Characterization and taxonomic description of five *Mycoplasma* serovars (serotypes) of avian origin and their elevation to species rank and further evaluation of the taxonomic status of *Mycoplasma synoviae*. *Int. J. Syst. Bacteriol.,* Vol. 32(1): 108-115.

Kahane I., Granek J. & Reisch-Saada A. (1984) The adhesions of *Mycoplasma gallisepticum* and *M. pneumoniae*. *Ann. Microbiol*., Vol. 135A: 25-32.

Kempf I., Blanchard A., Gesbert F., Guittet M., Bennejean G. (1993) The polymerase chain reaction for *Mycoplasma gallisepticum* detection. *Avian Pathol.,* Vol. 22: 739-750.

Khan M.I. (2002) Multiplex PCR of avian pathogenic mycoplasmas. In *Methods in Molecular Biology*: *PCR Detection of Microbial Pathogens*, Vol. 216: pp. 223. Edited by K. Sachse, & J. Frey. Humana Press Inc., Totowa, N.J.

Khan M.I., Kirkpatrick B.C. & Yamamoto, R. (1989) *Mycoplasma gallisepticum* species and strain specific recombinant DNA probes. *Avian Pathol*., Vol. 18: 135–146.

Khan M.I., Lam K.M. & Yamamoto R. (1987) *Mycoplasma gallisepticum* strain variations detected by SDS-polyacrylamide gel electrophoresis. *Avian Dis*., Vol. 31: 315–320.

Kleven S.H. & Fan H. (1998) Pen trial studies on the use of live vaccines to displace virulent *Mycoplasma gallisepticum* in chickens. *Avian Dis*., Vol. 42: 300–306.

Kleven S.H. (1997) Changing expectations in the control of *Mycoplasma gallisepticum*. *Acta. Vet. Hung*., Vol. 45: 299-305.

Kleven S.H. (1998) Mycoplasmosis. In *A Laboratory Manual for the Isolation and Identification of Avian Pathogens,* 4th edition, pp.74-80. Edited by D.E. Swayne, J.R. Glisson, M.W. Jackwood, J.E Pearson & W.M. Reed. American Association of Avian Pathologists. Kennett Square, PA.

Kleven S.H. (2003) Mycoplasmosis. Introduction. In *Diseases of Poultry*, 11th Edition, pp. 719-721. Edited by Y. M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald, & D.E. Swayne. Iowa State University Press Ames, IA. Wiley-Blackwell.

Kleven S.H., Eidson C.S. & Fletcher O.J. (1978) Airsacculitis induced in broilers with a combination of *Mycoplasma gallinarum* and respiratory viruses. *Avian Dis*., Vol. 22: 707-716.

Kleven S.H., Fulton R.M., Garcia M., Ikuta V.N., Leiting A., Liu T., Ley D.H., Opengart K.N., Rowland G.N. & Wallner-Pendleton E. (2004) Molecular characterization of *Mycoplasma gallisepticum* isolates from turkeys. *Avian Dis.* Vol. 48: 562-569.

Kleven S.H., Morrow C.J. & Whithear K.J. (1988) Comparison of *Mycoplasma gallisepticum* strains by hemagglutination-inhibition and restriction endonuclease analysis. *Avian Dis*., Vol. 32: 731–741.

Krass C.J. & Gardner M.W. (1973) Etymology of the Term *Mycoplasma*. *Int. J. of Syst. Bact.,* Vol. 23(1): 62–64.

Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A. Lopez R., Thompson J.D., Gibson T.J. & Higgins D.G. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics,* Vol. 23(21): 2947-2948.

Larsen J.E., Lund O. & Nielsen M. (2006) Improved method for predicting linear B-cell epitopes. *Immunome Res.* 2(2)

Lauerman L.H. (1998) *Mycoplasma* PCR Assays. In *Nucleic amplification assays for diagnosis of animal diseases*, pp. 41-52. Edited by L.H. Lauerman. American Association of Veterinary Laboratory Diagnosticians, Auburn, AL.

Levisohn S. & Kleven S.H. (1995) *In vivo* variation of *Mycoplasma gallisepticum* antigen expression in experimentally infected chickens. *Vet. Microbiol.,* Vol. 45: 219-231.

Levisohn S. & Kleven S.H. (2000) Avian mycoplasmosis (*Mycoplasma gallisepticum*). *Rev. Sci. Tech.,* Vol. 19: 425-442.

