Molecular Characterization of *Mycoplasma Synoviae* Isolated From the Respiratory System and Joints of Chickens with Special Reference of *vlhA* Gene

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**ABSTRACT**

The present work was designed to make sequencing of *M. synoviae* isolates for identification of nucleotide differences in the *M. synoviae* vlhA gene which isolated from the respiratory system and joint from chickens. A total of 224 chicken samples (109 respiratory samples, 115 joint samples) collected from birds from different flocks showing respiratory manifestation and arthritis were examined. Identification of *M. synoviae* by PCR Using primers corresponding to the single copy preserved 5’ end for vlhA genetic factor, amplicons for 350~400 bp then, generated which 6 and 26 out of 224 samples were positive samples for *M. synoviae* from culture and tissue by PCR respectively. High-resolution melting curve analysis (HRM) for amplicons using SYBR green fluorescent dye of 9 selected isolates (5 respiratory isolates and 4 arthritic isolates) showed that different Melting temperature curves were ranged between 84.6-85.1°C for isolates MS from the respiratory samples and other melting temperature curves ranged between 73.6-74.6°C for isolates Ms from the arthritic samples. The results proved that, there was a broad concordance among nucleotide sequence of all isolates which isolated from the respiratory system except in one sample which observed the point mutations and the frame-shift mutation in some nucleotide differ than other respiratory isolates in addition there is complete concordance between nucleotide sequence of all isolates which isolated from joint. However, in comparison the Respiratory *M. synoviae* isolates with *M. synoviae* joint isolates, there was clear variation in nucleotide sequence which was confirm the result of HRM. Compared to vaccine MS-H strain arrangement, all isolates show clear variation from (MsH) vaccine strain. This study proved a difference between *M. synoviae* isolated from the respiratory system and Joint and live commercial vaccine (MsH) strain. Also, these informations showed that variations in the vlhA gene sequence could be introduced into the expressed vlhA gene amino acid codons and effective in pathogenesis rate in herds.

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1. **INTRODUCTION**

*Mycoplasma synoviae* is one of the most significant agents of disease that can be presented as joint and/or respiratory conditions. Although, the symptomatic bird shows respiratory problems (as cough, sneezing, aero sacculitis and sinusitis), impaired growth and synovitis. Chronic and asymptomatic infections are both more common and more important because of the losses they cause (Lockaby et al., 1998; Menegatti et al., 2010).

*M. synoviae* (MS) was known as a significant pathogen of domestic poultry worldwide, which
resulted in great financial losses in the concentrated poultry industry (Kleven et al. 1997) besides retarding growth in poultry and turkeys (Khiari et al., 2010).

*M. synoviae* infection most frequently manifests as a subclinical upper respiratory infection. However, respiratory infections can happen as a result of synergistic interaction among *M. synoviae* and other pathogens, and systemic infection naturally leads to infectious synovitis. *M. synoviae* can be spread either via direct contact or via eggs (Harada et al., 2009). In *M. synoviae*, Haemagglutinins account among the most significant surface proteins involved in colonization and virulence of avian Mycoplasmas (Narat et al., 1998; Benčina et al., 2001). Haemagglutinins are determined by sequences of a multigene family stated as variable lipoprotein hemagglutinin (vlhA) genes (Noormohammadi et al., 1997; Benčina et al., 1999). The expressed vlhA genetic factor 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 occurred directly after amino acid residue 344 (Noormohammadi et al., 2000). Both MSPA and MSPB are surface exposed proteins and show high frequency antigenic variation (Noormohammadi et al., 1997; Noormohammadi et al., 1998). Such a gene replacement mechanism, also known as gene conversion, permits a single strain of *M. synoviae* to create a huge number of variants by recruiting new sequences from a great pseudogene reservoir. This pseudogene reservoir could be limited to a restricted part of the genome, which provides an optimal environment for site-specific recombination (Khiari et al., 2010). Furthermore, the approach did not detect whether the nucleotide variation noticed relates to genomic rearrangements that commonly occur within strains (Noormohammadi et al., 2000). The inter-strain variety at the vlhA expression site, including major variances in the predicted secondary structures of the stated adhesions (May and Brown, 2011).

