MOLECULAR CHARACTERIZATION OF *MYCOPLASMA SYNOVIAE* IN CHICKENS IN SOUTH AFRICA USING SINGLE-STRANDED CONFORMATION POLYMORPHISM AND HIGH-RESOLUTION MELTING CURVE ANALYSIS OF THE *vlhA* GENE

By

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Magister Scientiae (Veterinary Tropical Diseases)

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2012

DECLARATION

I declare that this dissertation hereby submitted to the University of Pretoria for the degree of MSc (Veterinary Tropical Diseases) has not been previously submitted by me or anyone for the degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly cited.

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Raphela Mashikoane Pinky Jane

Pretoria, ___/___/2012

This dissertation forms part of the requirements for a web-based MSc degree research project in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

These projects carry a weight of approximately 100 credits, and are therefore smaller than projects required for a research-based MSc degree with a weight of 240 credits.

It would be appreciated if reviewers could evaluate the dissertation in that context.

ACKNOWLEDGEMENTS

I would like to thank:

My supervisor Prof. Estelle Venter for accepting me as her student, for her patience, for believing in me and encouraging me to go on even when times were hard, for helping me all the way with my project, and also for helping me to produce the quality research proposal and dissertation. I would not have finished my MSc degree if it was not for you.

God Bless!

My co-supervisor Dr. S Bisschop for helping me to produce the quality research proposal and for providing with me samples.

Ms Anna-Mari Bosman for being there for me throughout my project and making my project her priority. I would not have finished my project if it was not for you.

Dr. Kgomotso Sibeko for helping me with the real-time PCR.

Ms. Rebone Mahalare for helping me with preparing of agarose gels.

Mr. Johan Gouws for providing me with the samples.

Ms. Rina Serfontein for helping me with bursaries and registration.

Mr. Phokela Segobola for being there for me and helping me, emotionally and mentally. When I needed someone, you were always there for me. God Bless!

My family for understanding why I registered for this degree and encouraging and supporting me.

Last but not least, my son Phenyo Raphela, for encouraging me to think positive and work hard to invest for his future.

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ABBREVIATIONS

DNA	Deoxyribonucleic acid
DVTD	Department of Veterinary Tropical Diseases
ELISA	Enzyme linked immunosorbent assay
HI	Haemagglutination-inhibition test
HRMC	High resolution melting curve analysis
IFA	Indirect Fluorescence Assay
Mg	*Mycoplasma gallisepticum*
Ms	*Mycoplasma synoviae*
MS-H	*Mycoplasma synoviae* vaccine strain
MSPA	Major surface protein A
MSPB	Major surface protein B
NAD	Nicotinamide adenine dinucleotide
OBP	Onderstepoort Biological Products
ORF's	Open reading frames
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RSA	Rapid serum agglutination assay
RT-PCR	Real-time polymerase chain polymerase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSCP	Single stranded conformation polymorphism analysis
Tm	Melting temperature

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ABSTRACT

Mycoplasma synoviae (Ms) causes respiratory infection and synovitis in chickens and turkeys and is an economically important pathogen of poultry worldwide. It is critically important to characterize Ms strains, especially in countries in which poultry flocks are vaccinated with the live attenuated Ms strain MS-H. Vaccination with this vaccine may cause sero-conversion and persistence of the vaccine strain in the respiratory tract and will frequently result in positive Ms cultures and PCR results. Vaccination of flocks therefore complicates the diagnosis of Ms by the presence of detectable antibodies in the blood. Many diagnostic techniques cannot distinguish between the vaccine strain and wild type strain. Single stranded conformation polymorphism (SSCP) and real-time PCR with high melting curve (HRM) analysis can discriminate between the different Ms strains obtained from the field and also distinguish them from the live vaccine

strains. These techniques provide effective tools for the further study of the epidemiology and spread of Ms strains in chickens in South Africa.

This project was undertaken to establish whether SSCP and HRM analyses can be used effectively to discriminate between Ms field isolates and the vaccine strain. *Mycoplasma synoviae* DNA was extracted from samples and conventional PCR was performed using oligonucleotide primers complementary to the single-copy conserved 5' end of the variable lipoprotein and haemagglutinin encoding gene (*vlhA*). Twenty six samples were separated by agarose gel electrophoresis and prepared for SSCP and real-time PCR and HRM curve analysis.

Results obtained from SSCP were compared to real-time PCR/HRM. Differences obtained by SSCP and melting curve analysis between different isolates were confirmed by sequencing. Results obtained from the different techniques differentiated the strains from the vaccine strain (isolate Ms10), which had a different melting temperature to the others and a different band pattern on the SSCP gel. These results confirmed that HRM and SSCP methods can be used to detect and discriminate between *Mycoplasma synoviae* field isolates and the vaccine strain.

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Mycoplasma species were first described in chickens during the 1930s by Nelson (1939) and the chronic respiratory disease caused by mycoplasma was described in 1943 by Delaplane and Stuart. Infectious synovitis was described in 1938 in turkeys by Dickson and Hinshaw and also described by Olson *et al.,* (1954). The successful cultivation of *Mycoplasma synoviae* (Ms) from chickens and turkeys was reported in the 1950s and the similarities between the organism in chickens and turkeys were noted by Dickson and Hinshaw in 1938. During 1950s researchers Markham and Wong (1952), and Van Roekel and Olesiuk (1953) reported the successful cultivation of the organisms from chickens and turkeys and suggested they were the members of the pleuropneumonia group. During the 1970's and 1980's, infectious synovitis was described in chickens by Mohammed and co-workers (1986).

Mycoplasma synoviae is the smallest and simplest bacteria and lacks a cell wall (Kleven, 1997). It is a major pathogen of chickens and turkeys and causes respiratory tract infection and synovitis worldwide (Kleven, 1997). Most of the damage resulting from *Mycoplasma* species infections in humans and animals is due to host immune and inflammatory responses rather than to direct deadly effects of *Mycoplasma* virulence factors (Razin *et al*., 1998). Different Ms strains are characterised by differences in infectivity, tissue tropism and pathogenicity (Razin *et al*., 1998).

Most mycoplasmas affecting animals depend on adhesion to host tissues for colonization and infection, therefore their adhesion is the major virulence determinants and adherence-deficient mutants are avirulent (Rottem, 2003). It is important for mycoplasmas to escape the host immune system, therefore they reside intracellularly, but it is not their preferred location in the body and they are mainly found extracellular. Another survival strategy is molecular mimicry and phenotype plasticity (Markham *et al.*, 1994). *Mycoplasma* species is pleomorphic and lacks the ability of Gram staining, therefore the characterisation of mycoplasmas based on morphological characteristics is non-effective. This also has the limitation in the ability to

establish if the flock is vaccinated or infected with the wild type strain (Boguslavsky *et al.,* 2000).

Molecular methods are widely available and allow sensitive and specific diagnosis of infectious agents whose conventional isolation and identification is difficult and often unsuccessful, but the use of these molecular techniques is restricted to specialized laboratories (Kempf, 1998). Recently several PCR assays have been used for the amplification of the conserved *vlhA* gene of Ms (Amy *et al* 2010) which replaced most of the serological methods and are more effective than most of the traditional techniques, e.g. culture methods, that are time consuming and costly. The strain identification of Ms from PCR can be confirmed by sequencing (Garcia, 2005).

Mycoplasma has been indicated in the literature as a continuous problem in the poultry industry, leading to economic losses worldwide. For example, the loss of 127 million eggs in the USA in 1984, and ten years later *Mycoplasma* infection had led to a loss of 34 thousand tons of broilers. Different geographical areas have been studied but no information has been documented for incidences in South Africa, therefore the diversity of the organism in SA is not known (Boguslavsky *et al.,* 2000).

1.2 Literature review

1.2.1 Classification of *Mycoplasma synoviae*

Mycoplasma is the general name for the group of prokaryotes lacking a cell wall (Razin *et al.,* 1998). They utilize sterols to strengthen the outermost trilaminar membranes which act as barriers that protect the contents of the mycoplasma cell. Mycoplasmas are the smallest self-replicating organisms and are distinguished phenotypically from other bacteria by their pleomorphic morphology, size and total lack of cell wall (Razin *et al.,* 1998). They are classified in the: phylum: Firmicutes, order: Mycoplasmatales, family: *Mycoplasmataceae* and genus-*Mycoplasma* (Lockaby *et al*., 1999). The class Mollicutes includes the mycoplasmas, the ureaplasmas, the achoeplasmas and the spiroplasmas. These organisms are mostly parasitic or commensals of plants, insects, and animals (Lockaby *et al*., 1999).

1.2.2 Morphology and genome structure

Mycoplasma synoviae is phenotypically characterized by a small size (0.3-0.8 µm) and its lack of a cell wall. The cell membrane is rich in protein components that consist of adaptive

lipoproteins. It lacks flagella and therefore is non-motile (Razin *et al*., 1985). *Mycoplasma synoviae* has lost all genes required for amino acid and cofactor synthesis, synthesis of the cell wall and lipid metabolism. It also does not have a urea cycle and therefore the substrates and cofactors [nicotinamide adenine dinucleotide (NAD) and amino acids] needed must be taken from the host or artificial culture (Razin *et al*., 1985).

Related sequences of a multigene family referred to as *vlhA* genes in Ms encode for haemagglutinins. In avian mycoplasmas, genes encoding for these immunogenic and surface exposed proteins contribute considerable to antigenic variability (Bencina *et al.,* 2002). Mycoplasmas are thought to colonize mucosal surfaces more efficiently and become more virulent, by alternating the composition of their surface proteins. These haemagglutinins are the most important surface proteins involved in colonization and virulence of avian mycoplasmas (Bencina *et al.,* 2002).

Mycoplasma synoviae has two major surface antigens that are encoded by a single gene, the *vlhA* gene, the product of which is cleaved post-translationally to yield a lipoprotein, referred to as Major Surface Protein B and a haemagglutinin, referred to as Major Surface Protein A (Noormohammadi *et al.,*1998).

The major surface protein A (MSPA) and major surface protein B (MSPB) are phase variable and involved in adhesion of the organism to erythrocytes (Noormohammadi *et al.,* 1998). The *vlhA* gene from Ms was characterized, and polypeptides were expressed from non-overlapping 5′ and 3′ regions in *Escherichia coli*. The *vlhA* 5′ region of the expression product reacted with specific reagents against MSPB, while that of the 3′ region reacted with specific reagents against MSPA. The amino acid sequence was analyzed and the results showed that a characteristic signal peptidase II cleavage site, and the presence of the acylation site was confirmed by identification of a lipid-associated membrane protein, similar in molecular mass to MSPB. The *vlhA* gene sequence was analyzed further and revealed a high identity with a gene of the member of a large translated family, the *Mycoplasma gallisepticum* pMGA1.7 (Noormohammadi *et al.,* 1998). Noormohammadi *et al.,* (1998) further analysed the Ms genome and showed that the *vlhA* gene may have been transferred horizontally.