Levisohn S., Rosengarten R. & Yogev D. (1995) *In vivo* variation of *Mycoplasma gallisepticum* antigen expression in experimentally infected chickens. *Vet. Microbiol.,* Vol. 45: 219-231.

Ley D.H. (2003) *Mycoplasma gallisepticum* infection. In: *Diseases of poultry*, 11th Edition: pp. 722-744. Edited by Y.M. Saif, H.J. Barnes, J.R. Glisson, A.M. Fadly, L.R. McDougald & D.E. Swayne. Iowa State University Press, Ames, IA.

Ley D.L. (2008) Mycoplasmosis: *Mycoplasma gallisepticum* infection. In *Disease of Poultry*,11th Edition, pp. 807-834. Edited by Y.M. Saif, A.M. Fadly, J.R. Glisson, L.R. McDougald, L.K. Nolan & D.E. Swayne. Blackwell Publishing, Ames, IA.

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

Ley D.H., McLaren J.M., Miles A.M., Barnes H.J. & Franz G. (1997b) Transmissibility of live *Mycoplasma gallisepticum* vaccine strains ts-11 and 6/85 from vaccinated layer pullets to sentinel poultry, and identification by random amplified polymorphic DNA (RAPD) analysis. *Avian Dis.,* Vol. 41: 187–194.

Ley D.H. & Yoder H.W. (1997) *Mycoplasma gallisepticum* infection. In *Diseases of Poultry*, 10th edition, pp. 194-207. Edited by B.W. Calnek. Iowa State Univ. Press, Ames, IA.

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

Lysnyansky I., Garcia M. & Levisohn S. (2005) Use of mgc2-polymerase chain reaction-restriction fragment length polymorphism for rapid differentiation between field isolates and vaccine strains of *Mycoplasma gallisepticum* in Israel. *Avian Dis.,* Vol. 49: 238-245.

Lysnyansky I., Gerchman I., Perk S. & Levisohn S. (2008) Molecular characterization and typing of enroflozacin-resistant clinical isolates of *Mycoplasma gallisepticum*. *Avian Dis*., Vol. 52(4): 685-689.

Mallinson E.T. & Rosenstein M. (1976) Clinical, cultural, and serological observations of avian mycoplasmosis in two chicken breeder flocks. *Avian Dis*., Vol. 20: 211-215.

Maniloff J. (1978) Molecular biology of *Mycoplasma*, In *Microbiology-1978*. pp. 390-393. Edited by D. Schlessinger. American Society for Microbiology, Washinton, D.C.

McArthur M.W. & Thornton J.M. (1991) Influence of proline residues on protein conformation*. J. Mol. Biol*., Vol. 218(2): 397-412.

Melzak K.A., Sherwood C.S., Turner R.F.B. & Haynes C.A. (1996) Driving forces for DNA adsorption to silica in perchlorate solutions. *J. Colloid Interface Sci. (USA),* Vol. 181: 635-44.

MERCK Animal Health (2009) MYCOVAC-L®, viewed 14 May 2012, http://www.merck-animal-health-usa.com/products/130_120692/productdetails_130_121193.aspx

Miles R.J. (1992) Cell nutrition and growth. In *Mycoplasmas: Molecular biology and pathogenesis*, pp. 23-40. Edited by J. Maniloff, R.N. McElhaney, L.R. Finch & J.B. Baseman. American Society for Microbiology Press, Washington, DC.

Moretti S.A. (2009) Unpublished data: Study of *Mycoplasma gallisepticum* in southern Africa. (Available via correspondence)

Morowitz H.J. & Wallace D.C. (1973) Genome size and life cycle of the *Mycoplasma*. *Ann. N.Y. Acad. Sci.,* Vol. 225: 63-73.

Morowitz H.J. (1985) The completeness of molecular biology. *Isr. J. Med. Sci*., Vol. 20(9): 750–753.

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

Nascimento E.R., Yamamoto R. & Khan M. (1993) *Mycoplasma gallisepticum* F-vaccine strain-specific polymerase chain reaction. *Avian Dis*., Vol. 37: 203-211.

Nascimento E.R., Yamamoto R., Herrick K.R. & Tait R.C. (1991) Polymerase chain reaction for detection of *Mycoplasma gallisepticum*. *Avian Dis*., Vol. 35: 62-69.

Neimark H.C. (1986) Origins and evolution of wall-less prokaryotes. In *The Bacterial L-Forms*, pp. 21-42. Edited by S. Madoff. Marcel Dekker Inc., New York.

