*Pourbakhsh / Archives of Razi Institute, Vol. 69, No. 1, June (2014) 1-14*

DOI: 10.7508/ari.2014.01.001

***Review Article***

# Molecular characterization of *Mycoplasma synoviae* isolates from commercial chickens in Iran

**Pourbakhsh*, S.A.**

*Mycoplasma Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran*

Received 20 Oct 2013; accepted 05 May 2014

**ABSTRACT**

Detection of *Mycoplasma synoviae* (MS) by culture and polymerase chain reaction (PCR) has been reported from commercial chicken farms in different provinces of Iran. In some reports the phylogenetic analysis of MS isolates based on 16S rRNA and variable lipoprotein hemagglutinin (vlhA) genes have been carried out. The PCR product containing partial 16S rRNA genes of Iranain isolates was sequenced, and compared with 16S rRNA gene of MS sequences which were available in GenBank. Variations, polymorphisms, and differences between nucleotides of all isolates were observed. Phylogenetic analysis of these sequences showed that all MS isolates from Iran were most closely related to sequences of MS from Brazil. Sequence analysis of the N-terminal end of the hemagglutinin encoding gene vlhA were also used as an alternative for the detection and initial typing of field strains of MS in commercial poultry. The results showed that there was a complete concordance between all Iranian isolates nucleotide sequence and the 5́-vlhA region sequence remained unchanged in all MS isolates and demonstrated differentiation between Iranian isolates and live commercial MS-H vaccine strain. More recently, the single-copy domain of the conserved region of vlhA gene in MS was sequenced, analyzed and verified to type MS field isolates in Iran and live vaccine MS-H strain. In addition, a restriction fragment length polymorphism (RFLP) method was established based on single nucleotide polymorphism that existed in all field isolates of Iran to differentiate between these field isolates and MS-H. This PCR-RFLP method allowed differentiating all MS field isolates from the vaccine strain.

***Keywords:*** *Mycoplasma synoviae, 16S* rRNA sequences, vlhA gene, Phylogenetic analysis, RFLP, commercial chickens, Iran

## INTRODUCTION

Avian mycoplasmas occur in a variety of bird species. Avian mycoplasmosis is caused by several pathogenic mycoplasmas. The most important mycoplasmas for chickens and turkeys are *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Mycoplasma meleagridis*. Besides, *Mycoplasma iowe* is an emerging pathogen in turkeys, but of little concern for chickens. Among these pathogenic avian mycoplasmas, MG and MS are the most important and they are the only ones listed by OIE. MS is recognized as an important pathogen of domestic poultry worldwide, which causes great economic losses in the intensive poultry industry (Kleven *et al* 2008) and retarded growth in chickens and turkeys (Khiari *et al* 2010). Although respiratory infections with MS are generally considered to be subclinical (Van Eck *et al*

*Author for correspondence.Email: a.pourbakhsh@rvsri.ac.ir

1980), an increasing number of reports have documented economic losses attributable to respiratory infections (Morrow *et al* 1990, Lockaby *et al* 1998, Kang *et al* 2002) and arthropathic strains (Landman & Feberwee 2001, van Beek *et al* 2002, Kleven *et al* 2003). However, respiratory diseases can occur as a result of a synergistic interaction between MS and other pathogens, and systemic infection typically leads to infectious synovitis and airsacculitis. MS may be transmitted either laterally via direct contact or vertically via eggs (Harada *et al* 2009). The eggshell pathology and the concomitant egg production losses that result from it further highlight the economic significance of MS in commercial poultry. (Feberwee *et al* 2009). As the vertical transmission plays a major role in spread of MS in chickens the most effective method of control is the regular flocks monitoring and eliminating of positive breeder flocks (Kleven *et al* 2003). In Iran, a live commercial vaccine has been increasingly used in MS infection control programs of breeder farms. In countries that poultry flocks are vaccinated with the live MS strain (MS-H), differentiation of vaccine and field strains are of critical importance. The aim of this article was molecular characterization and differentiation of MS isolates from commercial chickens in Iran and comparison of these isolates with the vaccine strain.

**History.** The microorganisms of the class Mollicutes (Mycoplasma) were first identified in 1898 as the etiologic agent of the bovine contagious pleuropneumonia (BCPP) and thereafter, all similar agents were named pleuropneumonia-like (PPLO-like) organisms. Avian mycoplasmosis was primarily described in turkeys in 1926 and in chickens in 1936. Delaplane & Stuart (1943) referred to it as chronic respiratory disease (CRD) of poultry. Markham & Wong (1952) associated the etiologic agent of CRD to the pathogen responsible for the infectious sinusitis of turkeys. It was then considered as a member of the PPLO group and later named as MG (Nascimento *et al* 2005). Infectious synovitis caused by MS was described thereafter (Olson *et al* 1956, Kleven 1997). The first reports of MS infection with arthritic involvement date from the decades of 50 and 60 in broiler flocks, but it was only in the 70´s that the respiratory disease caused by MS was described (Nascimento *et al* 2005).

**Etiology.** Mycoplasmas are members of the class Mollicutes, Order I, Mycoplasmatales. Genus I, Mycoplasma, has more than 120 species; a DNA G+C content of 23-40%, a genome size of 600-1350 kb, require cholesterol for growth, occur in humans and animals, and have a usual optimum growth temperature of 37°C. Genus II, Ureaplasma is differentiated on the basis of hydrolysis of urea. Acholeplasmas are classified in Order III, Acholeplasmatales, family I, Acholeplasmataceae, genus I, Acholeplasma. They are characterized by lack of a growth requirement for cholesterol (Razin *et al* 1998). Phylogenetic analysis of the 16S ribosomal RNA gene has been used to analyze genetic relationships among mycoplasmas (Weisburg *et al* 1989). Mycoplasma is a trivial designation to the prokaryotes belonging to the class Mollicutes (mollis=soft and cutes=skin), i.e., bacteria that lack cell wall, which make them resistant to antimicrobials that act on this cell structure, such as penicillin. They have been considered extracellular agents, and can be considered as typical surface parasites, but scientists nowadays admit that some of them are obligatory intracellular parasites, whereas all other mycoplasmas are facultative intracellular organisms (Razin *et al* 1998). More detailed information on mycoplasma taxonomy and pathogenicity can be found elsewhere (Yamamoto 1990, Razin *et al* 1998). Mycoplasmas are very small prokaryotes devoid of cell walls, bounded by a plasma membrane only (Figure 1). This accounts for the "fried egg" type of colony morphology, resistance to antibiotics that affect cell wall synthesis, and complex nutritional requirements (Razin *et al* 1998). Mycoplasmas tend to be quite host-specific; some infect only a single species of animal, while others may have the ability to infect several different host species. They are found in humans, many animal species, plants, and insects. In general, mycoplasmas

