Molecular characterization of *Mycoplasma synoviae* isolated from broiler chickens of West Azarbaijan province by PCR of *vlhA* gene

Abolfazl Ghaniei

Department of Poultry Diseases, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

**Abstract**

*Mycoplasma synoviae* (MS) is a pathogen responsible for respiratory and locomotor disorders and causes major economic losses in poultry industry. Early and accurate diagnosis of MS infection plays a major role in control of the infection. This study was conducted to characterize Iranian field isolates of MS isolated from broiler chickens of West Azarbaijan province (Northwest of Iran), and differentiate them from vaccine strain MS-H. Two encoding genes, 16S rRNA and *vlhA* were employed. PCR results using primers related to 16S rRNA and *vlhA* genes were analyzed and compared. Out of 21 field samples, eight samples (38.0%) were positive using both sets of primers. Amplified products of *vlhA* gene were sequenced for MS strain identification. The results showed that Iranian field isolates of MS had high nucleotide and amino acid similarity. Iranian field isolates were distinct from vaccine strain MS-H. Results presented in this study showed that characterization of field isolates of MS by sequencing of *vlhA* gene and is beneficial for strain typing and differentiating them from vaccine strain. To our knowledge, this is the first study characterizing *vlhA* gene of MS isolates from broiler chickens in the West Azarbaijan province.

© 2016 Urmia University. All rights reserved.

**Key words:** Broilers Iran *Mycoplasma synoviae* *vlhA*

---

*Nosicif مولکولی جدایه های مایکوپلاسما سینوویه جدا شده از جوجه های گوشتی آذربایجان غربی به روش زنگ‌چرخ PCR*
Introduction

*Mycoplasma synoviae* (MS) is an important poultry pathogen; causing infectious synovitis and respiratory disease. Most frequently, respiratory involvement occurs as subclinical upper respiratory disease in which many birds are infected lifelong and become carriers. It may be transmitted laterally via direct contact and vertically via eggs. Rapid and accurate identification of MS isolates are of great importance in control of the infection. In this regard, molecular assays such as polymerase chain reaction (PCR) have been applied. Earlier MS specific PCRs were based on the 16S rRNA gene. Recently, other genes such as variable lipoprotein hemagglutinin (vlhA) are used.

Genome of MS encodes many proteins, however, only expression of a few of them have been documented. Hemagglutinins account among the most important surface proteins involved in colonization and virulence of avian mycoplasmas. In MS, hemagglutinins are encoded by related sequences of a multigene family referred to as vlhA genes. It was found that vlhA antigenic variation was achieved by the vlhA gene conversion in which a pseudogene sequence replaced the previously expressed sequence in the vlhA gene. Recently, sequence analysis of the single-copy conserved region of the MS vlhA gene has been used for investigations of MS strains and epidemiological analyses. The PCR based mutation detection techniques provide useful and cost-effective alternatives for the direct analysis of genetic variation.

In countries, that poultry flocks are vaccinated with the live MS strain MS-H, such as Iran, gene sequencing and strain typing of MS isolates are of critical importance, due to differentiation between field and vaccine isolates. The main purpose of the present study was to characterize Iranian field isolates of MS and differentiate them from vaccine isolates.

Materials and Methods

**Samples.** A total number of 21 broiler chicken farms of older than three weeks of age in West Azarbaijan province (Northwest of Iran) were sampled from April 2014 to January 2015. All samples were obtained from unvaccinated flocks. Four out of 21 samples were taken from apparently healthy flocks and 17 from flocks with respiratory involvement. From each farm, five swab samples obtained from the choanal cleft and trachea and suspended in 1.5 mL of phosphate-buffered saline and considered one sample.

**DNA extraction.** Each sample (1 mL) was centrifuged for 30 min at 14,000 g at 4°C. The supernatant was removed and the contents were dissolved in 25 µL deionized water. Samples were boiled for 10 min and then placed on ice for 10 min. Afterwards, they were centrifuged at 14,000 g for 5 min. The supernatant containing DNA was used as template in amplification reaction.

**Polymerase chain reaction.** In this study, a 530 base pair portion of avian mitochondrial DNA was amplified using 12S rRNA primers to rule out false negative results.