1.2.3 Epidemiology

1.2.3.1 Prevalence

Mycoplasma synoviae infection is common in most poultry producing countries and is widespread in layer chickens. The spread of infection is rapid between chicken houses on a farm, the clinical signs are variable and the mortality rate is usually less than 10% (Mc Mullin *et al.,* 2004; Barua *et al.,* 2006). The prevalence in South Africa, consisting of different geographical areas, is not known.

1.2.3.2 Hosts affected

Mycoplasma synoviae is highly infectious to poultry and the infection is a problem in broilers and layer industries. It often also appears in turkey flocks and chickens and turkeys are the major hosts of Ms infection in most poultry producing countries, especially commercial layer flocks (McMullin *et al.,* 2004). Other types of birds e.g. ducks, geese, guinea fowl, pigeons, pheasants, and quail are also susceptible to Ms infection (McMullin *et al.,* 2004).

Outbreaks of infectious synovitis occur mostly in chickens at 4-6 weeks and in turkeys at 10-12 weeks. Natural infection is seen mostly from one week of age in chickens and usually between 10-24 weeks in turkeys (McMullan *et al.,* 2004).

1.2.3.3 Route of transmission

Transmission of Ms occurs through the respiratory tract and transmission may either be lateral via respiratory aerosol or direct contact or vertical. Fomite transmission between farms may be possible but survival of the organism is probably poor. *Mycoplasma synoviae* can survive up to three days on feathers (Christensen *et al*., 2007). Vertical transmission plays a major role in Ms infection of chickens. The cycle can only be broken after birds are depleted and clean out is done properly. Broilers infected with Ms can only cause re-infection in the same flocks as well as to other flocks via lateral transmission and the cycle can be broken during a proper clean out programme (McMullin *et al.,* 2004). Chickens can be infected with Ms via lateral infection which occurs by contact of birds in the same unit, or farming complexes. None or only a few birds develop joint lesions when infected through the respiratory tract (Kleven, 2003).

1.2.4 Prevention and control

Prevention involves the establishment of mycoplasmas free breeding flocks. This can be done by treating infected hatching eggs with the antibiotic such as Tylosin to decrease the organism in the eggs. Before purchasing chicks from a hatchery, one should confirm that they are free from chronic respiratory disease and not only of clinical signs. Chicks should be raised at a place where there are no mycoplasma infected birds and on a mycoplasma clean farm (Kahn & Line 2011).

Good biosecurity and sufficient isolation of the external environment to prevent airborne infections from infecting healthy flocks; disposing of dead birds by incineration and deep burial or by means of special disposal pits; vaccines that are free from contamination with mycoplasmas should be used. Construction of the houses must be done in such a way to prohibit the entrance of any type of wild birds, wandering animals, and visitors to the farm to come in contact with flocks. Workmen should shower and use special clothes and strict biosecurity measures should be adopted (Kahn & Line 2011).

1.2.4.1 *Mycoplasma synoviae* vaccine strain MS-H

Mycoplasma synoviae vaccine strain MS-H is a live, attenuated, temperature-sensitive Ms vaccine strain which is used to control virulent Ms infection in commercial chicken flocks (Noormohammadi *et al.,* 2000). This vaccine strain was shown to colonize the upper respiratory system and to induce an antibody response in turkeys and chickens. Even at the maximum release dose, MS-H was not found to cause the air sac, joint, or tracheal lesions that are normally caused by the wild-type Ms infection (Noormohammadi *et al.,* 2000). Histopathologic examinations of vaccinated turkeys and chickens after exposure to a virulent Ms challenge revealed that administration of the vaccine by aerosol, but not eye drop, at the dose recommended for chickens, protected the birds against microscopic lesions and colonization of the virulent Ms in the trachea (Noormohammadi *et al.,* 2000).

Mycoplasma synoviae both as synovial infection and as a respiratory infection causes economic losses to the poultry industry and therefore effective vaccines against Ms are required. Inactivated Ms vaccines have been used; however they are expensive due to the large amount of antigenic material needed to trigger a sufficient immune response (Witviet *et al.,* 1999). Inactivated vaccines also have to be manually applied by the parental route which requires individual handling of each animal and is labor intensive. Live attenuated vaccines are more

desirable because they are self-replicating and therefore less antigenic material is required to trigger an immune response. They closely mimic natural infection and therefore give better protection (Witviet *et al.,* 1999).

1.2.5 Diagnosis

1.2.5.1 Clinical signs

Clinical signs are the first indicator of Ms infection. *Mycoplasma synoviae* infections can progress as either acute or chronic systemic disease with symptoms of arthritis, synovitis and bursitis in chickens and turkeys. Slow growth, pale combs and lameness are the earliest observable signs in a flocks affected with infectious synovitis. Then the feathers become ruffled and the comb shrinks as the disease progresses (Kleven, 2003). Bluish red comb may also appear in some cases. Swellings of the joints (tibio-tarsal joints and tarso-metatarsal joint) as well as breast blisters are also commonly observed. Affected birds become progressively exhausted, listless, dehydrated, and emaciated. At autopsy, when the joints and tendon sheaths are opened, chickens frequently have viscous creamy to grey exudates in the joints and tendon sheaths. Swollen livers, spleens and kidneys have also been seen (Kleven, 2003).

In birds with respiratory infection, there may be no apparent clinical signs notice (Kahn & Line 2011). Ms-induced airsacculitis may occur secondary to poor ventilation or respiratory infections e.g. Newcastle disease and infectious bronchitis. In many cases air sac lesions resolve after 1-2 weeks (Kahn & Line 2011).

1.2.5.2 Confirmation by laboratory tests

Culture

Mycoplasma requires a unique medium formulation to grow Nicotinamide adenine dinucleotide (NAD) is required for the growth of cells and swine serum is also preferred for induction of the medium as a growth requirement. Sterile cotton swabs are collected from tracheal, choanal cleft, synovial or air sac lesion, and inoculated into broth medium. It is a fastidious organism therefore requires 4-5 days of growth (Kleven, 2003). The culture is normally confirmed by IFA test especially when contamination with other microorganisms is prevalent (Kleven, 2003).

From live birds, samples are taken from fresh or frozen carcasses. Swabs are usually taken from the choanal cleft, oropharynx, oesophagus, trachea and conjuctiva (OIE *Terrestrial Manual*

2008). From dead birds, samples may be taken from the nasal cavity, infraorbital sinus, trachea, or air sacs. Infraorbital sinuses and joint cavities can also be used for aspiration of exudates. Dead-in-shell embryos or chickens or poults can also be used for sampling of Ms. Samples can also be taken from the inner surface of the vitelline membrane, and from the oropharynx and air sacs of the embryo (OIE *Terrestrial Manual* 2008).

Microscopy

Mycoplasma synoviae appear as pleomorphic coccoid bodies approximately 0.2 *μm* in diameter on slides stained by the Giemsa method. The cells are round or pear shaped with granular ribosomes; lacking cell walls and are bound by a triple layer membrane. Ruthenium red and negative staining is maybe used to demonstrate the extracellular surface layer (Kleven, 2003).

Serology

Due to the problems encountered with the growth of the organism, serological detection is usually used as a screening tool but should be followed with either culture or PCR (or both) for confirmation. Serological diagnosis of infectious synovitis and assessment of effective vaccination continue to rely on rapid slide agglutination (RSA) as a screening test and haemagglutination-inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) for laboratory confirmation. These tests are also used for flock monitoring. Problems are encountered with each of these tests related to their diagnostic performance, sensitivity, specificity, cost and or availability (Jeffery *et al*., 2007).

a. Rapid serum agglutination test

Rapid serum agglutination antigens are available commercially and are stained with crystal violet dye. Serum is mixed with stained Ms antigen on a clean white tile or glass plate. Agglutination is indicated by flocculation of the antigen within 2 min. Positive sera should be retested and if they still react strongly, they are considered to be positive. One of the disadvantages is that cross-reaction between Ms and Mg antigens can occur (OIE *Terrestrial Manual* 2008).

b. Haemagglutination-inhibition

The test requires a satisfactory haemagglutinating Ms antigen, obtained from either a fresh broth culture or a concentrated washed suspension of the mycoplasma cells in PBS, washed fresh chicken or turkey RBCs, as appropriate, and specific antibodies to the Ms antigen in the test sera (OIE *Terrestrial Manual* 2008). The haemagglutination-inhibition (HI) test has been used as a serological confirmatory test. This test requires technical skill to perform, it is time consuming and the test is less sensitive than the RSA test. The test reagents are also not commercially available. The results may be variable among laboratories performing the haemagglutination inhibition test. The HI test is very reliable when performed by experienced technologists using good antigen. Inter-laboratory comparison is required as the HI is a subjective test (Browning *et al*., 2000).

c. Enzyme-linked immunosorbent assay

Several commercial Mg and Ms ELISA kits are available (Czifra *et al*., 1993). The ELISA's rely predominantly on whole bacterial cells as a source of antigen. Studies of Ms ELISA's conducted by Opitz *et al.,* (1983); Patten *et al.,* (1984) and Higgins & Whithear, (1986), used crude membrane preparations produced by either osmotic lysis or sonication of whole cells. Although these ELISA systems are rapid and sensitive, they are relatively expensive to produce and, in some cases, have limited specificity (Kleven & Yoder, 1989). In addition, more recent studies on Ms surface proteins have highlighted the variable expression of major antigens of this organism and thus suggested that it may be difficult to ensure consistency of antigen preparations from cultures of Ms (Noormohammadi *et al.,* 1997).