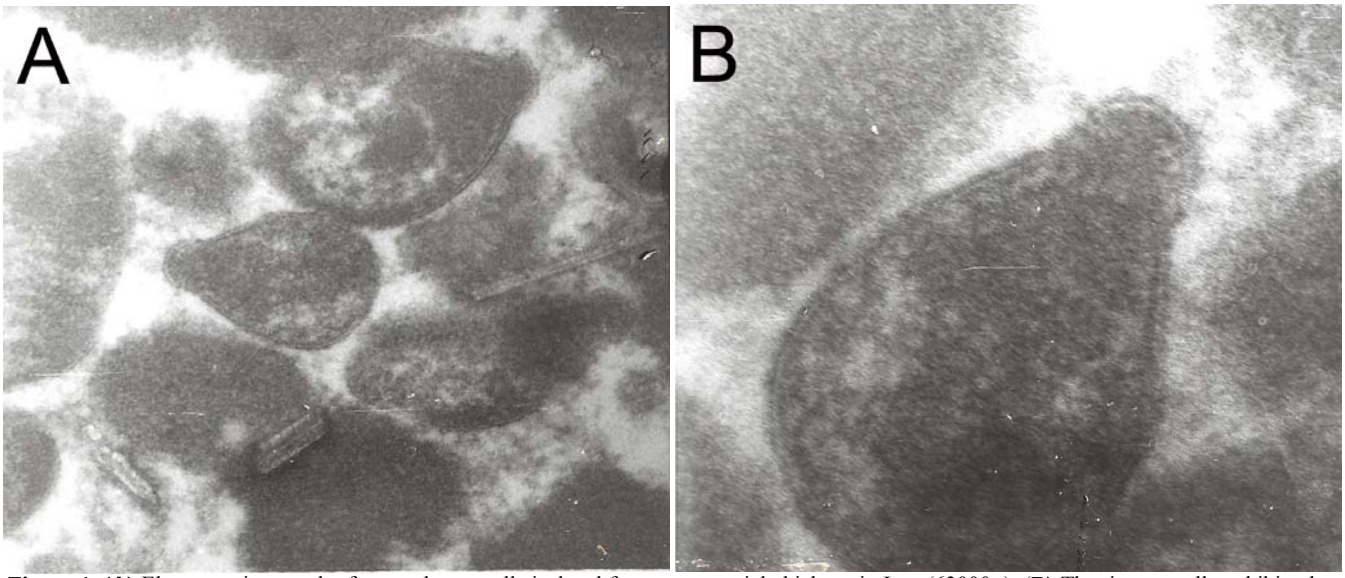


**Figure 1. (A)** Electron micrograph of mycoplasma cells isolated from commercial chickens in Iran (63000×). **(B)** The tip organelle exhibits the rod structure surrounded by a lucent space enclosed by the mycoplasma membrane (180000×).

colonize mucosal surfaces and most species are noninvasive. Some species, including *Mycoplasma gallisepticum* (Weisburg *et al* 1989), are now known to have the ability to penetrate cells. The mycoplasmas are distinguished from other prokaryotes by the total lack of a cell wall and by their minute dimensions. In fact, they can be considered as the smallest self-replicating organisms. Some mycoplasmas, like *M. genitalium*, have a genome of about 600 kb, estimated to comprise less than 500 genes (Razin 1985). These organisms are, therefore, attractive candidates for genome sequencing, an initial step already taken towards achieving the ambitious goal of complete definition in molecular terms of the machinery of a self replicating organism (Morowitz *et al* 1984). *M. genitalium* carries the smallest genome known so far for a self-replicating organism, a genome of only 580 kb. Craig Venter and his associates have found that the minimal set of genes essential for life of *M. genitalium* consists of 381 genes (Razin *et al* 1998).

**Isolation.** Mycoplasma species from avian sources generally require a protein-rich medium containing 10-15% added animal serum. Further supplementation with some yeast-derived component is often beneficial. Growth of MS requires the addition of nicotinamide adenine dinucleotide (NAD). A medium described by Frey (Frey *et al* 1968) or a medium described by Bradbury (Bradbury *et al* 1977) is commonly used for the cultivation of avian mycoplasmas. Mycoplasma organisms tend to grow rather slowly, usually prefer 37-38 °C, and are rather resistant to thallium acetate and penicillin, which are frequently employed in media to retard growth of contaminant bacteria and fungi. Colonies form on agar media after 3-10 days at 37 °C; however. Nonpathogenic species such as *M. gallinarum* and *M. gallinaceum* may develop colonies within 1 day (*M. gallinarum* and *M. gallinaceum* are frequently isolated as contaminants during attempts to isolate pathogenic avian mycoplasmas). Typical colonies are small (0.1-1.0 mm), smooth, circular, and somewhat flat with a denser central elevation. Variations in colony morphology have been described, but cannot be relied upon to differentiate the various species. Individual cells vary from 0.2 to 0.5 µm and are basically coccoid to coccobacillifonn, but slender rods, filaments, and ring forms have been described. Fermentation of carbohydrates is variable, but all species may be divided into those that ferment glucose with acid production and those that do not. Glucose is frequently added to broth media to enhance growth of

the carbohydrate-fermenting species and to provide an indication of growth when glucose fermentation produces acid in media containing added phenol red. Phosphatase activity is often present, as is arginine decarboxylase. Most species that do not ferment glucose use the amino acid arginine as their major source of energy. *M. iowae* and some other species, however, ferment glucose and hydrolyze arginine. One useful characteristic of MG, *M. meleagridis* and MS is hemagglutination of erythrocytes from chickens or turkeys. Hemagglutinating antigens are used for hemagglutination-inhibition serologic tests for these three pathogenic species.

**Vaccination.** To reduce the impact of MS on the poultry industry, a live commercially vaccine (Vaxsafe MSH, Bioproperties Ltd., Ringwood, Victoria, Australia) was developed in Australia. The temperature-sensitive MS-H vaccine strain was produced by mutagenizing an Australian field isolate with N-methyl-N9-nitro-N-nitrosoguanidine (Morrow *et al* 1998). Its safety and efficacy have been established under laboratory (Markham *et al* 1998a, Markham *et al* 1998b) and field (Markham *et al* 1998c) conditions. Vaccine doses of $4.8\times10^5$ccu/ml were protective (Jones *et al* 2006a), protective immunity was detected after 3–4 weeks post vaccination (Jones *et al* 2006b) and persisted for at least 40 weeks (Jones *et al* 2006c). The vaccine has been shown to be effective in reducing apical egg shell abnormalities caused by MS infection. Factors other than the temperature-sensitive phenotype appear to be involved in the attenuation of the MS-H vaccine strain (Noormohammadi *et al* 2003). This vaccine has received wide use in Australia and several other countries, but it is not licensed in many countries, including the United States. The safety and efficacy of MS-H vaccine also have been assessed in turkeys (Noormohammadi *et al* 2007). Markham et al. (1998) showed that after vaccination under field conditions, a small number of the vaccine strains reverted to non–temperature-sensitive forms, hence more effective tools to discriminate vaccine from field strains are needed.

**Serologic monitoring.** Serologic monitoring of MS infection in chicken breeder farms of Iran has been carried out routinely and regularly since 1986. Although serological screening is still in widespread use but may not detect subclinical MS infections, and monitoring programmes that depend solely on detecting seroconversion may be inadequate and nonspecific (Ewing *et al* 1998, Kleven *et al* 2001). Traditionally, MS has been diagnosed by serological screening or by culture method followed by identification of the Mycoplasma. Serological screening is relatively inexpensive but is not always sufficiently specific or sensitive while culture can be insensitive and time consuming (Kleven *et al* 2003).

