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Full Article

The vlhA gene sequencing of Iranian *Mycoplasma synoviae* isolates

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ABSTRACT

Mycoplasma synoviae expressed variable lipoprotein haemagglutinin (VlhA) is believed to play a major role in pathogenesis of the disease by mediating adherence and immune evasion. The aim of this study was sequencing Iranian *M. synoviae* isolates for the detection of nucleotide variation in the *M. synoviae* vlhA gene. Using oligonucleotide primers complementary to the single-copy conserved 5´ end of vlhA gene, amplicons of ~400 bp were generated from 10 *M. synoviae* isolated from commercial broiler chicken farms in Iran, afterward the conserved domain of the vlhA gene of *M. synoviae* was sequenced and analyzed for Iranian isolates. The results showed that, there was a complete concordance between all Iranian isolates nucleotide sequence (1-386 nt). In comparison with vaccine MS-H strain sequence, all Iranian isolates; entire vlhA sequence downstream of nucleotide 386 was different. It was also observed in all Iranian *M. synoviae* isolates, point mutations and frame-shift mutation. This study was demonstrated a difference between Iranian isolates and live commercial vaccine MS-H strain. Furthermore, these data indicated that changes in the vlhA gene sequence could introduce into the expressed vlhA gene amino acid codons and effective in pathogenesis rate in flocks.

Keywords: *Mycoplasma synoviae*, Haemagglutinin, VlhA gene, Sequence analysis, Vaccine MS-H strain, Iran

INTRODUCTION

Mycoplasma synoviae (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). *M. synoviae* infection most frequently manifests as a subclinical upper respiratory infection. However, respiratory diseases can occur as a result of a synergistic interaction between *M. synoviae* and other pathogens, and systemic infection typically leads to infectious synovitis. *M. synoviae* may be transmitted either laterally via direct contact or vertically via eggs (Harada *et al* 2009). In *M. synoviae*, haemagglutinins are encoded by related sequences of a multigene 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

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virulence of avian mycoplasmas (Bencina *et al* 2002, Narat *et al* 1998). The uniquely expressed vlhA gene of *M.synoviae* 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 *M.synoviae* 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).

More recently, sequence analysis of the single-copy conserved region of the *M. synoviae* vlhA gene has been used for investigations of *M. synoviae* strains and epidemiological (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 analysed (Jeffery *et al* 2007). The vlhA gene sequencing and analysis of *M. synoviae* isolates is of critical importance, particularly in countries that poultry flocks are vaccinated with the live *M. synoviae* strain MS-H. The main purpose of the present study was to perform sequencing Iranian *M. synoviae* isolates for the detection of nucleotide variation in the *M. synoviae* vlhA gene.

MATERIALS AND METHODS

Sampling. Samples were collected from 3 central province of Commercial broiler chicken farms (Tehran, Markazi and Qazvin). Most of the samples were obtained from flocks with clinical signs of infection by *M.synoviae*. A total of 10 field samples obtained from trachea and the lung/air sac. Samples were collected on cotton swabs by opening the trachea and vigorously rubbing the mucosa with the tip.

***M. synoviae* isolates.** The *M. synoviae* isolates for which the vlhA sequences (encoding MSPB) were determined are described in Table.1 *M.synoviae* strains were grown on a modified Frey's medium containing 12-15% swine serum.

DNA Extraction. DNA extraction was accomplished using phenol-chloroform method.

Amplification with specific primers (PCR). In this study tow published primer sets were used for the specific detection of genus and species of *M.synoviae*. For genus Mycoplasma as follow: MYF: 5'-GCTGCGGTGAATACGTTCT-3', MYR: 5'- TCCCC ACGTTCTCGTAGGG -3'. The 163 bp fragments were amplified (Kojima et al 1997). In *M.synoviae* species as follow: MSCons-F: 5'- TACTATTAGCAGCTAGTG C-3', MSCons-R: 5'- AGTAACCGATCCGCTTAAT-3'. The 350-400 bp fragments of *M.synoviae* vlhA gene were amplified (Jeffery et al 2007). The vlhA-PCR mix was performed in a total volume of 25µL per sample, containing 2.5 µl of 10 X PCR buffer (Roch Diagnostics-corporation, Indianapolis, USA), 4 µl of 25 mM $MgCl_2$, 0.3 µl of 10 mM dNTPs, 0.3+0.3 µl each primer, 1 U Taq DNA polymerase (Roch Diagnostics-Corporation, Indianapolis, USA). Consequently 15.2 µl of deionized distilled water and 1.7 µl of extracted DNA as template was carried out.The vlhA-PCR reaction was conducted in a Gradient Mastercycler (Eppendorff, Germany) as follows: In genus: 7.5 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at

56 °C and 1 min at 72 °C, with a final extension cycle of 5 min at 72 °C.