Immunological tests

a. Growth inhibition test

The growth of mycoplasma is inhibited by specific antisera. This enables species to be identified. The test is insensitive and not routinely used. Sera must be high-titred, mono-specific and prepared in mammalian hosts as poultry sera do not always efficiently inhibit the growth of mycoplasmas. Pure cultured organisms must be used and several dilutions should be tested. The growth rate of the organism may also influence growth inhibition (Clyde, 1983).

b. Indirect immunoperoxidase test

The principle is the same as the Indirect Fluorescence Assay test (which uses two antibodies; the unlabeled first (primary) antibody specifically binds the target molecule, and the secondary

antibody, which carries the fluorophore, recognises the primary antibody and binds to it) (Rosendal *et al.,* 1972) except that the binding of specific antibodies to colonies *in situ* is detected by adding an anti-rabbit immunoglobulin that has been conjugated to the enzyme peroxidase. A positive reaction is developed by adding an appropriate substrate which colours colonies after oxidation. An immunobinding procedure can be used for rapid identification of mycoplasmas in broth medium and clinical specimens in which the test colonies are blotted onto nitrocellulose and then tested in a similar manner. Polyclonal anti-sera should be used for serotyping isolates by the immunoperoxidase test. The immunoperoxidase test has an advantage over immunofluorescence in that the immunoperoxidase does not require an expensive fluorescence microscope (Kotani *et al*., 1985) and it is also useful for identifying mycoplasmas which form colonies poorly. This method has, however, the following disadvantages: the immunobinding technique is not highly sensitive compared to the agar isolation method. Fixing mycoplasma cells sometimes decreases staining specificity and enhances cross-reactivity between species (Imada *et al.,* 1982).

Molecular identification

a. Restriction fragment length polymorphism

Restriction fragment length polymorphism (RFLP) is based on patterns of restriction enzyme digested fragments of DNA on gel electrophoresis. It can be used to identify different *Mycoplasma* species and strains. Restriction fragment length polymorphism has been described by Morrow *et al*., (1990) to classify Ms strains and is currently used in the classification of new field strains of Ms. However, since it requires isolation and culture of the organism and extraction of genomic DNA, RFLP is time consuming another disadvantage may be genomic rearrangements that may occur in progenies of a single Ms isolate (Noormohammadi *et al.*, 2000).

b. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method was used by Marois *et al.,* (2001) to differentiate strains based on protein profiles. This method is rapid, sensitive, and cost effective compare to single stranded conformation polymorphism (SSCP), and it does not discriminate variant populations present in a sample.

c. Polymerase chain reaction

The polymerase chain reaction (PCR) is one of the most useful methods for the detection of Ms and it has previously been used by Jeffery *et al.,* (2007) for the detection of Ms. The PCR can be used without the need to isolate Ms and is sensitive, specific and produces results within one day. The PCR involves the amplification of the *vlhA* gene of Ms. It produces good quality amplified products that can be further analyzed by other PCR-based techniques, such as electrophoretic methods for mutation detection e.g. RFLP and SSCP.

PCR and DNA sequence analysis were used to analyze the N-terminal end of the haemagglutinin encoding *vlhA* gene as an alternative for the detection and initial typing of field strains of Ms in commercial poultry (Hong *et al.,* 2004).

Mycoplasma synoviae obtained from various sources as well as other avian mycoplasmas and other bacterial species were tested and the *vlhA* gene-targeted PCR assay was highly specific in the identification of Ms. Sequence analysis of the amplified products also confirmed the potential use of the N-terminal region of the *vlhA* gene for typing Ms strains directly from clinical samples (Hong *et al.,* 2004).

Other PCR applications e.g. Multiplex PCR, PCR-RFLP, arbitrary by primed PCR (Fan 1995), and random amplification of polymorphic DNA analysis (RAPD) PCR's with high sensitivity have been published for Ms as well as for other pathogenic mycoplasmas (Sachse *et al.,* 1999). PCR with arbitrary primers and RAPD PCR's are used for strain differentiation which is very useful to study the epidemiology of diseases (Lauerman *et al.,* 1993; Kiss *et al.,* 1997).

d. Real-time PCR

The advantage of real-time PCR is the speed with which samples can be analyzed. Compared to other methods e.g. Southern blot, the analysis of the gene copy number is much faster and simpler (Coleman *et al.,* 2006). The advantage of real-time PCR over traditional PCR is the ability to measure the kinetics of the reaction in the early phase of PCR, and to detect of mutations without requiring electrophoresis (Coleman *et al.,* 2006).

e. Single-strand conformation polymorphism

Single-strand conformation polymorphism (SSCP) is used for the discrimination of variants or strains of a given organism. It is technically simple and is used for the detection of mutations and sequence variants (Figure 1) (Gasser *et al.*, 2007).

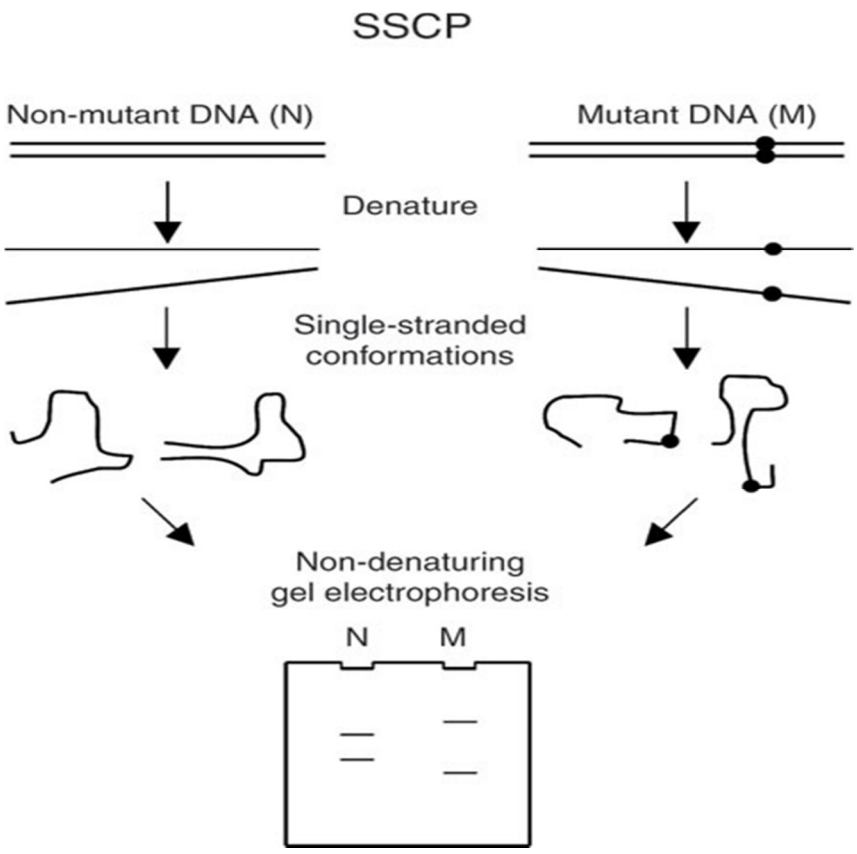

Figure 1 A point mutation (represented by a dot on a DNA strand) leads to the formation of different single-strand conformations of the mutant DNA (M) compared with the non-mutant molecule (N), resulting in differential mobilities in a non-denaturing gel matrix A (Gasser *et al.*, 2007).

The sensitivity of the previously used mutation detection methods e.g. RFLP have been affected by factors such as the type of the substitution, length of the fragment examined, the local base sequence, the G+C content of the DNA fragment, and the location of the sequence variation relative to the end of the fragment which can be overcome by SSCP. The sensitivity of SSCP to detect single base pair mutations ranges from 35% to nearly 100%. Sensitivity could also improve by running the SSCP gel under different conditions. Because of these characteristics, SSCP has been described as a method of choice (Coleman *et al.,* 2006).

f. High resolution melting curve

High resolution melting curve (HRM) analysis is used to characterize DNA samples according to their dissociation behavior as they transition from double stranded DNA to single stranded DNA during temperature change (Figure 2).

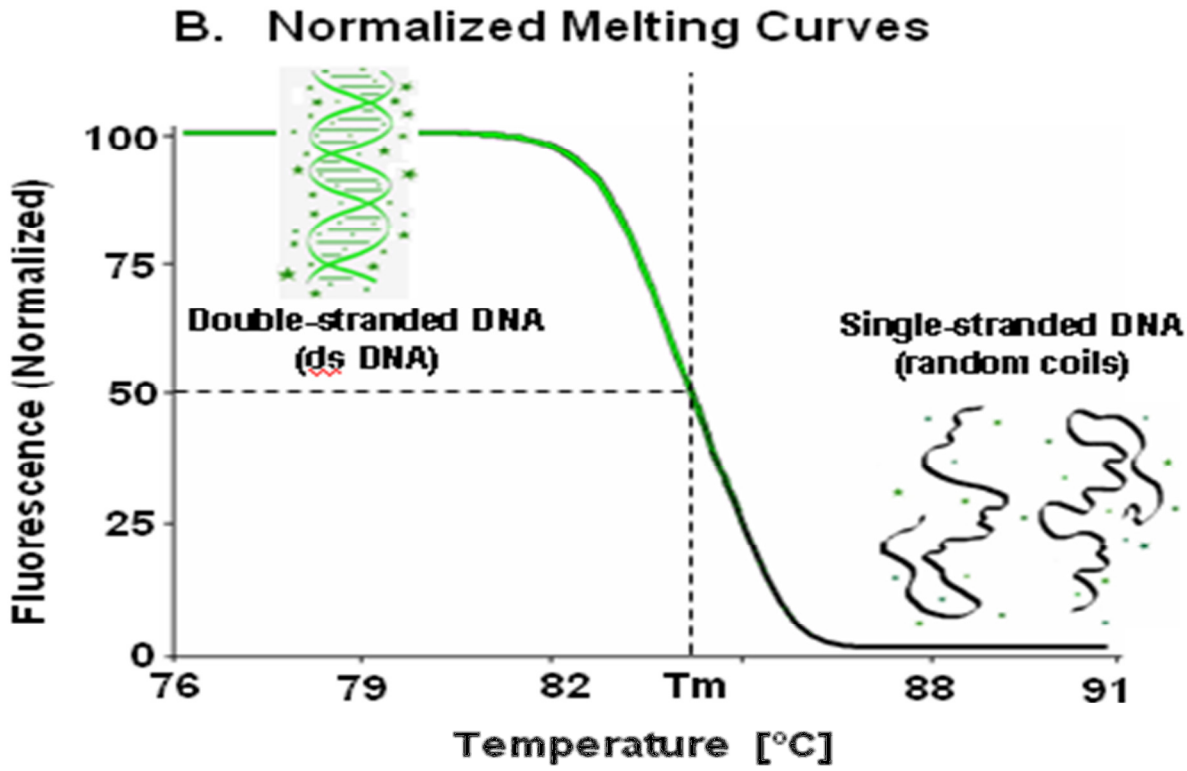

Figure 2 The basic principle of HRM curve analysis, from the double stranded DNA to the single stranded DNA represented by the curve (Wittwer *et al.,* 2003).