Corresponding functional variances in the extent to which they agglutinated erythrocytes, a quantitative substitution for VlhA-mediated cytoadherence, were also obvious by (May and Brown, 2011). The vlhA gene- targeted PCR is valuable tool for detection and initial typing of *M. synoviae* and could be applied in preliminary identification of *M. synoviae* isolates directly from clinical samples (Hong et al., 2004). High-resolution melting curve study (HRM) without using a fluorescence hybridization probe has been presented newly as a rapid technique for genotyping and mutation scanning of *M. synoviae* (Gundry et al., 2003). The introduction of novel fluorescent dyes, for example LCGreenl (Wittwer et al., 2003) and SYTO 9 green (Monis et al., 2005; Krypuy et al., 2006). The advantage of real time PCR over traditional PCR is the ability to measure kinetics of the reaction in the early phase of PCR, and to detect of mutations without requiring electrophoresis (Coleman and Tsongalis, 2006).

The PCR based SSCP or HRM curve analyses of vlhA afford high resolution mutation discovery tools for the detection and identification of *M. synoviae* strains. Especially, the (HRM) curve analysis is a rapid and effective technique which can be performed in a single test tube in less than 2 h (Jeffery et al., 2007). HRM curve analysis could differentiate between Ms isolates, the reference strain in addition to the vaccine strains. Melting temperature for the reference strain Ms9 was observed at 84.24 °C, for the other field isolates (Ms1, Ms2, Ms3, Ms4, Ms5, Ms6, Ms7, Ms8, Ms17) the melting temperature ranged from 83.81-85.01 °C (lower than the vaccine strain) and 85.62 °C (higher than the reference strain in addition to field isolates) in vaccine strain Ms10. More recently, sequence study of the single copy preserved region of the *M. synoviae* vlhA genetic factor has been used for surveys of *M. synoviae* strains and epidemiological (Noormohammadi et al., 2000; Benčina et al., 2001; Hong et al., 2004; Hammond et al., 2009; Slavec et al., 2011). PCR-based mutation discovery techniques provide useful and cost effective replacements for the direct analysis of genetic variation, mainly when large numbers of samples are to be analysed (Jeffery et al., 2007). Gene sequencing of vlhA genetic factor and analysis of *M. synoviae* isolates is very important, especially in countries where poultry were vaccinated with the live *M. synoviae* strain MS-H.

The chief purpose of the present study was to make sequencing of isolates of *M. synoviae* for the discovery of nucleotide variation in the *M. synoviae* vlhA gene which isolated from respiratory system and Joint. The main aim of the current study was to compare SYBR green (HRM) curve studies for the detection of inter-strain nucleotide variations in the *M. synoviae* vlhA gene, as replacements for sequencing, and to measure their usefulness for routine analytical and epidemiological applications.

2. MATERIALS AND METHODS
2.1 Sample collection:
Samples were gathered from poultry herd’s with clinical signs of infection by M. synoviae.

1-A total of 160 field samples (100 samples obtained from trachea, the lung and air sac and 60 samples obtained from synovial fluid and tendon sheath). Tracheal samples were collected on cotton swabs by opening the trachea and vigorously rubbing the mucosa with the tip or take one tracheal ring and Joint samples taken by opening of joint under aseptic condition and take swabs /part of tendon sheath.
2-Sixty four clinical samples (9 tracheal ring and 55 synovial fluid) used for comparison between culture method and PCR

2.2. Isolation of M. synoviae:
The collected samples pre inoculated in a saline solution supplied with penicillin G for 15-30 min before cultivation on the selective medium to decrease the contamination of the samples then, which inoculated into closed sterilized tubes containing 2-3 ml of a modified Frey’s broth medium containing 12-15% swine serum then incubated at 37C for 3-5 days and observed every day for acidity specified by change of the color of medium from red to yellowish- orange (Kleven, 1997). The positive inoculated tubes were cultivated on Frey’s agar medium for 2-3 weeks in a closed container supplied with damp cotton for increasing the humidity and lighted candle for CO2 production and O2 exhaustion in the container air. The incubated plates were examined every day for any bacterial growth.