**Culture and PCR techniques.** As the vertical transmission plays a major role in spread of MS in chickens the most effective method of control is regular flock monitoring and eliminating of positive breeder flocks. Reliable and rapid diagnosis is needed to prevent dissemination of infection (Lockaby *et al* 1998; Kleven 2003). In Iran, for flock monitoring rapid serum agglutination (RSA) is used as screening test. MS strains can vary in antigenic make up and have the ability to alter the expression of major surface antigenic proteins affects the sensitivity and specificity of serologic monitoring systems (Adair *et al* 1990, Avakian *et al* 1990, Noormohammadi *et al* 1997). Various serological, bacteriological and molecular methods are used to confirm reactors detected by RSA. Some PCRs are based on the 16S rRNA gene (Garcia *et al* 1995, Lauerman *et al* 1993), some are in-house tests (Lauerman, 1998), and others are commercially produced kits. Sample collection is of importance because MS tend to disappear from tissues of infected birds after a few weeks. MS is a fastidious organism that requires medium enriched with many factors. Some factors especially yeast extract and serum may vary in their ability to support growth. MS is not stable at pH 6.8 or lower and it is sensitive to temperatures greater than 39ºC and dehydration. During transportation, chilling of specimens should be considered because MS die

rapidly at room temperature. Additions of more antibiotics are ineffective in controlling bacterial contamination and in most cases filtration is needed. Cultures are incubated for more than a few hours, after the phenol red indicator has changed to yellow (pH<6.8), may no longer be viable. Incubation of agar should be placed in a closed container to prevent dehydration of agars (Kleven *et al* 1998, 2003). Culture of Mycoplasma is laborious and can take 3–4 weeks, and even then, the result can be negative or be hampered by mixed infections (Zain *et al* 1995, Bradbury *et al* 1977). The attempts to eradicate the MG infection in Iran poultry farms have been commenced earlier than MS. Furthermore the culture of MS is more difficult than MG, so the reports of isolation and molecular detection of MG are more than MS. There are only four reports about isolation and molecular identification of MS from different provinces of Iran (Ghaleh Golab Behbahan *et al* 2005, Pourbakhsh *et al* 2010, Haghbin Nazarpak *et al* 2010, Bayatzadeh *et al* 2011). The PCR method of Garcia et al. (1995) used by Galeh Golab Behbahan et al. (2005) selectively amplify a 780 base pair DNA fragment within the 16S rRNA gene of MG, MS and *M.iowae* and for specific detection of MS another method ( RFLP) is needed. But the PCR used in other studies of Iran (Pourbakhsh *et al* 2010, Haghbin Nazarpak *et al* 2010) is species specific (Lauerman *et al* 1993) and has been used in recent years by other workers successfully (Buim *et al* 2010). This specific PCR amplifies a 207 bp region of the 16S rRNA gene of MS. Ghaleh Golab Behbahan et al. (2005) in Fars province identified 100 Mycoplasma isolates by PCR and RFLP and among them they pointed to 4 isolates of MS obtained by culture. The media that they used for MS culture were described previously by Frey et al. (1968). They did not explain the details of MS culture more. In 2010, Haghbin Nazarpak et al. isolated MS in broiler breeder farms by culture and PCR techniques. They have studied 30 specimens which obtained from flocks with positive serum reactions by culture and PCR techniques. After collecting of choanal cleft and tracheal swabs in PPLO broth, a short-term incubation was occurred and then filtered a serial passage was performed. Along the fourth passage, all of the specimens cultured on PPLO agar, in addition, original specimens used to extract of DNA and MS-PCR technique. They have shown that 15/30 was positive by culture (approved by of standard MS specific antiserum) and 25/30 was positive by PCR. These observations highlight the higher sensitivity of PCR rather than culture. These results may be observed because of PCR ability to detect DNA after mycoplasma´s death. However in order to achieve a high level of positive results in culture the proper swabbing and handling of specimens for prevention of harmful effects on MS and preparing the suitable environment for growth of MS are needed (Haghbin Nazarpak *et al* 2010). Pourbakhsh et al. (2010) have used the MS specific PCR and culture methods in order to detect of MS from breeder farms where located in Tehran province. A total of 475 samples including choanal cleft, trachea, ovary and /or joint cavities from 23 broiler breeder farms of Tehran area were collected. Samples were cultured in PPLO broth media supplemented for MS isolation. The bacteria DNAs were extracted by phenol/chloroform method. Out of 475 samples, 146 cultures were shown positive and typical Mycoplasma colonies, 85 samples were also identified MS based on agglutination test with specific MS antiserum and the PCR method. In addition to these 85 samples that were positives in both culture and PCR, 37 samples that had not grown in Mycoplasma media were positive in MS specific PCR. A total of 292 samples were negatives in both culture and PCR methods. 122 positive samples out of 475 samples (25.7%) were belonged to 7 breeder farms (30.4%) (Pourbakhsh *et al* 2010). Culture method can be costly and time-consuming, and can also be inconclusive because of low sensitivity (Ewing *et al* 1998). But culture should not be ignored because it is needed for some research projects and even for diagnosis aims, for example without enrichment culture in order to prepare the PCR samples, many false negative PCR results

might occur (Ben Abdelmoumen Mardassi *et al* 2005). Other studies modifications of Jordan's medium (Kleven *et al* 1998) were applied (Pourbakhsh *et al* 2010, and Haghbin Nazarpak *et al* 2010). They successfully isolated 85 MS bacteria by culture method and identified them using PCR assay. MS Culture and isolation are not easy and almost are not successful in all the Iranian poultry laboratories. Marois et al. (2000) in comparison the PCR and culture methods showed that in the experimental infection, 10/96 and 46/96 samples of food, drinking water, feathers, droppings or dust were positive by culture and Mycoplasma-PCR respectively. In field conditions, the number of positive results for environmental samples were respectively 7/28 and 17/28. PCR-based tests are now routinely used for detecting pathogenic avian mycoplasmas. Furthermore the specific PCR can be used to identify the mycoplasma isolate after appear positive result in culture. For eradication of MS infection, rapid and accurate identification of MS is of great importance and molecular methods such as the PCR have been developed to improve this. Earlier MS specific PCRs were based on the 16S rRNA gene (Lauerman *et al* 1993, Garcia *et al* 1995) and recently, some have been based on haemagglutinin genes (Hong *et al* 2004, Ben Abdelmoumen Mardassi *et al* 2005). Specific MS species published primers by Lauerman et al. (1993) were used (Pourbakhsh *et al* 2010, and Haghbin Nazarpak *et al* 2010). They indicated sensitivity for their MS PCR of 82% and a specificity of 100% as determined by comparison with culture, serology, epizootiology, and history. For increasing the sensitivity of the PCR they suggested extraction of the sample RNA and performing reverse transcription of the rRNA. These MS primers were selected from the 16S rRNA gene, and each cell is reported to have 104 ribosomes (Lauerman *et al* 1993). However it is suggested to apply other specific species PCR based on haemagglutinin genes. It is also recommended to use the enrichment step for better results of MS PCRs, which consists of the incubation of specimens for 24 h prior to detergent treatment in the DNA extraction stage. This is certainly due to an increase of the DNA starting material and the reduction of PCR inhibitors present in the original samples (Ben Abdelmoumen Mardassi *et al* 2005). Because of its sensitivity and specificity, PCR should circumvent the overgrowth problems encountered with culturing of field samples (Ewing *et al* 1998). These results strongly support the use of PCR assay (Lauerman *et al* 1993) as an efficient alternative or supplement to culture and serological identification of the MS infection, especially in breeder chicken flocks.