Single stranded DNA collects fluorescence signals with much greater optical and thermal accuracy. The melt curve plots the transition from high fluorescence of the initial pre-melt phase through the sharp fluorescence decrease of the melt phase to basal fluorescence at the post-melt phase (Coleman *et al.,* 2006). Fluorescence decreases as the DNA intercalating dye is released from double-stranded DNA as it dissociates (melts) into single strands. The midpoint of the melt-phase, at which the rate of change in fluorescence is the greatest, defines the melting temperature (Tm) of the particular DNA fragment (Coleman *et al.,* 2006).

Single strand conformation polymorphism and melting curve analysis provide cost effective alternatives for the direct analysis of genetic variations and the detection of mutation of Ms. These two PCR-based mutation detection techniques are more sensitive and practical than RFLP particularly when a large number of samples need to be analyzed (Jeffery *et al.,* 2007).

g. *Sequencing*

Sequencing is explained as the process of determining the order of the nucleotide bases along a DNA strand. The automated sequencing is based on the use of dideoxynucleotides which are

tagged with different coloured fluorescent dyes. All four reactions occur in the same tube and are separated in the same lane on the gel. Each labelled DNA fragment passes a detector at the bottom of the gel, the colour is recorded and the sequence is reconstructed from the pattern of colours representing each nucleotide in the sequence (Rizzo & Buck 2012)

1.2.6 Background of the study

Vaccination of flocks complicates the diagnosis of Ms by causing the development of detectable antibodies in the blood and also frequently resulting in positive mycoplasma cultures and PCR results. Many diagnostic techniques cannot distinguish between vaccine strain and natural infection. Single stranded conformation polymorphism and real-time PCR can discriminate between the different Ms isolates obtained from the field and also distinguish them from the live vaccine strain. These provide effective tools for the further study of the epidemiology and spread of Ms strains in chickens in South Africa.

1.2.7 Objectives

We aimed to use SSCP and HRM analysis to characterize Ms strains in SA, and compare these molecular techniques with gene targeted sequencing. The objectives for this study were thus as follows:

- Amplification of the *vlhA* gene by conventional PCR
- Single stranded conformation polymorphism analysis of the Ms *vlhA* genes of different field isolates
- Real-time PCR and melting curve analysis of the Ms *vlhA* gene of different fields isolates
- Sequencing and sequence analysis of the Ms *vlhA* genes of these isolates

1.2.8 Hypothesis

The SSCP and HRMC will reliably discriminate between the different MS isolates obtained from the field and also distinguish them from live vaccine strains.

CHAPTER 2
MATERIALS AND METHODS

2.1 *Mycoplasma synoviae* samples

A total of 26 samples were obtained for this study [i.e. 9 field samples, 1 reference strain, 2 vaccine strains, 1 swab, 2 organs (trachea), and 11 extracted DNA samples] (Table 1). Isolates obtained from field samples (n=9) were obtained from the Bacteriology laboratory of the Department of Veterinary Tropical Diseases (DVTD), Faculty of Veterinary Science (FVS), University of Pretoria (UP). Other field samples included one swab specimen, eleven broth, and two organ (trachea) specimens (all not cultured before) and one reference and two vaccine strains were collected from the Poultry Reference Centre, FVS, UP as controls to validate results (Table 1). Water was included as negative control.

Table 1 Samples of *Mycoplasma synoviae* used in this study

Sample number	Sample reference number
A. Isolates from field samples	
Ms1	B2182/07*
Ms 2	B2182/07*
Ms 3	B2214/07(2023)*
Ms 4	B2214/07(2024)*
Ms 5	B312/08*
Ms 6	B434/08*
Ms 7	B85/09*
B. Swab specimen (field isolates)	
Ms 11	Ms (swab)011688
C. Agar specimens (field isolates)	
Ms 12	MG(poultry trachea "dead"H (B279)-1)
Ms 13	MG(poultry trachea "dead"H (B279)-2)
D. Extracted DNA from Molecular Diagnostic Services (MSD, supplied by Dr. Denis York)	
Ms 14	Ms (ext1)
Ms 15	Ms (ext2)
Ms 16	Ms (ext3)
Ms 17	Ms (ext4)*
Ms 18	Ms (ext5)
Ms 19	Ms (ext6)
Ms 20	Ms (ext7)
Ms 21	Ms (ext8)
Ms 22	Ms (ext17)
Ms 23	Ms (ext18)
Ms 24	Ms (ext19)
E. Organ specimen (field isolates)	
Ms 25	Ms 589/10(H9)
Ms 26	Ms 589/10(H5)
F. Reference and vaccine strains and controls	
Ms 8	Ms, NCTC(10124)*
Ms 9	Ms, ATCC(25204)*
Ms 10	Ms, Vaxsafe*

* 11 samples selected on real-time PCR results for SSCP and sequencing analysis

2.2 Genomic DNA extraction

Different extraction methods were used for the different sample types used in this study:

a. **Swabs** were prepared by adding 1 ml of phosphate buffered saline (PBS) (134 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to the swab and vortexing for 2 min.

b. **Agar samples** were prepared by cutting the block of agar with a DNA/RNA free scalpel blade, and melting it at 96 °C.A volume of 1 ml PBS was added to the melted agar.

c. For the preparation of **organ samples (tracheal)**, 3 small pieces of organs (0.25 x 0.25 x 0.25 cm) were cut, put into 2ml tubes and frozen in liquid nitrogen. The tissues were crushed and a small piece (0.25 x 0.25 x 0.25 cm) was transferred to another 2 ml tube and 1 ml of PBS was added.

d. DNA extraction was completed by using the commercially available (QIAamp® DNA Mini Kit, Whitehead Scientific, South Africa) according to the manufacturer's instructions. To extract DNA a volume of 200 µl of the prepared samples was used. DNA was eluted into 100 µl of AE buffer (QIAamp® DNA Mini Kit, Whitehead Scientific, South Africa) and the concentration was determined by spectrophotometry (Beckman Coulter ™ DU® 530, Beckman Coulter, South Africa) and agarose gel electrophoresis (Sambrook *et al.,* 1989). The DNA concentration of the isolates ranged from 0.8-6.5 ng/µl and the isolates were divided into six different groups according to their DNA concentration.

2.3 Conventional polymerase chain reaction

The conventional PCR primers described by Jeffery *et al.,* (2007) were used in this study. PCR was performed using primers that amplified a 350-400 bp fragment of the *vlhA* gene. The reaction mixture consisted of Platinum Quantitative PCR Supermix-UDG (Invitrogen, Applied Biosystems, South Africa), 20 pM of each primer

[Link (5' - TACTATTAGCAGCTAG TGC-3') and

MSCons-R (5' - AGTAACCGATCCGCTTAAT -3')] (Inqaba Biotech, South Africa) DNA template.

The concentration range of the DNA template was between 20.30-93.01 ng/µl and 2.5 µl purified DNA to a final volume of 25 µl was used. The thermocycler programme was as follows, starting with 2 min at 96 °C; 10 min at 94 °C; and 40 cycles of 96 °C for 15 sec, 54 °C for 15 sec, 72 °C for 20 sec (Perkin Elmer 9600 Thermocycler (Applied Biosystems, South Africa). The PCR amplicons were verified using 1% agarose gel electrophoresis containing ethidium bromide (10 mg / ml) and documented on a Kodac Documentation system (Kodac, New York). PCR was conducted in groups accordingly to DNA concentration (see 2.2).

2.4 Real-time PCR and high resolution melting curve analysis

The26 isolates were subjected to real-time PCR. The LightCycler®V 2,0 (Roche Diagnostic, SA) instrument were used. The total volume of the reaction was 20 µl and consisted of 2 µl Master 9 SYBR Green 1 dye (Roche Diagnostic, South Africa), 0.5 µl forward primer (Link), 0.5 µl reverse primer (MSCons-R) (Fermentas, Inqaba Biotech, South Africa), and 1 *µl* DNA The concentration varied from 21-93 ng/µl to the final volume of 20 µl. The negative control consisted of the complete PCR mixture without the DNA. Real-time PCR was performed and the melting curve analysis (LightCycler®, Roche) was generated with the following programme: Pre-incubation: 96 °C for 2 min, Amplification: 96 °C for 15 sec, 54 °C for 15 sec, 72 °C for 20 sec. Melting curves: 99 °C for 0 sec, 70 °C for 15 sec, 99 °C for 0 sec at 0.2 ramp rate and Cooling: 42 °C for 30 sec.

2.5 Single Stranded Conformation Polymorphism analysis

Single stranded conformation polymorphism was used to analyze PCR products of the *vlhA* gene from the Ms isolates used in the study (Jeffery *et al.,* 2007). A 10% polyacrylamide gel was used for analysis [14 ml of 30% acrylamide/(10%) bisacrylamide, 37.5:1 (2.6% C) (Bio-Rad laboratories, South Africa), 2.1 ml of 10 x Tris-borate/ ethylenediaminetetra-acetate (0.045 M Tris-borate, 0.5 x 0.001 M EDTA, (pH 8.0), 11.5 ml ddH_2O, 14 ml of a freshly made 0.28% ammonium persulphate (APS)(BHD), AnalaR, Merck, South Africa and 33.5 µl N,N,N`,N`-tetramethyla-ethylenendiamin (TEMED) (Merck, South Africa) (0.08%)]. PCR products obtained were loaded on the gel and the gel was subjected to electrophoresis for 16 hours at 72V and 72 °C. Gels were stained for 15 min using ethidium bromide (0.5 µg/ml), destained in water and then photographed using Kodac Documentation system (Kodac, New York).

2.6 Sequencing and nucleotide sequence analysis

From results obtained by the real-time PCR as well as the melting curve analysis, 11 samples (with different Tm) were selected for sequencing (Table 1) to confirm the HRM results. Samples were sequenced by Inqaba Biotechnical industries (Pty) Ltd (Pretoria, South Africa) and analysed at the Department of Veterinary Tropical Diseases. Sequence data for the *vlhA* gene was assembled and edited to a total length of 1.652 bp by using GAP 4 of the Staden package (version 1.6.0 for Windows) (Bonfield *et al.,* 1995, Staden, 1996, Staden *et al.,* 2000). The assembled sequences were aligned with related sequences obtained from GenBank (http://www.ncbi/blast) (FM164367 *Mycoplasma synoviae*st B10307 (UK), FN666085 *Mycoplasma synoviae*st B9895 (Netherlands), AJ580991 strain B15402 (Hungary), AB501271 strain MS-H (Japan) using ClustalX (version 1.81 for Windows). The alignment was manually shortened to the size of the shortest sequence.

The same set of primers used for conventional PCR was used for sequencing of the *vlhA* gene in order to be able to target the different promoters on the *vlhA* gene and to sequence the whole *vlhA* gene (Fermentas, Inqaba Biotech, South Africa).