2.3. PCR of amplification:
DNA Extraction: DNA extraction was extracted (from 19 suspected positive M. synoviae culture from 160 sample) and 64 tissue samples used for comparison between culture method and PCR using using QIAamp DNA Mini Kit following the guidelines of the instructions of the manufacturer. Two oligonucleotide primers Connection (5'-TACTATAGCAGCTAGTGC-3') and MSCons-R (5'-AGTAACCCGATCCGTATAT-3') were designed and used to amplify 350–400bp of the single copy preserved 5 end of the vlhA genes (Jeffery et al., 2007) from various M. synoviae strains. The amplification reactions were done in a total volume of 25µl containing 12.5µl of Emerald Amp GT PCR master mix (2x premix), 1µl of each primer (20 pmol), 4.5µl of nuclease free water besides 6µl of the template of DNA .Cycling conditions of the primer during PCR was 94 °C at 5 min , afterward 35 cycles of temperature 94 °C at 30 sec, 54°C at 45 sec and temperature 72 °C at 45 sec, in addition to a final extension cycle of temperature 72 °C at 10 min .All PCR products were then purified by using the QIAquick PCR purification kit (Qiagen) before nucleotide sequencing .

Agarose gel electrophoresis: The amplified products were then electrophoresed through a 1.5% gel of agarose and then detected by staining with ethidium bromide.

DNA Molecular weight marker: The ladder (100 bp) was mixed mildly by pipetting up and down. Six µl of the needed ladder were directly loaded.

HRM curve analysis: Amplification of target sequences was done on Real time PCR machine (Stratagene MX3005P)). Each 25µl reaction consisted of 12.5 µl of 2x QuantiTect SYBR Green PCR Master Mix, 0.5µl of each primer (50pmol) (Link and MSCons-R), 4.5µl of RNase Free Water and7µl of extracted M. synoviae genomic DNA. The reaction mix was incubated at 94 C for 5min and then exposed to 40 cycles of 95C for 30 sec, 54 for 30 sec and 72C for 40 sec. After achievement of 40 PCR cycles enter in one cycle of dissociation curve cycle were 95C for1 min , 54 for 30 sec and 95C for 30sec (HRM) curve study was done by using the application.

2.4. Sequencing and nucleotide sequence study: The resulted PCR products were purified by means of the QIAquick PCR purification kit (Qiagen Inc. Valencia CA) following the manufacturer's instruction then, sent to MWG Biotech Company (Germany) for sequencing. To validate the PCR results and to assess sequence variations between M. synoviae isolates, incomplete vlhA genetic factor from 9 isolates were sequenced and sequences were examined for both DNA strands by using the same primers as for PCR and Blasted in GenBank (early collected, and edited using DNAsis Max 3.0 software.

2.5. DNA similarity
The nucleotide sequences were compared with each other and with those previously accessible in GenBank (accession no AF464936, AF035624, GU084388, KC832809, GU084386, AY913824, AY913826, KP316021, AY913823, AY907705, AY907706, AY907704, AY913825, AF314229, AF314228 and AF314230). The percent of resemblance of all the M. synoviae vlhA gene sequences was determined using The CLUSTALW multiple sequence alignment program, version 1.83 of Meg Align module of Laser
gene DNAStar software Pairwise, which was designed by (Thompson, 1994). Sequence alignments and phylogenetic comparisons of the aligned sequences for the gene were also performed with the MegAlign module of LasergeneDNAStar software to determine nucleotide and sequence of amino acid similarities and relationships. All respiratory isolates were examined showing 100% similarity except one sample show 97.9% similarity with other respiratory isolates and all arthritis isolates were examined showing 100% resemblance, but there is clear variation between respiratory and arthritis isolate and subsequently isolates, MS-H vaccine and other strain from gene bank were designated for subsequent phylogenetic analysis.