**Phylogenetic analysis of 16S rRNA gene.** An alternative method of conventional culture and serological identification are the specific DNA detection methods based on sequencing. Several methods were cited by researchers like Kempf (1998), Ramirez (2008), Sprygin (2010), and in addition, a manual published by Lauerman (1998) contains a validated PCR assay for MS, and other avian Mycoplasmas based on unique sequences contained in the 16S rRNA gene (OIE Terrestrial Manual 2008). All of the available molecular methods for evaluating phylogenetic relationships (e.g., Nucleic acid probes; DNA-DNA and DNA-rRNA hybridization, 16S-23S-5S rRNA and protein sequencing, 16S rRNA oligonucleotide cataloging, enzymological patterning, etc.) have advantages in spite of their limitations. In general, macromolecular sequences seem preferred because they permit quantitative inference of relationships. Moreover, because they accumulate, sequences are most useful in the long term (Lane *et al* 1985). The genome of Mollicutes is distinguished by its small size and low guanine and cytosine (G + C) content. The first region to be studied is that coding for ribosomal RNAs (rRNAs). These choices are intelligible because: rRNAs are major and ubiquitous cell products, and the genes responsible for their synthesis as well as the factors regulating their expression are major topics for research. Also, rRNAs are highly conserved molecules, so that variations in their nucleotide sequences may be significant in phylogenetic analysis (Bayatzadeh *et al* 2011). In 1989,

a phylogenetic analysis based on 16S rRNA sequences was reported for the mollicutes by Carl Woese (Weisburg *et al* 1989). The comparison of rRNA sequences is a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, and other organisms (Weisburg *et al* 1991). 16S rRNA sequence comparison from bacteria is one of the most authoritative and precise methods for determining phylogenetic interrelationships (Clarridge 2004). The 16S rRNA gene, a common molecular marker of Eubacteria, was shown to be a valuable complementary marker for species identification using different molecular biological techniques such as denaturing gradient gel electrophoresis (McAuliffe *et al* 2005), restriction analysis (Stakenborg *et al* 2005), pulse field electrophoresis, polymorphic amplification of random primers (Marois *et al* 2001) and gene sequencing (Gray *et al* 2005). Several thousands of bacterial 16S rRNA sequences have been deposited in data bases, and many Mycoplasma sequences are now available in GenBank or in the European Molecular Biology Laboratory (EMBL) data banks for nucleotide sequences. Molecular phylogeny of Mycoplasmas based on 16S rRNA sequence data is most comprehensive (Pettersson *et al* 2000). According to the 16S rRNA based phylogeny; MS was descended from a common progenitor of the monophyletic clusters of the hominis group (Pettersson *et al* 2000). Ribosomal RNA has been used to develop identification systems for Mycoplasmas and sequencing of the 16s rRNA genes will be an extremely powerful tool in the classification of Mycoplasmas. Mycoplasmas in general show a more rapid evolutionary rate than other bacteria, which is reflected in variations of otherwise conserved nucleotides in Mycoplasma 16S rRNA (Weisburg *et al* 1989, Bayatzadeh *et al* 2011). Buim et al. (2010) showed different polymorphic patterns as polymorphisms and differences in nucleotides of MS 16S rRNA among strains from distinct Brazilian areas of poultry production. Ramirez et al. (2008) showed polymorphisms and sequence length variations in the *ISR* between the 16S and 23S rRNA genes in MS species. For the first time in Iran, Hosseini Aliabad et al. (2012) have analyzed the DNA sequence of MS isolates from Mazandran province poultry flocks and compare them with MS from other countries. For this purpose, they collected 32 samples from choanal cleft, trachea and air sac of flocks with respiratory symptom and cultured in specific PPLO medium. Ten out of the 32 samples were positive in culture and reacted with specific antiserum. The 10 positive samples were subsequently used for molecular study. The PCR product containing 16s rRNA genome that was generated with specific primers and produced a specific 207 base pair (bp) band was sequenced. Multiple alignment and phylogenic analysis of DNA sequences was performed with DNASTAR program. Alignment and comparison of the sequences in DANSTAR program revealed that 7 of 10 isolate are quite similar and 3 other isolate are different from group one. Identity plot (IP) of nucleic acids showed only 30% homology among two groups and phylogenic analysis arranged them in two distinct lineages. Phylogenetic analysis of DNA sequences of MS isolated from Mazandran province of Iran and 21 MS from the entire world revealed low homology among most of them. However, some of the Mazandran isolates showed high homology with MS isolated from Brazil In 2012, (Hosseini Aliabad *et al* 2012). Bayatzadeh et al. (2013) have performed the phylogenetic analysis of MS isolates derived from commercial chicken farms of Iran based on 16S rRNA gene. The partial sequence of 16S rRNA genes obtained from 19 isolates of central region of Iran (Tehran, Qazvin, Semnan and Markazi provinces) were amplified by PCR in 207bp fragment and then sequenced, after that compared with 16S rRNA gene of MS sequences which were available in GenBank. Sequences were aligned using ClustalW method (BioEdit 7.0) and Neighbor-joining algorithm (MEGA 5.0) was used to create phylogenetic tree. Phylogenetic analysis of these sequences showed that all 19 MS isolated from Iran were most closely related to sequences of MS from Brazil (ranging from >95 to 100% similarity). This relationship between sequences

of field isolates from Iran and those of Brazil maybe bring about the proximate poultry industry trade between two countries (importations of corn, soya, fish meal, chicken meat, etc. from Brazil) and similar aviculture condition that induced similar nucleotide variations. Presence of variations, polymorphisms, and differences between nucleotides of all isolates were observed. They suggested that the different molecular structure and heterogeneity among MS isolates may be explained by transmitted the mutations and variations between other countries by reason of high volume of trading or the spontaneous mutations by reason of region conditions (Bayatzadeh *et al* 2013). They concluded that future research should focus on determining the genes of surface/variable proteins and ISR gene sequences of MS isolates for use in phylogenetic analysis and the sequences of non-16S rRNA genes (Bencina *et al* 2001, Hong *et al* 2004, Ramirez *et al* 2008, Hammond *et al* 2009). Indeed, Future research should focus on analysis the longer fragments of 16S rRNA for use in phylogenetic analysis and the sequences of non-16S rRNA genes for further evaluation of the relationship between isolates (Bayatzadeh *et al* 2013).

The 16S rRNA gene sequencing showed the presence of variations, polymorphisms, and differences between nucleotides of the all isolates. Mycoplasmas, particularly, species with the smallest genomes, have highly mutation rates, suggesting that those are in a state of rapid evolution. Therefore, they may be useful systems for the study of molecular events during periods of rapid evolution (Razin *et al* 1998). The high mutation rate, as indicated by this hypothesis that many 16S rRNA sequences which are highly conserved in eubacteria are absent in Mycoplasmas (Rogser *et al* 1985). Spontaneous mutations may occur when a lineage remains in the same flocks for long periods (Buim *et al* 2010).