CHAPTER 3
RESULTS

3.1 Conventional polymerase chain reaction

Genomic DNA was successfully extracted from 26 samples. The concentration was determined by the spectrophotometer reading and ranged from 0.8-6.5 ng/µl. Because of the range in DNA concentration, the conventional and real-time PCR were conducted in groups according to the DNA concentration. The volumes of the DNA used in conventional and real-time PCR for each group were as follows:12.5 µl for Group 1 (isolates 13, 21, 26, 27), 10 µl for Group 2 (isolates 3, 6, 7, 23), 8 µl for Group 3 (isolates 2, 9, 10, 11, 12), 5 µl for Group 4 (isolates 4, 5, 8, 25, 16, 22), 3 µl for Group 5 (isolates 1, 15, 20, 24), and 1 µl for Group 6 (isolates 14, 17, 18, 19).

PCR products of the expected size range (350-400 bp) were generated. Samples with the highest concentration of DNA (1, 2, 3, 5, 6, 7, 10, 11, 12, 16, and 21) showed a clear reaction as indicated by single bands. Isolates with a lower DNA concentration (4, 8, 9, 13, 14, 15, 17, 18, 19, 20, 22, 23, 24, 25 and 26) did not show a visible amplicon on the agarose gel (Figure 3).

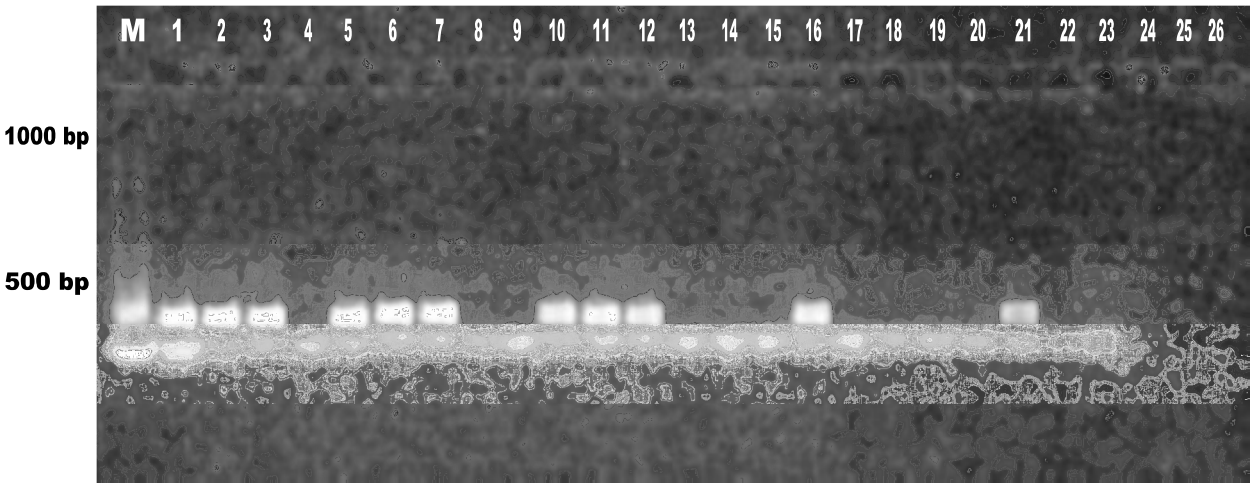

Figure 3 Agarose gel electrophoresis of PCR products of the *vlhA* gene from different Ms isolates. Lane 1 molecular weight marker, Lanes 2-27 isolates and Lane 24, negative control (water).

3.2 Real-time PCR and high resolution melting curve analysis of PCR products

Melting curve analysis of Ms isolates (n=26) indicated the presence of *Mycoplasma synoviae*. Melting peaks between 83.15-83.65 °C (large curves) and 81.42-82.33 °C (smaller curves) were observed (Figure 4). A shoulder peak was observed for the negative control at 75 °C (Figure 4).

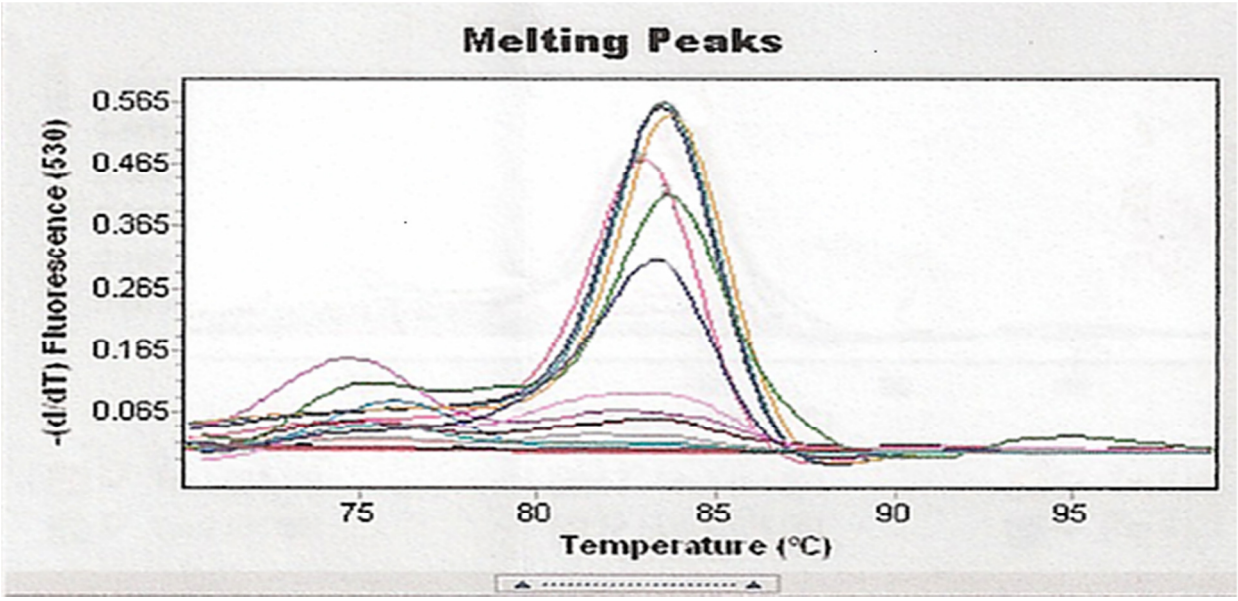

Figure 4 Melting peaks of PCR products at different temperatures.

The 11 selected isolates were re-analysed and the following melting temperatures were observed (Table 2). Melting temperature ranging between 84.24-84.98 °C for isolates Ms2-Ms9, a melting curve at 85.01 °C was observed in the analysis of isolate Ms1 and 85.62 °C in isolate Ms10. Isolate Ms17 showed two melting peaks, one of 83.81 °C and the other one at 88.75 °C. The negative (water) control showed a curve at 76.78 °C (Figure 5).

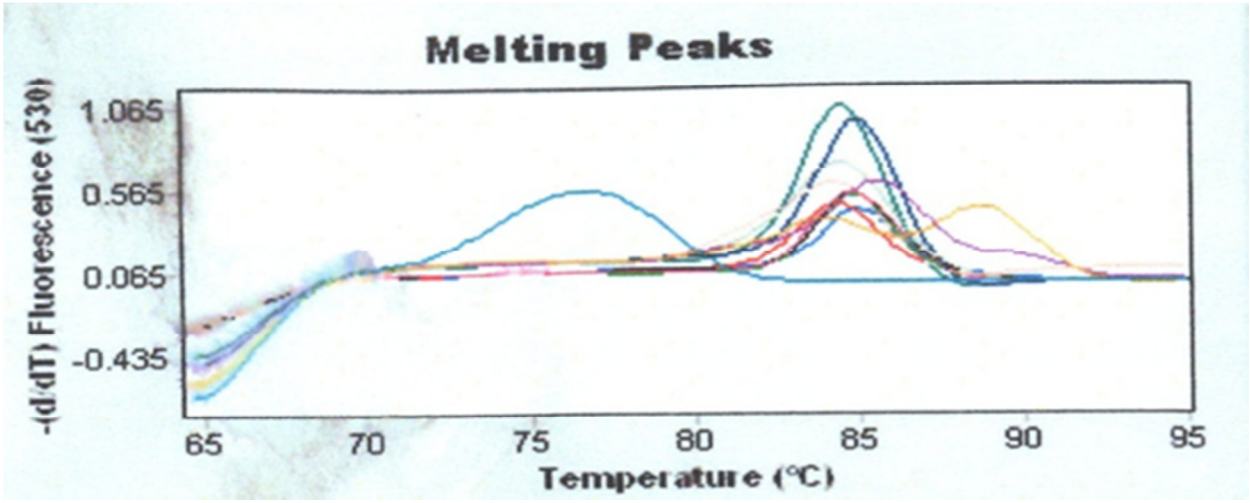

Figure 5 Melting peaks of the 11 isolates.

3.3 Distinct SSCP profiles represented by *Mycoplasma synoviae* isolates

SSCP gel electrophoresis profiles show little or no profile difference between isolates Ms1-Ms7 and only single bands were observed. The single bands (7) range in size of about 300-400 bp, and it appears that none of the bands are the same size. The profiles of Ms8-Ms10 and Ms17 were migrating differently and showed multiple bands, ranging in size of about 300-1300 bp.