For detection of MS genome in swab samples, 2 sets of primers were used. The first was 16S rRNA primers. 16S-F: 5’-GAAGCAAGATAGTGATATCA-3’ and 16S-R: 5’-GTCTTGTTCCGAGGATACCA-3’ previously designed by Lauerman et al., amplifying a 207 bp region of the 16S rRNA gene of MS. The PCR reactions were carried out in 25 µL volume of 2.5 µL of 10X PCR buffer, 0.5 µL of dNTP (10 mM), 1 µL of each primer (10 pmol µL⁻¹), 0.5 µL of Taq DNA polymerase (5U per µL), 0.5 µL of MgCl₂ (50 mM), 17 µL of deionized water and 2 µL of extracted DNA. Thermal condition of amplification included initial denaturation of 95 °C for 5 min, followed by 35 cycles of 94 ºC for 30 sec, 51 ºC for 30 sec and 72 ºC for 90 sec. Final extension was done in 72 ºC for 10 min.

The second specific MS primers, for amplifying vlhA gene, were as the following: vlhA-F: 5’- ATTACGAGCTAGTGCAATTCCGCTTAA -3’. The vlhA-PCR mix was performed in a total volume of 25 μL per sample, containing 2.5 μL of 10X PCR buffer, 0.5 μL of 50 mM MgCl₂, 0.5 μL of 10 mM dNTPs, 1 μL of each primer, 0.25 μL of Taq DNA polymerase (5U per µL). Consequently 17.25 μL of deionized distilled water and 2 μL of extracted DNA as template, were added. The vlhA-PCR reaction was conducted in Eppendorff thermal cycler (Eppendorf, Hamburg, Germany) as follows: 5 min at 94 °C, followed by 35 cycles of 60 sec at 94 °C, 60 sec at 53 °C and 1 min at 72 °C, with a final extension cycle of 10 min at 72 °C. Amplified products were stained using ethidium bromide (0.5 µg µL⁻¹) and subjected to agarose gel electrophoresis.

**Sequencing and data analysis.** Four PCR products of vlhA gene of MS isolates (MS01, MS06, MS07, and MS16) were submitted for sequencing to the Bioneer Inc. (Daejeon, South Korea) using vlhA primers as the sequencing primers. Nucleotide (nt) and predicted amino acid (aa) sequences data were aligned with clustal W alignment algorithms. The sequence alignments were checked by eye for ambiguities and errors by the examination of chromatograms. Phylogenetic analysis was conducted based on the nt sequences using a distance method and an un-weighted pair group with arithmetic mean and by calculating bootstrap values for 1000 replicates in MEGA software (Version 6.0; Biodesign Institute, Tempe, USA).

Results

Eight swab samples out of 21 (38.1%) were positive for MS using PCR of both primers (16S rRNA and vlhA) as diagnostic method for MS.
Since live MS vaccine was not used in these broiler flocks, amplified products of field strains were compared to vaccine strain (MS-H). Also, some published vlhA sequence of field strains were included in this comparison (Table 1).

Table 1. Published MS sequences of vlhA used for multiple alignment analysis.

| Name | Gene bank accession no. | Country of origin |
|------|-------------------------|-------------------|
| *MS01 | KT880075 | Iran |
| MS06  | KT880076 | Iran |
| *MS07 | KT880077 | Iran |
| MS16  | KT880078 | Iran |
| MSR036 | JX233544.1 | Iran |
| MSR37 | JX233546.1 | Iran |
| MSR050 | JX233549.1 | Iran |
| MSR-20 | JX960386 | Iran |
| MSR-25 | JX960390 | Iran |
| MSR-12 | JX960384 | Iran |
| MSR-15 | JX960385 | Iran |
| MSR-30 | JX960392 | Iran |
| MSR-11 | JX960383 | Iran |
| MSR-21 | JX960387 | Iran |
| MSR-7 | JX960383 | Iran |
| MS-H | AF464936.1 | Australia |
| B1185 | FM164346 | UK |
| B9504K261 | FM164372 | Germany |
| B9196798 | FM164349 | UK |
| J1585 | AJ580981 | UK |
| WUV1853 | AM998371 | USA |

* indicates MS field isolates of the current study; Superscript numbers indicate group number based on Bayatzadeh et al., classification.

Fig. 1. Nucleotide sequence alignment of vlhA genes of MS field isolates (MS01, MS06, MS07, and MS16 are isolates of this study; MS371 is from a study by Pourbalsh et al.,25; MS12, MS15, MS7, MS11, MS20, MS21, MS25, and MS30 are representatives of eight groups based on Bayatzadeh et al.,24) and vaccine strain MS-H. Note the 12 additional same nucleotides of MS isolates of the current study, MS371, and MS12, MS15 at the positions 100 to 111, which were not present in vaccine strain MS-H.
Table 2. Percentage of nucleotide and amino acid identities for the vlhA genes of 19 strains of *Mycoplasma synoviae*. MS01, MS06, MS07, and MS16 are sequences that identified in current study; MSR371 is representative of Iranian field isolates from Pourbakhsh *et al.* study.21