Sequencing. PCR products were purified from the agarose gel by High pure PCR product purification kit (Roche, Germany) according to the manufacturer's instruction and sent to MWG Biotech Company (Ebersberg, Germany) for sequencing. To justify the PCR results and to evaluate sequence variations among *M. synoviae* isolates from central regions of Iran, partial vlhA gene from 10 isolates were sequenced and sequences were determined for both strands of DNA and Blast in GeneBank (early assembled, and edited using DNAsis Max 3.0 software and afterward Blast in NCBI and aligning all the sequences manually by BioEdit 7.0). Bioedit 7.0 program was used for aligning all the sequences manually, and for deducing the amino acid sequences.

DNA similarity. The percent similarity of all the M. synoviae vlhA gene sequences was determined using the program Bioedit version 7.0. All Iranian isolates were then examined into those showing 100% similarity and afterward isolates and MS-H vaccine was selected for subsequent phylogenetic analysis.

Analysis of sequences. Dendrograms were constructed from alignments of the representative strains by the neighbour-joining method with 1000 bootstrap replicate analyses, using the Molecular Evolutionary Genetic Analysis (MEGA) software for sequence alignments.

RESULTS

Testing the primer pairs. Using the MSCons-F and MSCons-R primer a PCR product was obtained with all 10 samples tested. The amplicon sizes varied between 350 and 400 bp (Figure 1).

The vhlA gene sequence analysis on iranian *M. synoviae* isolates. Since the live *M. synoviae* vaccine was not used in these farms, PCR method was applied to the involvement of *M. synoviae* infection in trachea and the lung/air sac samples taken. Samples were collected from commercial broiler chicken farms in 3 central provinces of Iran (Tehran, Markazi and Qazvin) and the obtained results were compared with the vaccinal strain (The data obtained from GeneBank) on the basis of vlhA gene sequence analysis.

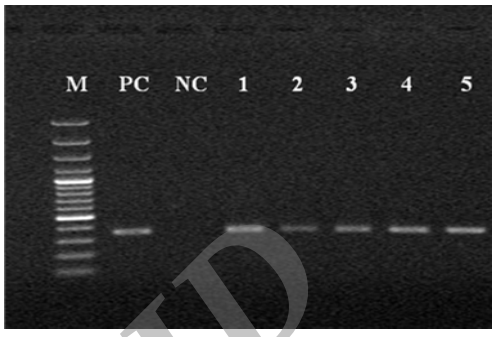

Figure 1. PCR electrophoresis gel demonstrating *M.synoviae* vlhA gene amplification with MSCons-F – MSCons-R primer; M: 100 bp DNA marker ladder; NC:Negative control; PC: Positive control (MS-NCTC 1012-05); Lanes: 1-5, Positive samples in the study (350-400 bp).

The conserved regions of vlhA gene were sequenced for all 10 isolates, and have been deposited with the DNA Data Bank of Iran under accession numbers JX233541–JX233550. The names and origins of these isolates are shown in Table 1. In this study, deletion of

Table 1. *M. synoviae* Iranian isolates used in this study.

Accession number in GeneBank	Isolate name	from	Base pair
JX233541.1	MSR640	Qazvin	357
JX233542.1	MSR382	Markazi	352
JX233543.1	MSR816	Markazi	353
JX233544.1	MSR836	Tehran	410
JX233545.1	MSR715	Qazvin	352
JX233546.1	MSR371	Tehran	349
JX233547.1	MSR811	Tehran	351
JX233548.1	MSR815	Markazi	355
JX233549.1	MSR850	Qazvin	352
JX233550.1	MSR761	Qazvin	353