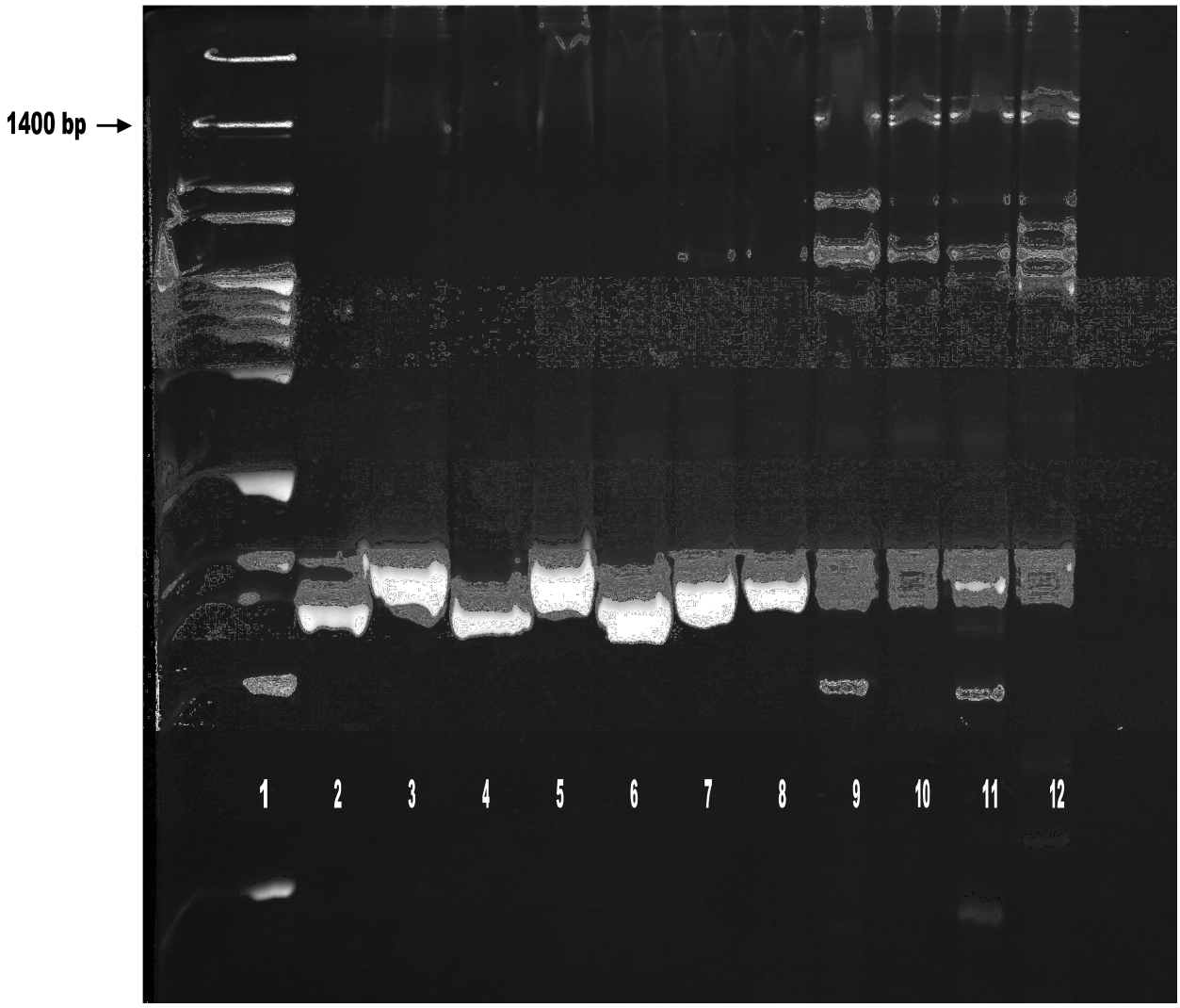

Figure 6 SSCP profiles of the PCR products from 11 eleven different *Mycoplasma synoviae* isolates. Lane 1 is the molecular marker, Lanes 2-8 are samples Ms1-Ms7, Lanes 9-11 are samples Ms8-Ms10; Lane 11is sample Ms17, Lane 12 is the negative control (water).

3.4 Nucleotide sequencing analysis of PCR products of the eleven *Mycoplasma synoviae* isolates

Sample Ms3 was used as a standard sequence to compare to all of the 11 samples as well as the sequences downloaded from GenBank. Sequence differences were observed (within the 11 samples) in the sequence data sets of Ms4, Ms9 and Ms10. The sequencing differences that occur between Ms9 and Ms10 were 100% similar and different to the standard isolate Ms3, the following nucleotide changes were observed: position 53 CT to AA; position 108 C-T; 114 C-T; position 119 G-A; position 147 T-C; position 153 G-A; position 308 A-G; 312 A-C (Figure 7).

The sequence differences observed in Ms4 were: position 108 C-T; 114 G-T; position 119 G-A; position 124 C-T.

No sequencing differences were observed in sequencing analysis from the other isolates (Ms1, 2, 3, 5, 6, 7, 8 and 17) and GenBank sequencing data (Figure 7).

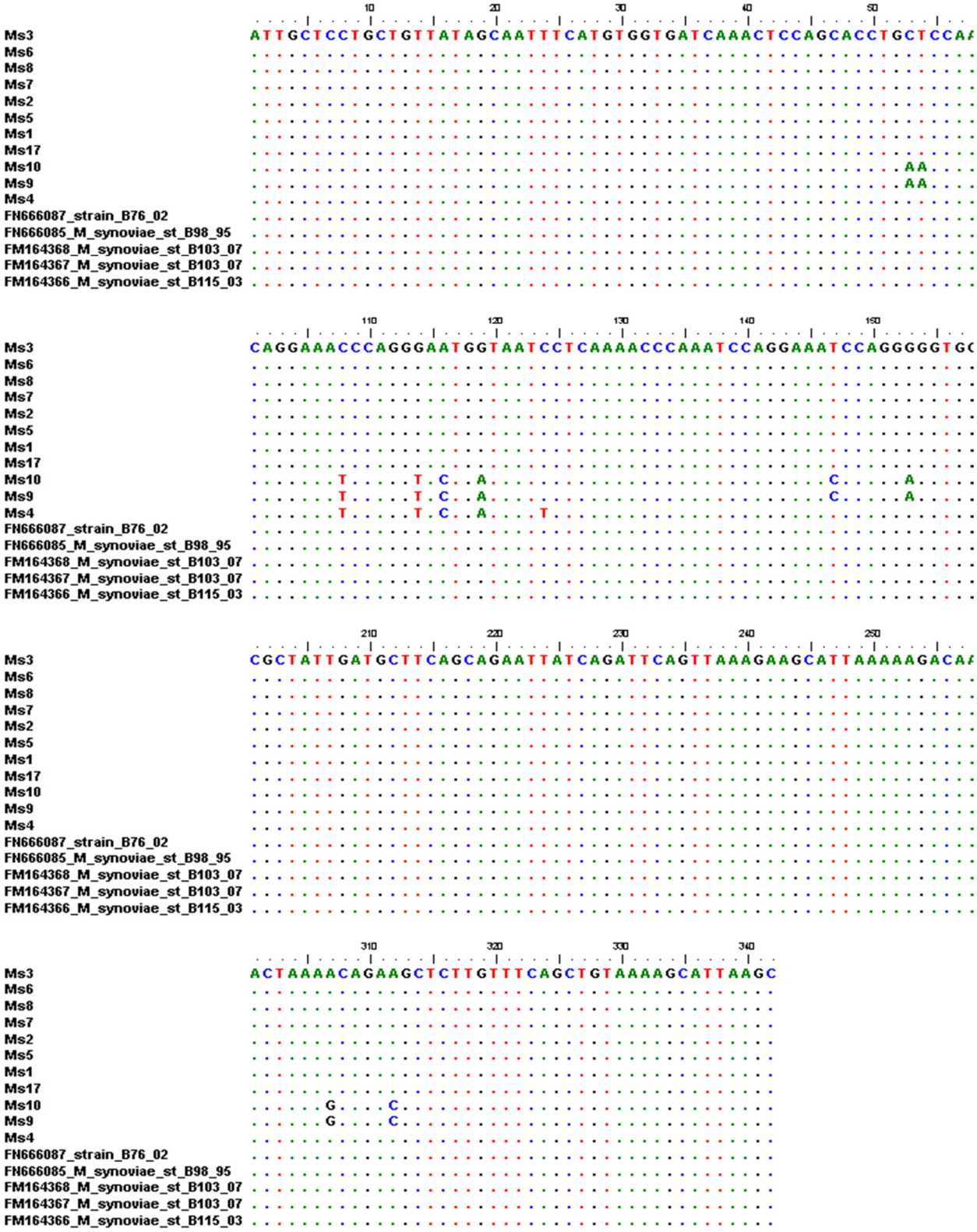

Figure 7 Nucleotide comparison of partial *vlhA* gene sequences amplified from different Ms isolates. Identity differences are shown by dots.

Table 2 Comparison of the Tm, SSCP and sequencing results of the Ms isolates used in this study

Isolates	Tm	Bands	Product size	Nucleotide
Ms1	85.01 °C	1	± 350	0
Ms2	84.88 °C	1	± 400	0
Ms3	84.39 °C	1	± 350	0
Ms4	84.92 °C	1	± 400	5
Ms5	84.91 °C	1	± 350	0
Ms6	84.51 °C	1	± 490	0
Ms7	84.98 °C	1	± 490	0
Ms8	84.56 °C	7	± 300-1300	0
Ms9	84.24 °C	5	± 300-1300	10
Ms10	85.62 °C	5	± 300-1300	10
Ms17	83.81°C	7	± 300-1300	0
Control	**76.78 °C**	**-**	**-**	**0**

CHAPTER 4
DISCUSSION AND CONCLUSION

Mycoplasma synoviae causes a respiratory tract infection in chickens and turkeys worldwide (Kang *et al.,* 2002). It can cause serious econ.omic losses, especially in infected layer flocks which may suffer from decreased egg production (Kang *et al.,* 2002). Vaccination of flocks against Ms complicates the diagnosis of Ms by causing the development of detectable antibodies in the blood and also frequently resulting in positive Ms cultures and PCR results. Most diagnostic techniques cannot distinguish between vaccine strain and natural infection (Kang *et al.,* 2002).

This study was undertaken in an attempt to discriminate between the different Ms isolates obtained from the field and also to distinguish them from the live vaccine strain by SSCP, real-time PCR and HRM curve analysis. Sequencing data were obtained to confirm the above results.

This study provides a direct comparison between SSCP and HRM curve analysis using SYBR Green 1 dye for detection of differences in the *vlhA* gene of Ms. The techniques of SSCP and HRM curve analysis allowed for the detection and discrimination of Ms isolates from field samples, reference strains and vaccine strains. The results showed that both SSCP and HRM curve analysis were capable of detecting variations of a few bp in PCR products of approximately 400 bp (Figure 7). The *vlhA* single-copy gene region targeted for PCR in this study is known to be conserved (Bencina *et al.,* 2001; Noormohammadi *et al.,* 2000, 2002; Hong *et al.,* 2004). Differentiation of field strains causing natural infection and the vaccine strains improves the diagnosis of Ms and therefore reducing economic problems.

Different isolates were collected, i.e. swabs, organs, broth, agar block, extracted DNA from Ms positive isolates, were received. The isolates were prepared differently (depending on the type of isolate) before extraction and the same method of DNA extraction was performed (see 2.2). Different concentrations of DNA per samples were obtained and this could be attributed to the type of isolate and the method used for the preparation of the sample. Different levels of infection of birds, sampled in the same way, also affected DNA concentration between samples, for example samples used in this study; Ms1-Ms7. A comparison between the preparation methods used was not done in this study.