| Strains  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| MS01     | 1   | 98  | 99  | 100 | 82  | 86  | 78  | 91  | 79  | 99  | 69  | 69  | 98  | 89  | 87  | 66  | 68  | 72  | 73  |
| MS06     | 2   | 99  | 98  | 98  | 83  | 87  | 79  | 92  | 78  | 98  | 69  | 69  | 98  | 88  | 88  | 66  | 68  | 72  | 73  |
| MS07     | 3   | 99  | 99  | 100 | 77  | 86  | 75  | 87  | 76  | 98  | 64  | 64  | 98  | 86  | 84  | 61  | 63  | 67  | 71  |
| MS16     | 4   | 100 | 99  | 100 | 83  | 81  | 84  | 82  | 88  | 100 | 59  | 59  | 100 | 100 | 85  | 63  | 64  | 100 | 84  |
| B1185    | 5   | 97  | 97  | 96  | 88  | 82  | 92  | 88  | 93  | 83  | 86  | 85  | 82  | 91  | 82  | 79  | 82  | 74  | 85  |
| B9504K261| 6   | 92  | 93  | 91  | 87  | 94  | 88  | 91  | 88  | 86  | 75  | 75  | 85  | 77  | 95  | 71  | 73  | 87  | 79  |
| B9196798 | 7   | 94  | 94  | 92  | 89  | 97  | 98  | 82  | 97  | 76  | 83  | 84  | 77  | 84  | 85  | 78  | 81  | 76  | 90  |
| J1585    | 8   | 95  | 95  | 93  | 88  | 98  | 96  | 94  | 82  | 90  | 75  | 75  | 90  | 81  | 94  | 72  | 72  | 85  | 77  |
| MS-H     | 9   | 88  | 88  | 86  | 90  | 96  | 98  | 99  | 95  | 76  | 84  | 83  | 77  | 86  | 84  | 81  | 85  | 75  | 90  |
| MSR371   | 10  | 100 | 99  | 99  | 100 | 97  | 92  | 94  | 95  | 100 | 69  | 72  | 100 | 90  | 88  | 67  | 68  | 79  | 76  |
| MSR20    | 11  | 95  | 96  | 94  | 87  | 96  | 97  | 96  | 96  | 97  | 95  | 99  | 69  | 77  | 73  | 90  | 93  | 67  | 80  |
| MSR25    | 12  | 96  | 96  | 95  | 87  | 96  | 97  | 96  | 96  | 97  | 96  | 100 | 72  | 77  | 74  | 89  | 92  | 68  | 68  |
| MSR12    | 13  | 100 | 99  | 99  | 100 | 97  | 92  | 93  | 94  | 87  | 100 | 95  | 96  | 90  | 88  | 67  | 68  | 79  | 75  |
| MSR15    | 14  | 97  | 96  | 95  | 100 | 95  | 92  | 92  | 96  | 92  | 97  | 93  | 93  | 97  | 79  | 76  | 73  | 67  | 80  |
| MSR30    | 15  | 92  | 92  | 91  | 89  | 94  | 97  | 94  | 97  | 95  | 92  | 96  | 95  | 92  | 93  | 73  | 74  | 90  | 79  |
| MSR11    | 16  | 96  | 96  | 95  | 89  | 91  | 98  | 90  | 98  | 91  | 96  | 94  | 93  | 96  | 84  | 99  | 98  | 70  | 77  |
| MSR21    | 17  | 96  | 96  | 94  | 89  | 90  | 99  | 91  | 96  | 92  | 96  | 95  | 94  | 96  | 84  | 98  | 98  | 67  | 80  |
| MSR7     | 18  | 96  | 95  | 95  | 100 | 97  | 97  | 97  | 97  | 96  | 97  | 98  | 97  | 96  | 95  | 91  | 99  | 98  | 70  |

| WVU1853  | 19  | 95  | 94  | 94  | 88  | 92  | 97  | 93  | 95  | 95  | 95  | 91  | 92  | 94  | 87  | 95  | 88  | 88  | 97  |

*Percentage of amino acid identity is in upper triangle; percentage of nucleotide identity is in lower triangle; Superscript numbers indicate group number based on Bayatzadeh *et al.* classification.*²⁴

Fig. 2. Phylogenetic tree of MS isolates based on the nucleotide sequence of vlhA gene. Branched distances correspond to sequence divergence.
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. In countries like Iran that poultry flocks are vaccinated with live MS-H vaccine, differentiating of field and vaccine strains has critical importance. Ghafouri et al., Ansari et al., Jamshidi et al., and Pourbakhsh et al. used vlhA based PCR for differentiation of Iranian field isolates of MS. In order to differentiate field and vaccine strains of MS, Bayatzadeh et al. analyzed and sequenced vlhA gene of 21 Iranian field isolates. They also used PCR-restriction fragment length polymorphism (RFLP) for characterization of isolates. They stated that DNA sequence analysis and PCR-RFLP were suitable tools for distinction between wild type and vaccine strains of MS. Amplification of haemagglutinin-encoding vlhA gene, sequencing and phylogenetic studies have been reported earlier by researchers to apperceive the relationships between the MS field and MS-H strain.