**Phylogenetic analysis of VIhA gene.** In MS, haemagglutinins are encoded by related sequences of a multigenes family referred to as variable lipoprotein hemagglutinin (vlhA) genes (Bencina *et al* 1999, Noormohammadi *et al* 1997). Haemagglutinins account among the most important surface proteins involved in colonization and virulence of avian mycoplasmas (Bencina *et al* 2001, Narat *et al* 1998). The unique expressed vlhA gene of MS yields a product that is cleaved post-translationally into an N-terminal lipoprotein (MSPB) and a C-terminal haemagglutinin protein (MSPA) (Noormohammadi *et al* 1998). Cleavage was found to occur immediately after amino acid residue 344 (Noormohammadi *et al* 2000). Both MSPA and MSPB are surface-exposed proteins and exhibit high frequency antigenic variation (Noormohammadi *et al* 1997, 1998). Such a gene replacement mechanism, also known as gene conversion, allows a single strain of MS to generate a large number of variants by recruiting new sequences from a large pseudogene reservoir. This pseudogene reservoir was found to be confined to a restricted region of the genome, providing an optimal environment for site-specific recombination (Khiari *et al* 2010). In addition, the approach does not determine whether the noclutide variation detected relates to genomic rearrangements that commonly occur within strains (Noormohammadi *et al* 2000). The inter strain diversity at the vlhA expression site, including major differences in the predicted secondary structures of their expressed adhesions (May *et al* 2011). Corresponding functional differences in the extent to which they agglutinated erythrocytes, a quantitative proxy for VlhA-mediated cytadherence, were also evident (May *et al* 2011). Recently, sequence analysis of the single-copy conserved region of the MS vlhA gene has been used for investigations of MS strains and epidemiological studies (Noormohammadi *et al* 2000, Bencina *et al* 2001, Hong *et al* 2004, Hammound *et al* 2009, Slavec *et al* 2011). PCR-based mutation detection techniques provide useful and cost-effective alternatives for the direct analysis of genetic variation, particularly when large numbers of samples are to be analyzed (Jeffery *et al* 2007). Polymerase chain reaction (PCR) and DNA sequence analysis of the N-terminal end of the hemagglutinin encoding gene vlhA have been used as

an alternative for the detection and initial typing of field strains of MS in commercial poultry (Hong *et al* 2004). The vlhA gene sequencing and analysis of MS isolates is of critical importance, particularly in countries that poultry flocks are vaccinated with the live *M. synoviae* strain MS-H. In Iran, Ansari et al. (2010) and Ghafori et al. (2011) using by vlhA-PCR and methods used for differentiation of *M. synoviae* studied MS isolates but they did not carry out sequences analysis for comparison Iranian isolates with MS-H strain. Ansari et al. (2010) have detected MS in clinical samples using the VIhA-PCR method. For serological screening test, they collected 373 serum samples from 25 breeder farms and rapid serum agglutination test conducted which revealed that 143 samples equivalent to 19 breeder farms were positive. For VIhA-PCR assay, 20 of the previously mentioned breeder farms were selected and sterile swab were collected from the palatine cleft, trachea, air sacs and lungs. Three swabs from 3 birds were placed in a test tube containing 1 ml of PBS and transferred to the laboratory for PCR test. The PCR product from specific primers showed 350-400 bp for all field isolates on electrophoresis gel in 8 farms. Recently, our grpup in Mycoplasma Reference Laboratory of Razi Institute have sequenced the vlhA gene of Iranian MS isolates and compared them with MS-H strain (Pourbakhsh *et al* 2013). They used PCR for detection of differences in a relatively short stretch of the MS vlhA gene. They showed that, there was a complete concordance between all Iranian isolates nucleotide sequence (1-386 nt) and the 5́-vlhA region sequence remained unchanged in all tested MS isolates. We have also demonstrated differentiate between Iranian isolates and live commercial vaccine strain (Pourbakhsh *et al* 2013). Furthermore comparison of the vlhA gene sequences of Iranian isolates and of MS-H strain indicated that Iranian isolates derived most probably from the same ancestor and separated from MS-H vaccine strain. These data showed that changes in the vlhA gene sequence can introduce into the expressed vlhA gene amino acid codons and translation. However, our group study was the first demonstration of diversity of the vlhA gene in Iranian MS isolates in comparison with MS-H strain (Pourbakhsh *et al* 2013). DNA sequence analysis and phylogenetic studies based on the haemagglutinin-encoding vlhA gene for apperceiving the true relationships between the MS field and MS-H strain have been reported earlier (Harada *et al* 2009, Ogino *et al* 2011). Harada et al. (2009) compared tested field isolates with the MS-H vaccine strain and found out that they neither had identical vlhA sequences nor were classified into the same Bercina's type or phylogenetic cluster. Ogino et al. (2011) showed the sequences and analyses of conserved domain of the variable lipoprotein and hemagglutinin (vlhA) gene of 19 field strains of MS isolated from chickens across Japan. They have also genotyped Japanese isolates of MS and carried out the rapid molecular differentiation of the isolates from the MS-H vaccine strain. Jeffery et al. (2007) showed that the polymorphism in the vlhA gene can be used for discriminating the vaccine strain from the field isolates obtained from a few countries (Jeffery *et al* 2007). Harada et al. (2009) believed that the vlhA sequence analysis are more effective in differentiating between MS strains and are particularly useful for discriminating the live vaccine strain from local field strains with identical vlhA sequences from Australia and even other countries if MS-H vaccination would become necessary in the future (Harada *et al* 2009). Ogino et al. (2011), with the alignment of MS-H and the nine representative Japanese strains, showed that the field isolates had 12 additional identical nucleotides, which were not present in MS-H and suggested this can be as a promising method for ensuring rapid identification of MS-H and field isolates. They found a single nucleotide polymorphism within conserve region in all the Japanese isolates, and they established a PCR method differentiate between isolates of MS and the live MS vaccine strain (MS-H). (Ogino *et al* 2011). The group demonstrated the complete sequence identity of the conserve region of the expressed vlhA gene of Iranian isolates with corresponding sequence of vaccine strain

(Pourbakhsh *et al* 2013). The 5́-vlhA region of vlhA gene in all isolates, contained at least 24 nucleotides was different in the sequence downstream of nucleotide 386. On the other hand, obtained isolates had vlhA sequences that were different in downstream of nucleotide 386 from vaccine strain. In comparison with MS-H, in all Iranian isolates examined in Pourbakhsh et al. (2013) study, some nucleotides had been substituted with other nucleotides. Thus, it is likely that the presences of nucleotide changes are because of uneven sequence between Iranian isolates and MS-H strain (Pourbakhsh *et al* 2013). This is reinforced by the DNA homologies that show the insertion/deletion and nucleotide changes because of Iranian isolates and in comparison with MS-H. In the 5́-vlhA region of Iranian isolates nucleotide sequence (1–386 bp), had point mutations content of 47 %, and while through the length of the region had frame-shift mutation content of only 53% (Pourbakhsh *et al* 2013). Bencina et al. (2001) expressed that changes in the vlhA gene sequence can introduce into the expressed vlhA gene amino acid codons and translation therefore these changes can be effective in pathogenesis rate in flocks, so that insertions/deletions which were observed in all the isolates examined in Pourbakhsh et al. (2013) study may be related to their pathogenicity. The alignment of MS-H and the 10 representative Iranian MS isolates showed that the Iranian isolates had 4 additional nucleotides that were identical in all isolates, which were not present in MS-H. In addition, deletion of 16 nucleotides was found in Iranian isolates when compared with MS-H. Furthermore, 24 nucleotides were found to be replaced by other nucleotides in all the isolates examined. The overall sequence similarity in Iranian partial vlhA gene sequences examined in this study was 81% between MS-H and the isolates. The overall examined sequence similarity in Iranian partial vlhA gene sequences was 81% between MS-H and the field isolates (Pourbakhsh *et al* 2013).