27 nucleotides was found in Iranian *M. synoviae* isolates when compared with MS-H. Furthermore, 24 nucleotides were found to be replaced by other nucleotides in all the isolates examined (Table 1, Figure 2 and Figure 4). The overall sequence similarity in Iranian partial vlhA gene sequences examined in this study was 81% between MS-H and isolates. We determined the vlhA sequences encoding MSPB

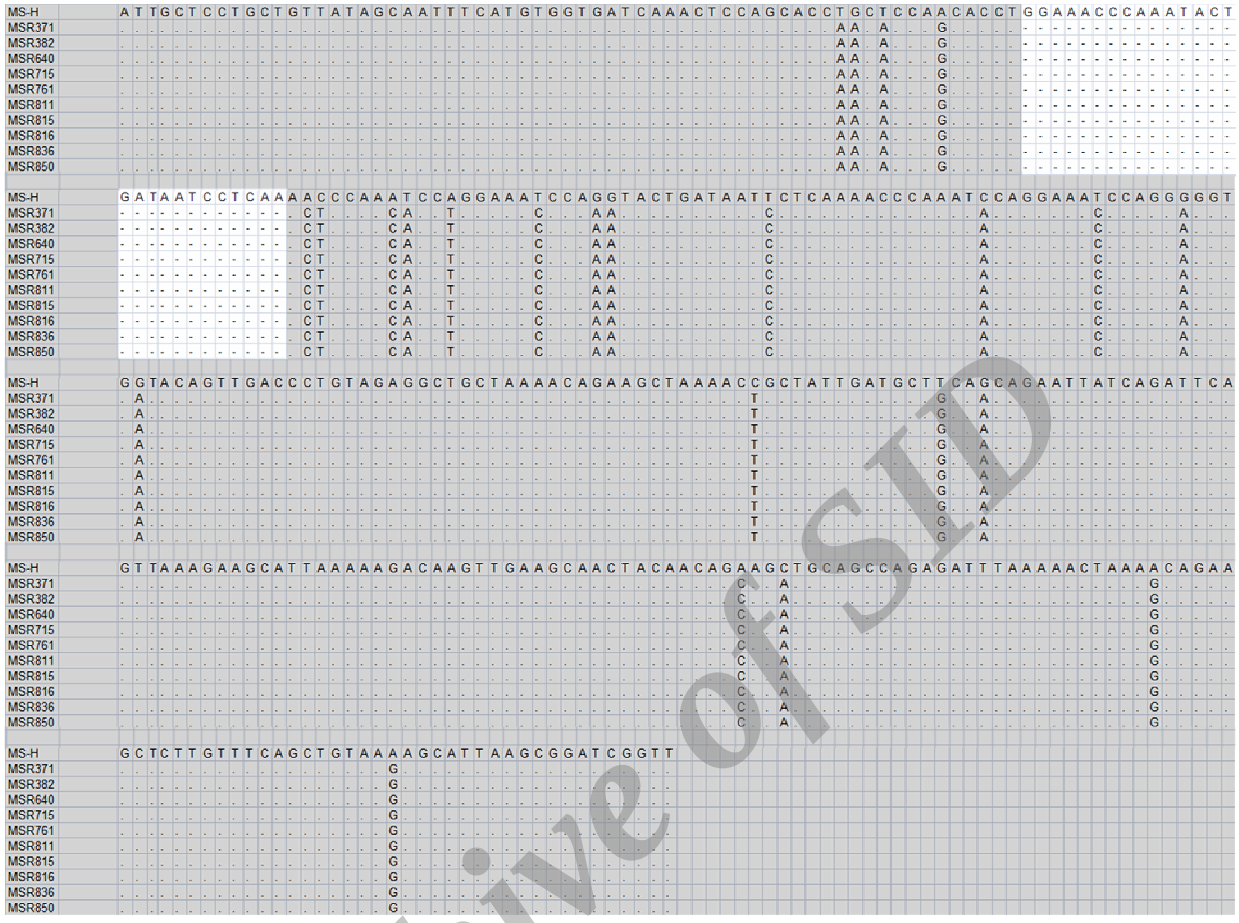

Figure 2. Comparison of the partial nucleotide sequence of the vlhA gene amplified from Iranian isolates with MS-H strain. Comparison of the deduced amino acid sequences of the N-terminal region of MSPB proteins predicted from the 5′-end vlhA sequences of 30 M. synoviae strains. The sequence in the first line was determined for MS-H strain and corresponds to nt 49-398 in the vlhA sequence (GeneBank accession number AF464936.1). Alignment of a partial vlhA sequence of 10 M. synoviae isolates starting with nucleotide position 1. Note that the corresponding starting positions resemble in all Iranian isolats and positions differ with MS-H strain, due to insertions or deletions shown in Fig. 3. The aligned sequences are varied at four positions that were identical in all isolates: deletion and nucleotide substitutions, whereas identity to the reference vlhA