3. RESULTS

Results of amplification of *Mycoplasma synoviae* (vlhA) coding genetic factor by using PCR: six isolates out of 19 suspected *M. synoviae* and 26 samples out of 64 samples were found positive for *M. synoviae* according to the vlhA genetic factor sequence available in GenBank, two oligonucleotide primers, Link and MSConsR, were designed and used for amplification of 350–400 bp of the single copy conserved 5 end of the vlhA genetic factor (Noormohammadi et al., 2000) as revealed in Figure 1,2,3, 4).

3.1. HRM curve outlines of the vlhA PCR products from *M. synoviae* isolates: Ten *M. synoviae* isolates (4 arthritis and 6 respiratory) were selected and subjected to (HRM) curve analysis as mentioned in Methods. All strains generated a major peak melting but 6 Melting temperature were ranged between 84.6–85.1°C for isolates MS isolated from respiratory samples and other 4 melting temperature curve ranged between 73.6–74.6°C for isolates Ms isolated from synovial fluid samples as shown in (Fig. 4 and Table 1).

Samples were gathered from marketable broiler and layer chicken farms and the results were compared with the vaccinal strain (The information gained from GeneBank) according to vlhA genetic factor sequence analysis. The preserved regions of vlhA gene were then sequenced for all 9 isolates, and have been placed with the DNA Data Bank which takes this accession number (KT957960-KT957961-KT957962-KT957963-KT957964-KT957965-KT957966-KT957967and KT957968).

All respiratory samples (resp2, 3, 4 and 5) show complete identity (100%) except resp1 strain which there were some bases variations at nucleotides positions 80, 81 (CT?AA), 135 (T?C), 141 (G?A), 166 (G?A), 187 (G?A), 206 (T?C), 268 (G?T) and 300 (C?A) in Resp1 isolates compared with other Respiratory isolates. Also there were deletion of 39 nucleotides from respiratory *M. synoviae* isolates when compared to MS-H vaccine and there were deletion of 57 nucleotides from respiratory isolates of *M. synoviae* when compared with Joint isolates of *M. synoviae* in addition there were some bases changes between Resp1 and Joint isolates at nucleotides positions 80, 81 (CT?AA), 135 (T?C), 141 (G?A), 187 (G?A), 189 (T?C), 206 (T?C) and 272 (C?A) and there were some bases changes between Resp2,3,4,5 and Joint isolates at nucleotides positions 166 (A?G), 189 (T?C), 268 (T?G) and 300 (A?C) shown at Figure 5.

All joint samples show complete similarty in between them, but there insertion of 18 nucleotides found in joint *M. synoviae* isolates when compared to MS-H vaccine shown at Figure 5.

The complete sequence similarity in partial sequences of vlhA genetic factor which were examined in this study was 92.9% between MS-H and joint *M. synoviae* isolates and 89.5% with respiratory *M. synoviae* isolates. As well as sequence similarity in partial sequences of gene of vlhA examined was 85.5% between joint *M. synoviae* isolates and respiratory *M. synoviae* isolates.
Figure 1. Gel electrophoresis demonstrating amplification of *M. synoviae* *vlhA* gene: Lane 1 and 17: 50 bp DNA ladder. Lanes: 2-5, 9-10, 11 and 21: Positive samples in the present study (350-400 bp).

Figure 2: PCR electrophoresis gel indicating *M. synoviae* *vlhA* gene amplification isolated directly from synovial fluid. Lane 11: DNA molecular weight ladder (100 bp) in (1) and Lane (8) in (2) Lane (2, 3, 4, 5, 6, 7, 8, 9, 10, 13, 14, 16, 17, 18, 19, 20 and 21): positive for *M. synoviae* in (1) and Lane (6, 7, 12 and 13): Positive for *M. synoviae* in (2). Lane (1, 2, 12 and 22): Negative for *M. synoviae* in (1) and Lane (1, 2, 4, 5, 9, 10, 11, 14, 15 and 16) in (2).