**PCR-RFLP based on single nucleotide polymorphism**. More recently, Bayatzadeh et al. (2014) sequenced, analyzed and verified the single-copy domain of the conserved region of vlhA gene in MS. In order to type the MS isolates of Iran, the restriction fragment length polymorphism (RFLP) method based on single nucleotide polymorphism that are existed in all isolates of Iran was also used. This PCR-RFLP allowed differentiating all MS field isolates from the vaccine strain (MS-H). Based on phylogenetic analysis, the isolates were assigned to 12 unique genotypes taking into account that within each group, DNA similarity was approximately 100%. Subsequent DNA sequence analysis of the PCR product based on percent similarity and evolutionary relationship appeared to be a useful tool for strain differentiation. Therefore the PCR-RFLP method and DNA sequence analysis would be useful tools to distinguish between MS isolates, whether they are derived from the vaccine or wild-type strain and also these teqniques have potential value of strain typing for epidemiological purposes of MS infection.

## Conclusion

This study based on analysis of the partial sequencing of 16S rRNA gene represent the information about the relationship between the MS isolates from Iran and Brazil poultry farms. These data provide further evidence, which despite the 16S rRNA genotype is conserved but present information about the heterogeneity, polymorphism and genetic diversity of MS isolates in different areas that may be explained by transmitted the mutations and variations between other countries by reason of high volume of trading, or the spontaneous mutations by reason of region conditions. DNA sequence analysis could determine the phylogenetic relationships between the MS strains and the phylogenetic tree showed which species were more closely related. This study confirms the potential value of strain typing for epidemiological purposes and suggests that phylogenetic studies are essential to understand the true relationships between strains. The data obtained by our team in Mycoplasma Reference Laboratory of Razi Institute indicate that sequencing and polymorphisms of the 5́-vlhA region might be very

useful for the isolates comparison with MS-H vaccine, as well as indicate that sequence analysis of the 5́-vlhA region has the potential to become a valuable tool for tracing spreading of MS isolates in poultry flocks. In the studies of Mycoplasma Reference Laboratory of Razi Institute using by vlhA gene analysis could successfully differentiate between the MS-H vaccine and Iranian isolates, these data provide novel information about vlhA diversification in Iranian MS isolates. Also, in all isolates, the vlhA gene sequence changes produced nucleotide diversity that can introduce different forms of the VlhA haemagglutinin. Upon the results, the research and assay based on the vlhA gene conversion events of the nucleotide sequence of Iranian isolates, uncovered more nucleotide variations through the length of the conserve region than the MS-H vaccine strain. Further studies to reveal other kinds of association between the pathogenicity of MS and the vlhA gene variations could provide key information to the industry in terms of epidemiology.

## Acknowledgments

We thank all the staff of the Mycoplasma Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran. This study was supported by of the Razi Institute and Education and Research Deputy of Jihad-Agriculture Ministry with the grant No. 2-18-18- 87080.

## References

Adair, B. M., Burns, K., McNulty, M. S., and Todd, D. (1990). A study of ELISA systems incorporating pooled viral and mycoplasma antigen preparations for antibody screening of chicken sera. *Avian Pathology* 19: 263-278.

Ansari, H., Pourbakhsh, S. A., Sheikhi, N., Bozorgmehri Fard, M. H. and Ashtari, A. (2010). Detection of *Mycoplasma synoviae* by vlha-PCR with special primers in clinical sample. *Veterinary Journal (Tabriz)* 4 (12): 673-682.

Avakian, A. P. and Kleven, S. H. (1990). The humoral immune response of chickens to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* studied by immunoblotting. *Veterinary Microbiology* 24: 155-169.

Bayatzadeh, M. A., Pourbakhsh, S. A., Homayounimehr, A.R., Ashtari, A. and Abtin, A.R. (2011). Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of *Mycoplasma synoviae* on broiler chicken farms. *Archive of Razi Institute* 66(2): 87-94.

Bayatzadeh, M.A., Pourbakhsh, S.A., Homayounimehr, A.R., Ashtari, A. and Abtin, A.R. (2013). Phylogenetic analysis of *Mycoplasma synoviae* isolated from commercial Iranian chicken farms compared with other GeneBank isolate sequences based on *16S rRNA* gene. *Avian Biology Research* 6(3): 233-238.

Bayatzadeh, M. A., Pourbakhsh, S. A., Ashtari, A., Abtin, A. R and Abdoshah, M. (2014). Molecular typing of Iranian field isolates *Mycoplasma synoviae* and their differentiation from the live commercial vaccine strain MS-H using *vlhA* gene. *British Poultry Research* 491-518.

Ben Abdelmoumen Mardassi, B., Ben Mohamed, R., Gueriri, I., Boughattas, S. and Mlik, B. (2005). Duplex PCR to differentiate between *Mycoplasma synoviae* and *Mycoplasma gallisepticum* on the basis of conserved species-specific sequences of their hemagglutinin genes. *Journal of Clinical Microbiology* 43(2): 948–958.

Bencina, D., Drobnic-Valic, M., Horvat, S., Narat, M., Kleven, S. H. & Dovc, P. (2001). Molecular basis of the length variation in the Nterminal part of *Mycoplasma synoviae* hemagglutinin. *FEMS Microbiology Letters* 203: 115–123.

Bencina, D., Narat, M., Dovc, P., Drobnic-Valic, M., Habe, F., Kleven, S. H. (1999). The characterization of Mycoplasma synoviae EF-Tu protein and proteins involved in hemadherence and their N terminal amino acid sequences. *FEMS Microbiology Letters* 173: 85-94.

Bradbury, J. M. (1977). Rapid biochemical tests for characteriution of the Mycoplasmataies. *Journal of Microbiology* 5: 531-534.

Buim, M., Buzinhani, M., Yamaguti, M., Oliveira, R., Mettifogo, E., Timenetsky, J. and Ferreira, A. (2010). Intraspecific variation in *16S rRNA* gene of *Mycoplasma synoviae* determined by DNA sequencing. *Comparative Immunology, Microbiology and Infectious Diseases* 33(1): 15-23.

Clarridge, J. E. (2004). Impact of *16S rRNA* gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Review* 17: 840-862.

Ewing, L., Cookson, K. C., Phillips, R. A., Turner, K. R. and Kleven, S. H. (1998). Experimental infection and transmissibility of *Mycoplasma synoviae* with delayed

serological response in chickens. *Avian Diseases* 42: 230–238.

Feberwee, A., Wit, J.J.D. and Landman, W. J. M. (2009). Induction of eggshell apex abnormalities by *Mycoplasma synoviae*: field and experimental studies. *Avian Pathology* 38: 77-85.

Frey, M.L., Hanson, R. P. and Anderson, D. P. (1968). A medium for the isolation of avian Mycoplasmas. *American Veterinary Research* 29: 2163–2171.

Garcia, M., Jackwood, M.W., Levisohn, S. and 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 Diseases* 39: 606-616.

Ghafouri S. A., Bozorgmehri fard M. H., Karimi V., Nazem shirazi M. H., Noormohammadi A. and Hosseini H. (2011). Identification and primary differentiation of Iranian isolates of *Mycoplasma synoviae* using PCR based on amplification of conserved 5' end of *vlha* gene. *Journal of Veterinary Research* 66(2): 117-122.

Ghaleh Golab Behbahan, N., Asasi, A. R., Afsharifar, A. R. and Pourbakhsh, SA. (2005). Isolation and detection of *Mycoplasma gallisepticum* by polymerase chain reaction and restriction fragment length polymorphism. *Iranian Journal of Veterinary Research of University Shiraz* 6: 35-41.

Gray, L. D., Ketring, K. L and Tang, Y. W. (2005). Clinical use of *16S rRNA* gene sequencing to identify *Mycoplasma felis* and *M. gateae* associated with feline ulcerative keratitis. *Journal Clinical Microbiology* 43: 3431–3434.