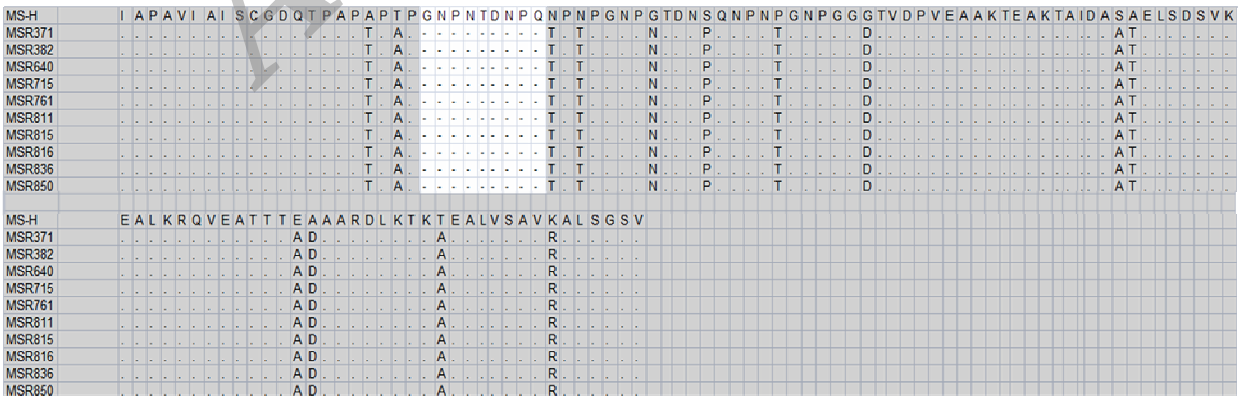

Figure 3. Comparison of the deduced amino acid sequences of the N-terminal region of MSPB proteins predicted from the 5'-end vlhA sequences of 10 M. synoviae isolates. The sequence in the first line was determined for the strain MS-H (GenBank accession number AF464936.1). In comparison with MS-H, in the sequence length of the Iranian isolates observed conversion into the expressed vlhA gene amino acid codons: before PRR region (PAP to PTP), into PRR region (T→A; N→T; G→N; S→P; P→T) and after PRR region (ASAE to AATE; TEAA to TADA).

proteins of all *M.synoviae* isolates (Figure 3). All isolates encoded a similar conserved end of the signal peptide (APAVIAISCG). The first amino acids of their deduced MSPB had an identical sequence with MS-H, except in 18 and 20 amino acids, in which the changed codon into region (CCTGCTCCAACA to CCAACACCAGCA) would alter the predicted sequence from PAPTP to PTPAP. In comparison with the MSPB sequence of MS-H, the MSPB of Iranian isolates had some deletion amino acids (GNPNTDNPQ) within Sequence length (Figure 3). The most notable conserved region was observed in the vlhA region encoding PRR (Figure 2 and Figure 3), the PRR sequences of all *M. synoviae* isolates examined in this study share into all the sequence with each other. In comparison with the MSPB sequence of MS-H, all Iranian isolates was observed in the vlhA region encoding PRR (Figure 2 and Figure 4) that there was some bases changes at nucleotides positions 58 (A→G), 92, 93 (AC→CT), 98, 99 (AT→CA), 102 (A→T), 108 (T→C), 112, 113 (GG→AA), 124 (T→C), 139 (C→A), 147 (T→C), 153 (G→A) and 158 (G→A) in Iranian isolates (Figure 2 and Figure 3).

Table 2. Total deduction from percent mutations in Iranian isolates sequence.