Broiler flocks of older than 3 weeks old with respiratory involvement were investigated to elucidate role of MS in respiratory complexes. Eight samples (38.1%) out of 21 were positive using both MS specific primers. Four apparently healthy flocks were also included in this survey. Two of them were positive, that emphasize role of MS as subclinical respiratory pathogen. Bayatzadeh et al., analyzed 43 broiler flocks for MS contamination. They noted 55.9% of swab samples were positive by PCR of 16S rRNA. In another study, 24 (55.0%) out of 43 samples of suspected flocks of three provinces of Iran were positive by PCR of vlhA. Results of above mentioned studies indicated relatively high prevalence of MS in poultry flocks of Iran.

Bayatzadeh et al., classified Iranian field isolates of MS to eight groups based on sequence similarity and phylogeny. Three out of four Iranian strains of current study including MS01, MS06, and MS07 had high sequence similarity with strains of group 3 (MSR12 is representative of group 3). MS16 had high sequence similarity with strains of group 3 and 4 (MSR15 is representative of group 4), (Table 2). Phylogenetic analyses based on nucleotide sequences also showed that Iranian field isolates of the current study clustered together with strains of group 3 and 4 (Fig. 2). It must be noted that nucleotide and amino acid sequence alignments of MS16 and MSR7 (representative of group 8 in Bayatzadeh et al. scheme) were the same (Table 2). However, phylogenetic analysis showed that they were distinct from each other.

Alignment of Iranian field isolates and MS-R (H) vaccine strain has critical importance. Ghafouri et al., Ansari et al., and Pourbakhsh et al. used vlhA based PCR for differentiation of Iranian field isolates in group 3 and 4. However, phylogenetic analyses based on nucleotide sequences also showed that Iranian field isolates of group 3 and 4 (MSR12 and MSR15) according to Bayatzadeh et al. scheme, also had G at this position.

Phylogenetic analysis of the vlhA gene of MS strains revealed that Iranian field isolates of current study clustered independently from the isolates of other countries and vaccine strain MS-H. Bayatzadeh et al. also cited that MS isolates of Iran are local strains.

This study certified the potential value of strain typing for epidemiological reasons and suggested that phylogenetic study of vlhA genes was essential to understand the true relationships between strains. Such investigations provide researchers with a better knowledge on the distribution, variability, and phylogenetic relationships of different MS isolated in Iran and other parts of the world.

Acknowledgments

This study was fully supported by grants (No. 92-D-002) from Vice Chancellor for Research and Technology of Urmia University that is acknowledged by the author.

References

1. Ferguson-Noel N, Noormohammadi AH. Mycoplasma synoviae Infection. In: Swayne DE (ED), Diseases of poultry. 13th ed. Hoboken, USA: Wiley-Blackwell 2013; 1687-1698.
2. Harada K, Kijima-Tanaka M, Uchiyama M, et al. 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 Dis 2009; 53: 538-543.
3. Lauerman LH, Hoerr FJ, Sharpston AR, et al. Development and application of a polymerase chain reaction assay for Mycoplasma synoviae. Avian Dis 1993; 37: 829-834.
4. Garcia M, Jackwood MW, Levisohn S, et al. Detection of Mycoplasma gallisepticum, M. synoviae, and M. iowae by multi-species polymerase chain reaction and restriction fragment length polymorphism. Avian Dis 1995; 39: 606-616.
5. Vasconcelos AT, Ferreira HB, Bizarro CV, et al. Swine and poultry pathogens: the complete genome sequence of two strains of Mycoplasma hyopneumoniae and a strain of Mycoplasma synoviae. J Bacteriol 2005; 187: 5568-5577.

6. Beric RL, Slavec B, Lavric M, et al. Identification of major immunogenic proteins of Mycoplasma synoviae isolates. Vet Microbiol 2008; 127: 147-154.