Noormohammadi A.H., Jones J.F., Underwood G. & Whithear K.G. (2002) Poor systemic antibody response after vaccination of commercial broiler breeders with *Mycoplasma gallisepticum* vaccine ts-11 not associated with susceptibility to challenge. *Avian Dis.,* Vol. 46(3): 623-628.

OIE Terrestrial Manual (2008) Avian mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae*). Viewed 15 May 2012, http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.05_%20AVIAN_MYCO.pdf

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,* Vol. 149: 2307-2316.

Papazisi L., Silbart L.K., Frasca 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,* Vol. 20: 3709-3719.

Papazisi L., Troy K.E., Gorton T.S., Liao X. & Geary S.J. (2000) Analysis of cytadherence-deficient, GapA-negative *Mycoplasma gallisepticum* strain R. *Infect. Immun.,* Vol. 68(12): 6643-6649.

Patterton H.G. & Graves S. (2000) DN*Assist*: the integrated editing and analysis of molecular biology sequences in Windows. *Bioinformatics,* Vol. 16(7): 652-653.

Peterson B.H. (1975) Concurrention infection of chicks with *M. synoviae* and *Pseudomonas* species. *Poult. Sci.*, Vol. 54: 1804-1805.

Pillai S.R., Mays H.L., Ley D.H., Luttrell P., Panangala V.S., Framers K.L. & Roberts S.R. (2003) Molecular variability of house finch *Mycoplasma gallisepticum* isolates as

revealed by sequencing and restriction fragment length polymorphism analysis of the *pvpA* gene. *Avian Dis.,* Vol. 47: 640–648.

Polak-Vogelzang A. A., Hagenaars R. & Nagel J. (1976) Evaluation of an indirect immunoperoxidase test for identification of *Acholeplasma* and *Mycoplasma*. *J. Gen. Microbiol.,* Vol. 106: 241-249.

Poole K. (2004) Efflux-mediated multiresistance in Gram-negative bacteria. *Clin. Microbiol. Infect.,* Vol. 10(1): 12–26.

Ramírez A.S., Naylor C.J., Pitcher D.G. & Bradbury J.M. (2008) High inter-species and low intra-species variation in 16S-23S rDNA spacer sequences of pathogenic avian mycoplasmas offer potential use as a diagnostic tool. *Vet. Microbiol.,* Vol. 128: 279-287.

Randall C.J., Siller W.G., Wallis A.S. & Kirkpatrick K.S. (1984) Multiple infection in young broilers. *Vet. Rec*., Vol. 114: 270-271.

Raviv Z. & Kleven S.H. (2009) The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. *Avian Dis.,* Vol. 53: 103-107.

Raviv Z., Callison S., Ferguson-Noel N., Laibinis V., Wooten R. & Kleven S.H. (2007) The *Mycoplasma gallisepticum* 16S-23S rRNA intergenic spacer region sequence as a novel tool for epizootiological studies. *Avian Dis.,* Vol. 51: 555-560.

Razin S. (1983a) Characteristics of the mycoplasmas as a group. In *Methods in Mycoplasmology*, Vol. 1, pp. 3–7. Edited by S. Razin & J.G. Tully. Academic Press, New York.

Razin S. (1983b) Introductory comments. In *Methods in Mycoplasmology*, Vol. 1, pp. 29-30. Edited by S. Razin & J.G. Tully. Academic Press, New York.

Razin S. (1994) DNA probes and PCR in diagnosis *of Mycoplasma* infections. *Mol. Cell. Probes*., Vol. 8: 497-511.

Razin S. (1997) Comparative genomics of mycoplasmas. *Wien. Klin. Wochenschr*., Vol. 109: 551-556.

Razin S. (2006) The genus *Mycoplasma* and related genera (Class Mollicutes). In *The Prokaryotes: Bacteria: Firmicutes, Cyanobacteria,* 11th Edition, pp. 879. Edited by M. Dworkin & F. Stanley. Springer, New York.

Razin S., Banai M., Gamleil H., Polliack A., Bredt W. & Kahane I. (1980) Scanning electron microscope of mycoplasmas adhering to erythrocytes. *Infect. Immun.,* Vol. 30(2): 538-546.

Razin S. & Tully J.G. (1983) Appendix. In *Methods in Mycoplasmology*, Vol. 1, pp. 495-499. Edited by S. Razin & J.G. Tully. Academic Press, New York.

Razin S., Yogev D. & Naot Y. (1998) Molecular biology and pathogenicity of mycoplasmas. *Microbiol. Mol. Biol. Rev.,* Vol. 62: 1094-1156.