Transversion mutation		**Transition mutation**	
Mutation	Number of nucleotides	Mutation	Number of nucleotides
A→T	1	A→G	3
T→A	3	G→A	6
A→C	3	T→C	3
C→A	2	C→T	2
C→G	-	**Frame-shift mutation**	
G→C	-	Mutation	Number of nucleotides
T→G	1	Deletion	27
G→T	-	Addition	-

DISCUSSION

Using PCR for detection of differences in a relatively short stretch of the *M. synoviae* vlhA gene and sequencing of the amplified product has also been described for detection of *M. synoviae* isolates variations. In this study showed that, there was a complete concordance between all Iranian isolates nucleotide sequence (1-386 nt) and the 5́-vlhA region sequence remained unchanged. These data indicated 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. This study demonstrated that a difference between Iranian isolates and live commercial vaccine strain. Furthermore, in comparison between the vlhA gene sequences of Iranian isolates and vaccinal MS-H strain that Iranian isolates derived most probably from the same ancestor and separate from MS-H vaccine strain. DNA sequence analysis and phylogenetic studies based on the haemagglutinin-encoding vlhA gene for apperceive the true relationships between the *M. synoviae* strains and MS-H strain, have been reported earlier (Harada *et al* 2009, Ogino *et al* 2011). Harada *et al.* (2009) was compared with the MS-H vaccine strain, tested field isolates neither had identical vlhA sequences nor were classified into the same Bercina's type or phylogenetic cluster. Ogino *et al.* (2010) conserved domain of the vlhA gene of *M. synoviae* was sequenced and analyzed for 19 field strains of *M. synoviae* isolated from chickens across Japan and also they was genotyped of Japanese field isolates of *M. synoviae* and rapid molecular differentiation from the MS-H vaccine strain. In Iran, using by vlhA-PCR, previous studies and methods used for differentiation of *M. synoviae* (Ansari *et al* 2010, Ghafori *et al* 2011), but do not sequences analysis for comparison Iranian isolates with MS-H strain. This study is the first demonstration of diversity of the vlhA gene in Iranian *M. synoviae* isolates in comparison with vaccine MS-H strain In this study have been showed that there was a complete concordance between all Iranian isolates nucleotide

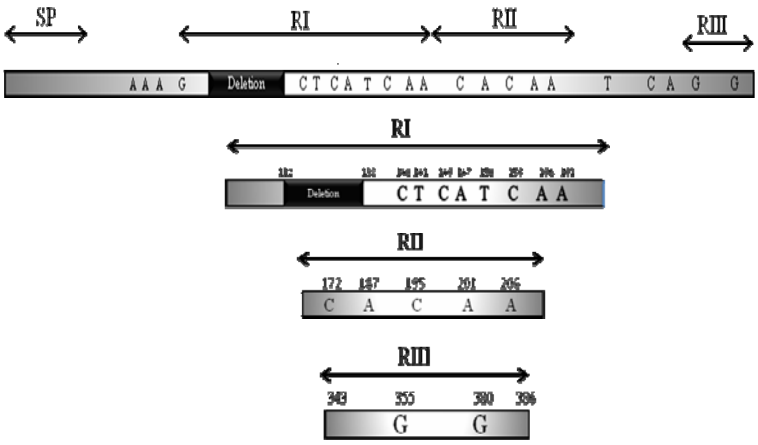

Figure 4. Schematic representation of the vlhA gene sequence of Iranian *M. synoviae* isolates. The above picture is schematic representation of sequence that its include RI, RII, RIII regions and positions of deletion and replace (in comparison with MS-H vaccine strain) in continuation shown separately, RI, RII, RIII regions.

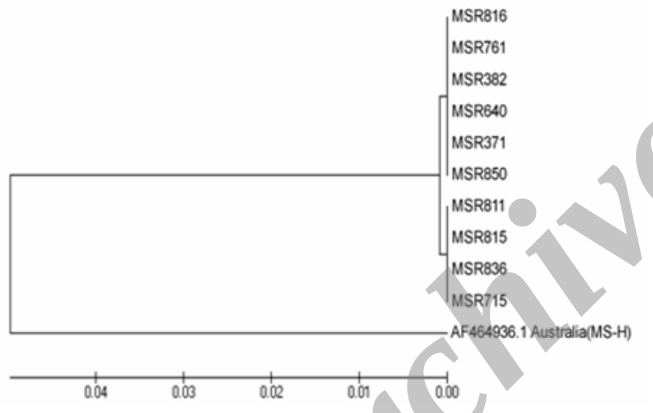

Figure 5. Phylogenetic tree constructed by neighbor-joining method based on the partial sequence of vlhA gene of M. synoviae. The sequences were obtained from the 10 samples in this study and sequence from AF464936.1 (MS-H strain). Values at nodes indicate bootstrap probabilities, as determined for 1000 resembling. Accession numbers are shown in Table 1.