From only some of the 26 samples single DNA bands could be observed when PCR products were subjected to agarose gel electrophoresis (Figure 3). The negative results could have been due to low concentration of DNA as these samples were positive using real-time PCR. The conventional PCR may therefore not be sufficiently sensitive to detect and differentiate strains of Ms. In future, the electrophoresis should be repeated using larger DNA volumes to eliminate the false negative results.

Fan *et al.,* (1995) described Ms by using an arbitrary-primed PCR for detection of strain variation. The results are however difficult to interpret and the approach does not allow determination of whether the profile variation detected relate to genomic rearrangements that commonly occur within single isolates (Fan *et al.,* 1995).

A real-time PCR/HRM was developed for the detection of Ms isolates from field samples (Jeffery *et al.,* 2007). The real-time PCR used one set of primers specific for a short region of the *vlhA* gene of the Ms isolate and the HRM of the PCR product used a high-resolution melt fluorescent dye (Jeffery *et al.,* 2007).

High resolution melting curve analysis discriminates DNA samples based on sequence, length, and GC-content. When there is a mutation in the sequence, it results in a different melting temperature and therefore different melting curves (Jeffery *et al.*, 2007). All HRM curve profiles (Figures 5 and 6) generated from Ms isolates, reference strains and vaccine strains used in this study were found to have a major peak and shoulder peaks (Table 2, sample Ms17) (Figure 6).

Of the 26 isolates subjected to HRM analysis, only 11 isolates showed clear variations in their melting temperature. As already mentioned in the results, the melting temperature of the isolates ranged from 83.81-85.62 °C. There was not much difference in the melting temperature of these isolates and their sequences did not differ much. Isolate 17 showed two peaks, one at 83.81 °C and the other one at 88.75 °C. The peak of 83.81 °C is close to the other isolates and could be the correct one while the other peak could have resulted from contamination with foreign DNA as no differences were observed in the sequencing results of this isolate (Figure 7). The shoulder peak for the negative control could have resulted from contamination, since such a peak was detected only in the negative control (which is the no-template control and contains water) or more likely from primer dimers (Figure 5). To exclude the possibility of contamination the testing could be repeated in future. The melting temperature of 76.78 °C of the shoulder peak from the negative control was lower than the melting peaks of other isolates.

SYBR Green 1 was used for the real-time PCR in this study. The results in the melting curve of the 11 isolates could also have been affected by this dye (Ma *et al*., 2006). Disadvantages of using SYBR Green is the detection of non-specific double-stranded reaction products which then result in increased background or false positives results. Although SYBR Green I intercalate into double-stranded DNA and is less expensive than probes, other dyes are available besides SYBR Green I that offer its benefits along with fewer of its disadvantages e.g. SYTO9 (Ma *et al*., 2006).

According to Tindall *et al*., (2009) HRM analysis is a prescreening method aimed at improving the turn-around time which, compared to gel-based methods, makes this method an ideal diagnostic or clinical tool. High resolution melting curve analysis allows for melt profiles of up to 96 or 384 bp PCR products to be achieved in minutes, compared to approximately 24 hr for most gel based methods and sequencing. Sensitivity, reproducibility, time and technical expertise are important factors to consider prior to embracing these newer technologies (Tindall *et al.,* 2009).

High-resolution melting curve analysis was able to differentiate all Mg strains when studied by Seyed *et al.,* (2010). Analysis of the nucleotide sequences of the amplicons from each strain revealed that each melting curve profile was related to a unique DNA sequence. The results presented in their study indicated that PCR followed by HRM curve analysis provides a rapid and robust technique for genotyping of Mg isolates using both Mg cultures and clinical swabs.

The present study, as compared to the above studies, also proved that HRM curve analysis was able to differentiate between Ms isolates, the reference strain and the vaccine strains. Melting temperature for the reference strain Ms9 was observed at 84.24 °C, for the other field isolates (Ms1, Ms2, Ms3, Ms4, Ms5, Ms6, Ms7, Ms8, Ms17) the melting temperature ranged from 83.81-85.01 °C (lower than the vaccine strain) and 85.62 °C (higher than the reference strain and the field isolates) in vaccine strain Ms10.

The results for SSCP gel electrophoresis profiles show little (only single bands, ranging in size from ± 300-400 bp) or no profile difference between isolates Ms1-Ms7 while the profiles of Ms8-Ms10 and Ms17, were migrating differently (multiple bands, ranging in size from ± 300-1300 bp) (Figure 6). Isolates Ms8 and Ms17 as well as Ms1 and Ms7 have different melting temperatures, although have the same number of bands on SSCP gels, as well as the same nucleotide sequences. Ms3 (field isolate) is used as standard isolate to compare to all the eleven

isolates. When Ms3 is compared to the reference strain Ms8 and Ms9 and the vaccine strain Ms10, their melting temperatures were different by a very small percentage i.e. Ms3 and Ms9 differed by 0.18%, Ms3 and Ms8 by -0.20% and Ms3 and Ms10 by -1.46%. The SCCP band profiles were also different i.e. Ms8 had 7 bands, Ms9 5 bands, Ms10 5 bands and Ms3 1 band.

When compare to sequencing data, the difference in the band patterns in the SSCP gel represents the difference in the nucleotide sequences. Isolates Ms9 and Ms10 did not have much difference in their melting temperature and their band patterns are exactly the same (Figure 6). These isolates could be identical notwithstanding the small difference in melting temperature. The interpretation of results, where very small differences in the nucleic acid sequences occur, should be interpreted with care. Ms3 had different melting temperature, band patterns as well as nucleotide sequences when compared to Ms9 and Ms10. Therefore, this results reveals clear discrimination between the Ms3 (field isolate), Ms9 (reference strain) and Ms10 (vaccine strain).

The reagents of SSCP are relatively inexpensive and the technique is easy to perform and does not require complicated equipment. Two advantages of SSCP are its very high resolving capacity and the bands can be excised for further analysis, e.g. by nucleotide sequencing. The SSCP has the disadvantage of being a time-consuming procedure and requires skill for the interpretation of results. In HRM curve analysis there is no option of excising the band, however, HRM curve analysis is rapid and convenient, and all relevant procedures including real-time PCR and melting-curve analysis can be performed in a single tube. It can be performed in an automated module therefore there is no need for extensive interpretation of results and the results can be observed within two hours. SYBR Green I was used in the current study for HRM curve analysis used with a roto-gene 6000 (Jeffery *et al.,* 2007).

Nucleotide sequencing was used to confirm the HRM curve analysis and SSCP results of the 11 isolates (Figure 7). The results revealed that the isolates were different by a few base pairs. The 350-400 bp PCR product of the *vlhA* gene of the eleven Ms isolates showing differences in the HRM curve peaks and different SSCP profiles (Ms5, Ms3, Ms6, Ms10 and Ms9, Ms1, Ms2, Ms4, Ms8, Ms7 and Ms17) were sequenced. Other sequences of the *vlh*A region of related isolates were retrieved from GenBank and compared to sequences obtained from Ms isolates from this study.

The sequences of most of the isolates are different in just a few base pairs (e.g. Ms4, Ms9 and Ms10). Differences between nucleotides sequences ranges from one base pair to almost 10 bp.

The lowest level of nucleotide variation detectable by HRM curve analysis and SSCP was five nucleotides between the strains.

The results revealed that sequence differences were observed in the sequence data sets of Ms4, Ms9 and Ms10. The *vlhA* sequences of Ms9 and Ms10 were identical and differed from the consensus sequence at the following positions: position 53 CT-AA; position 108 C-T; position 114 C-T; position 119 G-A; position 147 T-C; position 153 G-A; position 308 A-G; position 312 A-C. Isolate Ms10 is the vaccine strain (MS Vaxsafe) and Ms9 is a reference strain (Ms, ATCC (25204). They were both used as positive controls in the experiment and were both similar in their SSCP results. Their melting temperature differed by 1.38 °C. The results for sequencing in these cases confirm the SSCP results as these sequences were also 100% similar on the gel, with five bands (Figure 6).

The sequence differences observed in Ms4 were: position 108 C-T; 114 C-T; position 119 G-A; position 124 C-T. The nucleotide substitution is C-T which would not have much effect on the melting temperature of the sequence. No sequence differences were observed between sequences of the other isolates (Ms1, 2, 3, 5, 6, 7, 8 and 17) and sequence data obtained from GenBank. The melting temperature of each isolate differ by less than 2 °C, this was accepted as closely similar and the SSCP bands of isolates Ms1, 2, 3, 5, 6, 7, and 8, except Ms17, had almost similar band profiles which was considered as insignificant (Figure 7).

Currently, direct sequencing is widely used for detection of mutations, but the cost may be prohibitive and turnaround time is relatively long as compared to HRM (Jeffery *et al.,* 2007). Ishikawa *et al.,* (2010) developed a HRM assay and evaluated its effectiveness for screening for mutations in a library and compared their results with direct sequencing. Their results indicated that the HRM assay is as effective as direct sequencing (Ishikawa *et al.,* 2010).

A study by Jeffery *et al.,* (2007) also uses real-time PCR followed by melting-curve and SSCP analysis for detection and strain classification of Ms isolates. They were able to detect and distinguish between Ms isolates. This confirms the results obtained by this study.

Another approach for detection and discrimination between Ms isolates is PCR followed by sequencing of the amplified product described by Hong *et al.,* (2004). This approach is however considerably more time-consuming than HRM and requires more interpretation of results. SSCP

and nucleotide sequencing are also time-consuming and the analysis of the results is complicated and difficult to reproduce between different laboratories.

In this study, Real-time PCR, HRM curve analysis and SCCP techniques were able to discriminate between the field isolates strains, the reference strain and the vaccine strain. The results were confirmed by sequencing which also shows different number of nucleotides between the field isolates, the reference strain and the vaccine strain. Real-time PCR and HRM curve analysis were more effective compared to SCCP as indicated by significant differences in melting temperatures. In previous work by Jeffery *et al.,* (2007) real-time PCR and HRM was able to discriminate between all the Australian vaccine/field isolates and the overseas (USA) strains examined in their study. Their results confirms that HRM curve analysis provide effective tools for further study of the epidemiology and spread of Ms strains in chickens in South Africa.

REFFERENCES

Amy NZ, Kristina ML, Ziv R. (2010). Revised *Mycoplasma synoviae vlhA* PCRs. *Avian diseases.* 54: 1292-1297.

Anonymous. (2008). Avian mycoplasmosis (*Mycoplasma gallisepticum, Mycoplasma synoviae*), in *OIE Terrestrial Manual*, Chapter 2.3.5. Pages: 482-496.

Barua SR, Prodhan AM, Islam S, Chowdhury S. (2006). Study on *Mycoplasma gallisepticum* in chickens in selected areas of Bangladesh, Short communication. Chittagong Veterinary and Animal Sciences University, Pahartali, Chittagong, *Bangladesh Journal of Veterinary Medicine*. 4 (2): 141-142.