Haghbin Nazarpak, H. and Pourbakhsh, S. A. (2010). Isolation and detection of *Mycoplasma synoviae* from seropositive rapid reaction broiler breeder flocks by polymerase chain reaction and culture methods. *Journal of Veterinary Microbiology* 6(1):90-95.

Hammond, P. P., Ramirez, A. S., Morrow, C. J and Bradbury J. M. (2009). Development and evaluation of an improved diagnostic PCR for *Mycoplasma synoviae* using primers located in the haemagglutinin encoding gene *vlhA* and its value for strain typing. *Veterinary Microbiology* 136: 61–68.

Harada, K., Kijima-Tanaka, M., Uchiyama, M., Yamamoto, T., Oishi, K.., Areo, M. and Takahashi, T. (2009). Molecular Typing of Japanese Field Isolates and Live Commercial Vaccine Strain of *Mycoplasma synoviae* Using Improved Pulsed-Field Gel Electrophoresis and *vlhA* Gene Sequencing. *Avian Diseases* 53: 538–543.

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

Hosseini Aliabad, S. A., Pourbakhsh, S. A., Charkhkar, S., Bozorgmehri fard, M. H. and shikhi, N. (2012). Molecular study and phylogenetic analysis of *Mycoplasma synoviae* isolated from poultry flocks from Mazandran province of Iran. *African Journal of Biotechnology* 11(8): 2124-2129.

Jeffery, N., Gasser, R. B., Steer. P. A., Noormohammadi, A. H. (2007). Classification of *Mycoplasma synoviae* strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the *vlhA* gene single copy region. *Microbiology* 153: 2679-2688.

Jones, J. F., Whithear, K. G. Scott, P. C. and Noormohammadi, A.H. (2006a). Determination of the effective dose of the live *Mycoplasma synoviae* vaccine, Vaxsafe *MS* (strain MS-H) by protection against experimental challenge. *Avian Diseases* 50: 88–91.

Jones, J. F., Whithear, K. G., Scott, P. C. and Noormohammadi, A. H. (2006b). Onset of immunity with *Mycoplasma synoviae*: comparison of the live attenuated vaccine MS-H (Vaxsafe MS) with its wild-type parent strain (86079/7NS). *Avian Diseases* 50: 82–87.

Jones, J. F., Whithear, K. G., Scott, P. C. and A. H. Noormohammadi. (2006c). Duration of immunity with *Mycoplasma synoviae*: comparison of the live attenuated vaccine MS-H (Vaxsafe MS) with its wild-type parent strain, 86079/7NS. *Avian Diseases* 50: 228–231.

Kang, M. S., Gazdzinski, P. and Kleven, S. H. (2002). Virulence of recent isolates of *Mycoplasma synoviae* in turkeys. *Avian Diseases* 46: 102-110.

Kempf, I. (1998). DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. *Avian Pathol*ogy 27: 7–14.

Khiari, A. B., Guériri, I., Ben Mohammed, R., Mardassi, B. B. A. (2010). Characterization of a variant *vlhA* gene of *Mycoplasma synoviae*, strain WVU 1853, with a highly divergent haemagglutinin region. *BMC Microbiology* 10: 6-11.

Kleven, S. H. (1997). *Mycoplasma synoviae* infection. In Diseases of Poultry. 220–228.

Kleven, S. H. (1998). Mycoplasmosis. In: Swayne, D. E, Glisson, J. R, Jackwood, M. W., Pearson, J. E. and Reed, W. M. (Eds). A laboratory manual for the isolation and identification of avian pathogens (4th edn). Pp: 74-80. American Association of avian pathologists.

Kleven, S. H. (2003). *Mycoplasma synoviae* infection. In: Saif, Y. M., Barnes, H. J., Glisson, J. R., Fadly, A. M.,

McDougald, L. R. and Swayne, D. E. (eds) *Diseases of Poultry*, 11th edn, Iowa State Press, Ames. Pp: 756-766.

Kleven, S.H., and NFerguson-Noel. (2008). *Mycoplasma synoviae* infection. In: Diseases of poultry, 12th ed. Y. M. Saif, A. M. Fadly, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Blackwell Publishing, Ames, IA. Pp: 845–856.

Kleven, S. H., Rowland, G. N. and Kumar, M. C. (2001). Poor serologic response to upper respiratory infection with *Mycoplasma synoviae* in turkeys, *Avian Diseases* 48:719–723.

Landman, W. J. M. and Feberwee, A. (2001). Field studies on the association between amyloid arthropathy and *Mycoplasma synoviae* infection, and experimental reproduction of the condition in brown layers. *Avian Pathology* 30: 629-639.

Lane, D. J., Pace, B., Olsen, G. J., Stahlt, D. A., Sogint, M. L. and Pace, N.R. (1985). Rapid determination of *16S ribosomal RNA* sequences for phylogenetic analyses. *Proceeding of the National Academy of Science* 82: 6955-6959.

Lauerman, L. H. (1998). *Mycoplasma* PCR assays. In: L.H. Lauerman, Editor, Nucleic Acid Amplification Assays for Diagnosis of Animal Diseases, American Association of Veterinary Laboratory Diagnosticians, Auburn, Alabama Pp: 41–42.

Lauerman, L.H., Hoerr, F. J., Sharpton, A. R., Shah, S. M. and Van Santen, V. L. (1993). Development and application of a polymerase chain reaction assay for *Mycoplasma synoviae*. *Avian Diseases* 37: 829–834.

Lockaby, S. B., Hoerr, F. J., Lauerman, L. H. and Kleven, S. H. (1998). Pathogenicity of *Mycoplasma synoviae* in broiler chickens. *Veterinary Pathology* 35: 178-190.

Markham, J. F., Morrow, C. J. and Whithear, K. G. (1998a). Efficacy of a temperature-sensitive *Mycoplasma synoviae* live vaccine. *Avian Diseases* 42: 671–676.

Markham, J. F., Morrow, C. J., Scott, P. C. and Whithear, K. G. (1998b). Safety of a temperature-sensitive clone of *Mycoplasma synoviae* as a live vaccine. *Avian Diseases* 42: 677–681.

Markham, J. F., Scott, P. C. and Whithear, K. G. (1998c). Field evaluation of the safety and efficacy of a temperature-sensitive *Mycoplasma synoviae* live vaccine. *Avian Diseases* 42: 682-689.

Marois, C., Dufour-Gebert, F. and Kempf, I. (2000). Detection of *Mycoplasma synoviae* in poultry environment samples by culture and polymerase chain reaction. *Veterinary Microbiology* 73(4): 311-318.

Marois, C., Dufour-Gesbert, F., Kempf,I. (2001). Comparison of pulsed-field gel electrophoresis with random amplified polymorphic DNA for typing of *Mycoplasma synoviae*. *Veterinary Microbiology* 79: 1–9.

May, M. and Brown, D. R. (2011). Diversity of Expressed vlhA adhesin sequences and intermediate hemagglutination phenotypes in *Mycoplasma synoviae*. *Journal of Bacteriology* (45): 2116–2121.

McAuliffe, L., Ellis, R.J., Lawes, J. R., Ayling, R. D. and Nicholas, R.A. (2005). *16S rDNA* PCR and denaturing gradient gel electrophoresis; a single generic test for detecting and differentiating *Mycoplasma* species. *Journal Medicine Microbiology* 54: 731–739.