sequence (1-386 nt) and the 5́-vlhA region sequence remained unchanged in all *M. synoviae* isolates of which were examined in this study. 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 *M. synoviae* 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.* (2010), with the alignment of MS-H and the 9 representative Japanese strains isolated, showed that the some isolates in Japanese had 12 additional identical nucleotides, which were not present in MS-H and suggested them method can play a promising role in such studies by 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 *M. synoviae* and the live *M. synoviae* vaccine MS-H strain (Ogino *et al* 2011). This study demonstrated that in contrast, the complete sequence identity of the conserve region of the expressed vlhA gene of Iranian isolates with the corresponding sequence of vaccine strain, the 5́-vlhA region of vlhA gene in all isolates, contains at least 24 nucleotides same differ in the sequence downstream of nucleotide 386 and on the other hand, isolates obtained had vlhA sequences that differed downstream of nucleotide 386 from vaccine strain. In comparison with MS-H, in all Iranian isolate examined in this study, some nucleotides were substituted with other nucleotides. Thus, it is likely that the presence of nucleotide changes is a result of uneven sequence between Iranian isolates and MS-H strain; this is reinforced by the DNA homologies because comparison of Iranian isolates with MS-H, with the deletion and nucleotide changes, the similarity is 81%. In the 5́-vlhA region of Iranian isolates nucleotide sequence, has point mutations content of 47 %, and while through the length of the region has frame-shift mutation content of only 53 %. 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, in addition insertions/deletions were observed in all the isolates examined and may be related to pathogencity (Bencina *et al* 2001). In comparison with the MSPB sequence of

MS-H strain, the MSPB of Iranian isolates had some amino acid codons variation and deletional nine amino acids (GNPNTDNPQ), into PRR region. In this study, data indicated that changes in the vlhA gene sequence can influence in insertions/deletions rate and expressed amino acid codons of the vlhA gene. DNA sequence analysis was used to determine the phylogenetic relationships among the *M. synoviae* isolates and the tree showed which Iranian isolates were more closely related together. Therefore, if such a case is expected by epidemiologic background such as vaccination history and trend of *M. synoviae* in chicken flocks, it would be necessary that multiple strains per farms are carefully isolated and subjected to molecular investigations. It was suggested that the addition of DNA sequence analysis studies is essential to understand the true effects of vaccine MS-H strain on the Iranian isolates. The 5́-vlhA region is present in the *M. synoviae* genome as a single copy and does not change its sequence in clonal populations of *M .synoviae* (Noormohammadi *et al* 2000). This observation is crucial to strain identification as downstream of this region the sequence can change even in clonal populations of *M .synoviae* and therefore it cannot be considered a conserved sequence that characterizes individual strains (Noormohammadi *et al* 2000, Hammond *et al* 2009), thus sequencing the same region of the vlhA gene seemed to be useful for comparing local isolates with MS-H strain, this studies can be used to investigate whether *M. synoviae* isolates from diseased chickens have differed from the vaccine strain, too. Our data indicate that sequencing and polymorphisms of the 5́-vlhA region might be very useful for compare isolates 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 *M. synoviae* isolates in poultry flocks. In conclusion, the data of the present study provide novel information about vlhA diversification in Iranian *M. synoviae* isolates; the vlhA gene sequence changes produced nucleotide diversity. These data indicated that changes in the vlhA gene sequence could introduce into the expressed vlhA gene amino acid and effective in pathogenesis rate in flocks. This study using of vlhA gene analysis could successfully differentiate between the vaccine MS-H strain and the isolates. Therefore, more investigations based on the vlhA gene conversion event of the nucleotide sequence of Iranian isolates and uncover nucleotide variations through the length of the conserve region in the vaccine MS-H strain, which are increasingly used in *M. synoviae* control programs, could be applied**.** Another forms of association between the pathogenicity of *M. synoviae* and may provide key information to the industry in terms of epidemiology.

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