7. Bencina D. Haemagglutinins of pathogenic avian mycoplasmas. Avian Pathol 2002; 31: 535-547.

8. Noormohammadi AH, Markham PF, Whithear KG, et al. Mycoplasma synoviae has two distinct phase variable major membrane antigens one of which is a putative hemagglutinin. Infect Immn 1997; 65: 2542-2547.

9. Noormohammadi AH, Markham PF, Kanci A, et al. A novel mechanism for control of antigenic variation in the hemagglutinin gene family of Mycoplasma synoviae. Mol Microbiol 2000; 35: 911-922.

10. Bencina D, Drobnic-Valic M, Horvat S, et al. Molecular basis of the length variation in the N-terminal part of Mycoplasma synoviae hemagglutinin. FEMS Microbiol Lett 2001; 203: 115-123.

11. Hong Y, Garcia M, Leiting V, et al. Specific detection and typing of Mycoplasma synoviae strains in poultry with PCR and DNA sequence analysis targeting the hemagglutinin encoding gene vhA. Avian Dis 2004; 48: 606-616.

12. Development, evaluation of an improved diagnostic PCR for Mycoplasma synoviae using primers located in the hemagglutinin encoding gene vhA and its value for strain typing. Vet Microbiol 2009; 136: 61-68.

13. Slavec B, Lucijana Bercic R, Cizelj I, et al. Variation of vhA gene in Mycoplasma synoviae clones isolated from chickens. Avian Pathol 2011; 40(5): 481-489.

14. Jeffery N, Gasser RB, Steer PA, et al. Classification of Mycoplasma synoviae strains using single-strand conformation polymorphism and high-resolution melting-curve analysis of the vhA gene single copy region. Microbiology 2007; 153: 2679-2688.

15. Kleven SH, Jordan FTW, Bradbury JM. Avian Mycoplasmosis (Mycoplasma gallisepticum, M. synoviae). Manual of standard for diagnostic tests and vaccines. 7th ed. Paris, France: Office International des Epizooties; 2012; 6.

16. Ritchie PA, Anderson IL, Lambert DM. Evidence for specificity of psittacine beak and feather disease viruses among avian hosts. Virology 2003; 306:109-115.

17. Tamura K, Stecher G, Peterson D, et al. MEGA 6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 2013; 30(12):2725-2759.

18. Ghafoori SA, Bozorgmehri Fard MH, Karimi V, et al. Identification and primary differentiation of Iranian isolates of Mycoplasma synoviae using PCR based on amplification of conserved 5' end of vhA gene. J Vet Res 2011; 66(2): 117-122.

19. Pourbakhsh SA, Shokri GR, Banani M, et al. Detection of Mycoplasma synoviae infection in broiler breeder farms of Tehran province using PCR and culture methods. Arch Razi Inst 2010; 65(2): 75-81.

20. Haghbin Nazarpak H, Pourbakhsh S. Isolation and detection of Mycoplasma synoviae from seropositive rapid reaction broiler breeder flocks by polymerase chain reaction and culture methods. J Vet Microbiol 2010; 6(1): 90-95.

21. Ansari H, Pourbakhsh SA, Sheikh N, et al. Detection of Mycoplasma synoviae by vhA-PCR with special primers in clinical sample. Vet J 2010; 4(12): 673-682.

22. Jamshidi P, Ayazi M, Nazemshirazi MH. Differentiation of Mycoplasma synoviae strains in Iran by PCR. Life Sci J 2014; 11(3): 31-35.

23. Pourbakhsh SA, Maghami M, Ashtari A, et al. The vhA gene sequencing of Iranian Mycoplasma synoviae isolates. Arch Razi Inst 2013; 68(2): 117-124.

24. Bayatzadeh MA, Pourbakhsh SA, Ashtari A, et al. Molecular typing of Iranian field isolates of Mycoplasma synoviae and their differentiation from commercial live vaccine strain MS-H using vhA gene. Br Poult Sci 2014; 55(2): 148-156.

25. Bayatzadeh MA, Pourbakhsh SA, Homayounimehr AR, et al. Application of culture and polymerase chain reaction (PCR) methods for isolation and identification of Mycoplasma synoviae on broiler chicken farms. Arch Razi Inst 2011; 66(2): 87-94.

26. Maghami M, Pourbakhsh SA, Homayounimehr AR, et al. Diagnosis and detection of Mycoplasma Synoviae from commercial poultry flocks using polymerase chain reaction (PCR) based on amplification of vhA gene. Comp Pathobiol Iran 2013; 9(4): 837-846.

27. Ogino S, Munakata Y, Ohashi S, et al. Genotyping of Japanese field isolates of Mycoplasma synoviae and rapid molecular differentiation from the MS-H vaccine strain. Avian Dis 2011; 55: 187-194.