Bencina D, Drobnic-Valic M, Horvat S, Narat M, Kleven SH, Dovc P. (2001). Molecular basis of the length variation in the N terminal part of *Mycoplasma synoviae* haemagglutinin. *FEMS Microbiology Letter*. 203: 115-123.

Bencina D. (2002). Haemagglutinins of pathogenic avian mycoplasmas. *Avian Pathology.* 31: 535-547.

Bonfield JK, Smith KF, Staden R. (1995). A new DNA sequence assembly program. *Nucleic Acids Research.* 23: 4992-4999.

Browning GF, Whithear KG, Noormohammadi AH, Markham PF. (2000). Improving, Diagnosis of *Mycoplasma synoviae. Rural Industries Research and Development Corporation. Publication number: 00/078, Project number: UM-33J.*

Clyde WA. JR. (1983). Growth inhibition tests. *In:* Methods in Mycoplasmology, Vol. 1, Razin S. & Tully J.G., editors *Academic Press, New York, USA and London, UK.* Pages 405-410.

Coleman WB, Tsongalis GT. (2006). Molecular diagnostics for the clinical laboratories. 2nd edition, Human Press, Totowa, New Jersey. Pages 65-68 and 90-93.

Czifra G, Tuboly T, Sundquist BG, Stipkovits L. (1993). Monoclonal antibodies to *Mycoplasma gallisepticum* membrane proteins. *Avian Diseases*. 37 (3): 689-696.

Delaplane JP, Stuart HO. (1943). The propagation of a virus in embryonated chicken eggs causing a chronic respiratory disease of chickens. *Journal of the American Veterinary Medical Association* Res. 4: 325-332.

Dickson EM, Hinshaw WR. (1938). Treatment of infectious sinusitis of turkeys with argyrols and silver nitrate. *Journal of the American Veterinary Medical Association.* 93*:* 151-156.

Fan HH, Kleven SH, Jackwood MW. (1995). Studies of intraspecies heterogeneity of *Mycoplasma synoviae, M. meleagridis,* and *M. iowae* with arbitrarily primed polymerase chain reaction. *Avian Diseases*. 39: 766-777.

Gasser RB, Hu M, Chilton NB, Campbell BE, Jex AJ, Otranto D, Cafarchia C, Beveridge I, Zhu X. (2007). Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nature Protocols.* 1: 3121-3128.

Garcia M, Ikuta N, Levisohn S, Kleven SH. (2005). Evaluation and comparison of PCR methods for the detection of *Mycoplasma gallisepticum* infection in chickens. *Avian diseases*. 49: 152-132.

Hong Y, Garcia M, Leiting V, Bencina D, Dufour-Zavala L, Zavala, Kleven SH. (2004). Specific detection and typing of *Mycoplasma synoviae* strains in poultry with PCR and DNA sequence analysis targeting the haemagglutinin encoding gene *vlhA*. *Avian Diseases.* 48: 606-616.

Ishikawa T, Kamei Y, Otozai S, Kim J, Sato A, Kuwahara Y, Tanaka M, Deguchi T, Inohara H, Tsujimura T, Todo T. (2010). High-resolution melting curve analysis for rapid detection of mutations in a Medaka TILLING library. *Biomed Central Molecular Biology.* 11(70): 1-17.

Jeffery N, Gasser RB, Streer PA, Noormohammadi AH. (2007). Classification of *Mycoplasma synoviae* strains using single stranded conformation polymorphism and high-resolution melting curve analysis of the *vlhA* gene single copy region. *Microbiology*. 153: 2679-2688.

Kang MS, Gadzinski P, Kleven SH. (2002). Virulence of recent isolates of *Mycoplasma synoviae* in turkeys. *Avian Diseases*. 46:102-110.

Kempf I. (1998). DNA amplification methods for diagnosis and epidermiological investigations of avian mycoplasmosis. *Avian Pathology*. 27: 7-14.

Kahn CM, Line S. (2011). The Merck Veterinary Manual, 10th Edition. Whitehouse Station, N.J.: Merck & Company, Incorporation. Pages 2185-2336.

Kiss I, Matiz K, Kaszanyitzky E, Chavez Y, Johansson KE. (1997). Detection and identification of avian *Mycoplasma* by polymerase chain reaction and restriction fragment length polymorphism assay. *Veterinary Microbiology*. 58(1): 23-30.

Kleven SH. (1997). *Mycoplasma synoviae* infection. In: Calneck BW, Barnes HJ, Beard CW, MacDougald LR and Saif YM, *Diseases of Poultry*. *Iowa State University Press*, Ames. 9th edition. Pages 220-228.

Kleven SH. (2003). Recent developments in diagnosis and control. *In* World Poultry. *Mycoplasma* Special. Pages 8-9.

Kleven SH, Yoder HW (Jr.). (1989). Mycoplasmosis. In: A laboratory manual for the isolation and identification of avian pathogens. 3rd Edition. *American Association of Avian Pathologists*. Pages 57-62.

Kotani H, McGarrity GJ. (1985). Rapid and simple identification of Mycoplasmas by immunobinding. *Journal of Immunology Methods*. 85: 257-267.

Lauerman LH, Hoerr FJ, Sharpton AR, Shah SM, Van Senten VL. (1993). Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. *Avian Diseases*. 37: 829-834.

Lockaby SB, Hoerr FJ, Lauerman LH, Smith BF, Samoylov AM, Toivio-Kinnucan MA, Kleven SH. (1999). Factors associated with virulence of *Mycoplasma synoviae*. *Avian Diseases*. 43: 251-261.

Markham PF, Glew MD, Sykes JE, Bowden TR, Pollocks TD, Browning GF, Withear KG, Walker ID. (1994). The organization of the multigene family which encodes the major cell surface protein pMGA, of *Mycoplasma gallisepticum*. *FEMS Letter*. 352: 347-352.

Markham FS, Wong SC. (1952). Pleuropneumonia-like organisms in the etiology of turkey sinusitis and chronic respiratory disease of chickens. *Poultry Science*. 31: 902-904.

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

Ma H, Shei KJ, Chen G, QiaoXt, Chuang MY. (2006). Application of Real-time Polymerase Chain Reaction (RT-PCR). *The Journal of American Science*. 2(3): 1-15.

McMullin P. (2004). A Pocket Guide to Poultry Health and Disease. Sheffield, United Kingdom: 5M Enterprises Limited. Page 123.

Mohammed HO, Capener TE, Yamamoto R, McMartin DA. (1986). Prevalence of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in commercial layers in southern and central California. *Avian Diseases.* 30: 519-526.

Morrow CJ, Whithear KG, Kleven SH. (1990). Restriction endonuclease analysis of *Mycoplasma synoviae* strains. *Avian Diseases*. 34: 611-616.

Nelson JB. (1939). Growth of the fowl coryza bodies in tissue culture and in blood agar. *Journal of Experimental Medicine*. 69: 199-209.

Noormohammadi AH, Markham PF, Whithear KG, Walker ID, Gurevich VA, Ley DH, Browning GF. (1997). Mycoplasma *synoviae* has two distinct phase-variable major membrane antigens, one of which is a putative haemagglutinin. *Journal of infection and Immunology.* 65: 2542-2547.

Noormohammadi AH, Markham PF, Kanci A, Whithear KG, Browning GF. (2000). A novel mechanism for control of antigenic variation in the haemagglutinin gene family of *Mycoplasma synoviae*. *Molecular Microbiology*. 35: 911-923.

Noormohammadi AH, Browning GF, Jones J, Whithear KG. (2002). Improved detection of antibodies to *Mycoplasma synoviae* vaccine MS-H using an autologous recombinant MSPB enzyme-linked immunosorbent assay. *Avian Pathology.* 31: 611- 617.

Noormohammadi AH, Markham PF, Du¡y MF, Whithear KG, Browning GF. (1998). Multigene families encoding the major haemagglutinins in phylogenetically distinct Mycoplasmas. *Infectious Immunology*. 66: 3470-3475.

Olson NO, Bletner JK, Shelton DC, Munro DA, Anderson GC. (1954). Enlarged joint condition in poultry caused by an infectious agent. *Poultry Science*. 33: 1075.

Razin S. (1985). Mycoplasma adherence. In: The mycoplasmas. Vol IV. *Mycoplasma Pathogenicity*. S Rasin and MF Barile, editors, Academic Press, Orlando, FL. Pages 161-202.

Razin S, Yogev D, Naot Y. (1998). Molecular biology and pathogenecity of mycoplasmas. *Microbiology and Molecular Biology.* 62: 1094-1156.

Rizzo JM, Buck MJ. (2012). Key principles and clinical applications of "next-generation" DNA sequencing. *Department of Biochemistry and Center of Excellence in Bioinformatics and Life Sciences, State University of New York at Buffalo*, 701 Elicott St., Buffalo, NY 14203, United State of America. mjbuck@buffalo.edu. Pages 887-900.

Rottem S. (2003). Interaction of mycoplasmas with host cells. *Physiology Revision.* 83: 417-423.

Rosendal S, Black FT. (1972). Direct and indirect immunoflurescence of unfixed and fixed mycoplasma colonies. *Acta Pathologica et Microbiologica Scandanivica, Section B. Microbiology and Immunology*. 80(4): 615-622.

Sambrook J, Fritsch EF, Maniatis T. (1989). *Gel electrophoresis of DNA*. In: Molecular cloning a laboratory manual, 2nd edition, Cold Spring Harbor Laboratory Press, New York. Pages 6.3-6.17.

Seyed AG, Noormohammadi AH, Philip FM. (2010). Differentiation of *Mycoplasma gallisepticum* strains using PCR and high-resolution melting curve analysis. *Microbiology*. 156: 1019-1029.

Staden R. (1996). The Staden sequence analysis package. *Molecular Biotechnology*. 5: 233-241.

Staden R, Beal KF, Bonfield JK. (2000). The Staden package, 1998. *Methods Molecular Biology*. 132: 115-130.

Tindall EA, Petersen DC, Woodbridge P, Schipany K, Hayes VM. (2009). Assessing High-Resolution Melt Curve analysis for accurate detection of gene variants in complex DNA fragments. *Human Mutation*. 30(6): 876-83.

Van Roekel H, Olesuik OM. (1953). The etiology of chronic respiratory disease in poultry. *Annual Meeting of American Veterinary Medical Association.* 90th Proceedings. Pages 289-303.

Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. (2003). High-resolution genotyping by amplicon melting analysis using LC Green. *Clinical Chemistry*. 49: 853-860.