Morrow, C. J., Bell, I. G., Walker, S. B., Markham, P. F., Thorp, B.H. and Whithear, K.G. (1990). Isolation of *Mycoplasma synoviae* from infectious synovitis of chickens. *Australian Veterinary Journal* 67: 121-124.

Morowitz, H. J., Wallace, D. C. (1973). Genome size and life cycle of the mycoplasma. *Annual New York Academic Science* 225: 62-73.

Morrow, C. J., Markham, J. F. and Whithear, K. G. (1998). Production of temperature-sensitive clones of *Mycoplasma synoviae* for evaluation as live vaccines. *Avian Diseases* 42: 667–670.

Narat, M., Bencina, D., Kleven, S. H. and Habe, F. (1998). Hemagglutination-positive phenotype of *Mycoplasma synoviae* induces experimental infectious synovitis in chickens with a higher frequency than the hemagglutination-negative phenotype. *Infection and Immunity* 66: 6004-6009.

Nascimento, E. R., Pereira, V. L. A., Nascimento, M. G.F. And Barreto, M. L. (2005). Avian mycoplasmosis update. *Brazilian Journal of Poultry Science* 7(1): 1-9.

Noormohammadi, A. H., Hemmatzadeh, F. and Whithear, K. G. (2007). Safety and efficacy of the *Mycoplasma synoviae* MS-H vaccine in turkeys. *Avian Diseases* 51: 550–554.

Noormohammadi, A. H., Jones, J. F. Harrigan, K. E. and Whithear. K. G. (2003). Evaluation of the non-temperature-sensitive field clonal isolates of the *Mycoplasma synoviae* vaccine strain MS-H. *Avian Diseases* 47: 355–360.

Noormohammadi, A. H., Markham, P. F., Du¡y, M. F., Whithear, K.G. and Browning, G.F. (1998). Multigene families encoding the major hemagglutinins in phylogenetically distinct *Mycoplasmas*. *Infection and Immunity* 66: 3470-3475.

Noormohammadi, A. H., Markham, P. F., Kanci, A., Whithear, K. G.and Browning, G. F. (2000). A novel mechanism for control of antigenic variation in the haemagglutinin gene family of *Mycoplasma synoviae. Molecular Microbiology* 35: 911-923.

Noormohammadi, A.H., Markham, P. F., Whithear, K. G., Walker, I. D., Gurevich, V.A., Ley, D.H., Browning, G.F.(1997). *Mycoplasma synoviae* has two distinct phasevariable major membraneantigens one of which is a putative haemagglutinin. *Infection and Immunity* 65:2542-2547.

Ogino, S., Munakata, Y., Ohashi, S., Fukui, M., Sakamoto, H., Sekiya, Y., Noormohammadi, A.M. and Morrow, C.J. (2011). Genotyping of Japanese Field Isolates of *Mycoplasma synoviae* and Rapid Molecular Differentiation from the MS-H Vaccine Strain. *Avian Diseases* 55: 187–194.

OIE Terrestrial Manual. (2008). Avian Mycoplasmosis (*Mycoplasma gallisepticum, M. synoviae)*. In: Chapter 2.3.5. 482-496

Olson, N.O., Shelton, D.C., Bletner, J.K., Munro, D.A. and Anderson, G.C. (1956). Studies of infectious synovitis in chickens. *American* Journal of *Veterinary Research* 17: 747-754.

Pettersson, B., Tully, J. G., Bolske, G. and Johansson, K.E. (2000). Updated phylogenetic description of the *Mycoplasma hominis* cluster (Weisburg et al. 1989) based on *16S rDNA* sequences. *International Journal of Systematic Evolutionary Microbiol*ogy 50(1): 291–301.

Pourbakhsh, S. A., Maghami, M., Ashtari, A., Bayatzadeh, M. A. and Ahangaran, S. (2013). The *vlhA* gene sequencing of Iranian *Mycoplasma synoviae* isolates. *Archive of Razi Institute* 68: 117-124.

Pourbakhsh, S. A., Shokri, G. R., Banani, M., Elhamnia, F., Ashtari, A. (2010). Detection of *Mycoplasma synoviae* infection in broiler breeder farms of Tehran province using PCR and culture methods. *Archive of Razi Institute* 65(2): 75-81.

Ramirez, 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 offers potential use as a diagnostic tool. *Veterinary Microbiology* 128: 279–287.

Razin, S**.** (1985). Molecular biology and genetics of mycoplasmas (mollicutes). *Microbiological Reviews* 49: 419-455.

Razin, S., Yogev, D. and Naot, Y. (1998). Molecular biology and Pathogenicity of mycoplasmas. *Microbiology and Molecular Biology Reviews* 62: 1094-156.

Rogser, M. J., Simmons, J., Walker, R. T., Weisburg, W. G., Woese, C.R, Tanner, R.S., Robinson I. M., Stahl, D.A., Olsen, G., Leach, R.H., Maniloffi, J. (1985). Construction of the *Mycoplasma* evolutionary tree from 5S *rRNA* sequence data. *Evolution* 82:1160-1164

Slavec, B., Lucijana, Bercic, R., Cizelj, I., Narat. M., Zorman-Rojs I. O., Dovc, P. and Bencina. D. (2011). Variation of *vlhA* gene in *Mycoplasma synoviae* clones isolated from chickens. *Avian Pathology* 40(5): 481-489.

Sprygin, A. V., Andreychuk, D. B., Kolotilov, A. N., Volkov, M. S., Runina, I.. A., Mudrak, N. S., Borisov, A.V., Irza, V. N., Drygin, V.V. and Perevozchikova, N.A. (2010). Development of a duplex real-time TaqMan PCR assay with an internal control for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in clinical samples from commercial and backyard poultry. *Avian Pathol*ogy 39(2): 99-109.

Stakenborg, T., Vicca, J., Butaye, P., Maes, D., De Baere, T., Verhelst, R. et al. (2005). Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Mycoplasma* species. *BMC Infection Diseases* 5:46-52.

Van Beek, P., Feberwee, A., Fabri, T. and Heijmans, M.J.H.M. (2002). Longitudinal field study of the presence of *Mycoplasma synoviae* in meat-turkey flocks with arthritis. *Proceedings of the 4th International Symposlum on Turkey Diseases*. 177-178.

Van Eck, J. H., Van Kol, N. and Kouwenhoven, B. (1980). Egg production in relation to the results of a long term serological survey of 73 flocks of fowl. *Tijdschrift voor Diergeneeskunde* 105: 15-23.

Weisburg, W. G., Barns, S. M., Dale, A., Pelletier, D., Lane, J. (1991). *16S Ribosomal DNA* Amplification for Phylogenetic Study**.** *Journal of Bacteriology* 697-703.

Weisburg, W. G., Tully J. G., Rose D. L., Petzel, J. P, Oyaizu, H., Yang, D., Mandelco, L., Sechrest, J., Lawrence, T. G., van Etten. J., Maniloff, J. and Woese, C. R. (1989). A phylogenetic analysis of the mycoplasmas: basis for their classification. *Journal of Bacteriology* 171:6455- 6467.

Yamamoto, R. (1991). *Mycoplasma meleagridis* infection. In: Calnek, B. W., Burnes, H. J., Beard, C. W., Yoder, J. r. H. W. (Eds). *Diseases of poultry*. Iowa State University, Ames, Iowa, USA Press. Pp:212-23.

Zain, Z. M. and Bradbury, J. M. (1995). The influence of type of swab and laboratory method on the recovery of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in broth medium. *Avian Pathology* 24: 707-716.