Robertson B.D. & Meyer T. F. (1992) Genetic variation in pathogenic bacteria. *Trends Genet.,* Vol. 8: 422-427.

Rodwell A.W., & Mitchell A. (1979) Nutrition, growth and reproduction. In *The Mycoplasmas*, Vol. 1, pp. 103-109. Edited by M.F Barile & S. Razin. Academic Press, New York.

Rosengarten R. & Wise K.S. (1990) Phenotypic switching in mycoplasmas: phase variation of diverse surface lipoproteins. *Science,* Vol. 247: 315-318.

Rozen S. & Skaletsky H.J. (2000) Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Edited by S. Krawetz & S. Misener., pp. 365-386. Humana Press, Totowa, NJ.

Shah-Majid M. (1996) Effect of mixed infection of *Mycoplasma gallinarum* and Newcastle disease virus (F-strain) on the tracheal epithelium of village chickens. *Res. Vet. Sci*., Vol. 61: 176-178.

Shifrine M., Pangborn J. & Adler H.E. (1962) Colonial growth of *Mycoplasma gallisepticum* observed with the electron microscope. *J. Bacteriol*., Vol. 83(1): 187-192.

Simecka J.W., Davis J.K., Davidson M.K., Ross S.E., Städtlander C.T. K.H. & Cassell G.H. (1992) *Mycoplasma* diseases of animals. In *Mycoplasmas: Molecular biology and pathogenesis*, pp. 391-415. Edited by J. Maniloff, R.N. McElhaney, L.R. Finch & J.B. Baseman. American Society for Microbiology, Washington, DC.

Southern African Poultry Association (2010) Annual Statistical Reports-SAPA Industry Profile 2010: Animal health/ disease, viewed 21 August 2011, http://www.sapoultry.co.za/industry_profile.php

Swart C.W., Swart H.C., Coetsee E., Pohl C.H., van Wyk P.W.J. & Kock J.L.F. (2010) 3-D architechure and elemental composition of fluconazole treated yeast asci. *Scientific Res. Essays,* Vol. 22: 3411-3417.

Talkington D.F., Fallon M.T., Watson H.L., Thorp R.K. & Cassel G.H. (1989) *Mycoplasma pulmonis* V-1 surface protein variation: occurrence *in vivo* and association with lung lesions*. Microb. Pathog*., Vol. 7: 429-436.

Tully J.G. (1983) Bacterial and fungal inhibitors in *Mycoplasma* culture media. In *Methods in Mycoplasmology*, Vol. 1, pp. 205-210. Edited by S. Razin & J.G Tully. Academic Press, New York.

Tyler K.D., Wang G., Tyler S.D. & Johnson W.M. (1997) Factors affecting the reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *J. Clin. Microbiol*., Vol. 35: 339-346.

Vincze S., Klein G. & Altmann H. (1975) Dexoyribonuclease activity in the serum and spleen of rats with *Mycoplasma* induced arthritis*. Z. Rheumatol*., Vol. 34: 49-54.

Whithear K.G. (1996) Control of avian mycoplasmoses by vaccination. *Rev. Sci. Tech*., Vol. 15: 1527-1553.

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

Wise K.S., Yogev D. & Rosengarten R. (1992) Antigenic variation. In *Mycoplasmas: Molecular Biology and Pathogenesis*, pp. 473-489. Edited by J. Maniloff, R.N. McElhaney, L.R. Finch & J.B. Baseman. *American Society for Microbiology, Washington, DC.*

Woese C.R., Maniloff J. & Zablen L.B. (1980) Phylogenetic analysis of the mycoplasmas. *Proc. Natl. Acad. Sci. USA,* Vol. 77: 494-498.

Yogev D., Levisohn S., Kleven S.H., Halachmi D. & Razin S. (1988) Ribosomal RNA gene probes to detect intraspecies heterogeneity in *Mycoplasma gallisepticum* and *M. synoviae*. *Avian Dis*., Vol. 32(2): 220-231.

Yoshida S.A., Fujisawa A., Tsuzaki Y. & Saitoh S. (2000) Identification and expression of a *Mycoplasma gallisepticum* surface antigen recognized by a monoclonal antibody capable of inhibiting both growth and metabolism. *Infect. Immun*., Vol. 68(6): 3186-3192.

Zain Z.M. & Bradbury J.M. (1996) Optimising the conditions for isolation of *Mycoplasma gallisepticum* collected on applicator swabs. *Vet. Microbiol.,* Vol. 49: 45-57.