Fig. 3. PCR electrophoresis gel indicating *M. synoviae* *vlhA* gene amplification isolated directly from tracheal organs. Lane 4: DNA molecular weight ladder (100 bp). Lane (1, 2, 3, 5 and 6): Positive for *M. synoviae* Lane 7: Negative for *M. synoviae*
We detected the vlhA sequences encoding MSPB proteins of all *M. synoviae* isolates (Figure 6). All isolates encoded a similar preserved part of the signal peptide (LLLAASAVAIAPVIAISCQDGTPAP). The first amino acids of their MSPB had an identical sequence with MS-H, excluding 27 amino acids, where the changed codon into region (GCT to GAA) would modify the sequence which was predicted from PAP to PEP.

In comparison to MSPB sequence of (MsH), and the MSPB of respiratory isolates which observed that there were deletion in 13 amino acids (NPGTDNSQNPNPG) inside Sequence length also with the MSPB sequence for Joint isolates observed some insertion of 7 amino acids (GNPGTPG).

In comparison the MSPB sequence for respiratory isolates with the MSPB sequence of Joint isolates observed that the MSPB sequence of Joint isolates had insertion of 20 amino acids (GNPGTPGNPGTDNSQNPNPG) inside Sequence length.

In this study were complete similarity in protein sequencing of Joint isolates. Also were complete similarity in protein sequencing of respiratory isolates except respiratory1 which there were some nucleotide variation which lead to encoding different protein in 27 amino acids, in which changed sequence from PEP to PAP and in 55 amino acids (ETA to EAA), in 62 amino acids (KTA to KAA), in 68 amino acids (SAE to SVE), in 90 amino acids (ESA to EAA) and in 101 amino acids (AHA to ADA).

The sequence in first line was determined for MS-H strain (GenBank accession number AF464936.1). Alignment of a partial *vlhA* sequence of 9 *M. synoviae* isolates starting with nucleotide position 1. Note that the matching starting positions resemble in all isolates and positions vary with MS-H strain and between isolates with each other, because of insertions or deletions shown in Figure 5.
Fig. 5: Comparison of partial nucleotide sequence of the vlhA genetic factor amplified from different *M. synoviae* isolates. The computer software CLUSTALW fast was used in this comparison. Matching nucleotides and deletions are shown by ‘.’ and ‘-’ respectively. Comparison of the partial nucleotide sequence of vlhA genetic factor amplified from Respiratory and Joint isolates of MS-H strain.
Sequence analysis of \textit{vhlA} gene of \textit{M. synoviae} isolates. PCR technique was applied to the participation of \textit{M. synoviae} in trachea, the lung/air sac and synovial fluid samples.
4. DISCUSSION

This study delivers a direct comparison between nucleotide sequencing and (HRM) curve analysis using SYBR green dye for discovery of differences between the different MS isolates obtained from field and also to distinguish them from the live vaccinal strain in a comparatively short stretch of the \textit{M. synoviae} vlhA gene. The results showed that both nucleotide sequencing and (HRM) curve analysis were capable of detecting variations of 1 bp in PCR products of 400 bp. Nucleotide sequencing and SSCP are time-consuming measures and require skill for interpretation of results. On the contrary, the (HRM) curve analysis is rapid and convenient, and all relevant procedures including PCR and melting-curve analysis can be done in a single tube. An additional benefit of (HRM) curve analysis is that it can be done in an automatic module. High resolution melting curve analysis discriminates DNA samples based on sequence, length, and GC-content. When there is a mutation in the sequence, it results in a different melting temperature and therefore different melting curves (Jeffery et al., 2007). More significantly, within a single strain of \textit{M. synoviae}, the vlhA single-copy gene region targeted for PCR in this study is known conserved (Benčina et al., 2001; Noormohammadi et al., 2002; Hong et al., 2004).

This study defines, the use of real time PCR followed by melting curve for detection and strain classification of isolates of \textit{M. synoviae}. An arbitrary-primed PCR has formerly been described for detection of \textit{M. synoviae} strain variations (Fan et al., 1995), but the reproducibility of the method is still under question and the results are hard to be interpreted. Moreover, the approach does not determine whether the profile differences detect relates to genomic changes that occur commonly inside single isolates (Noormohammadi et al., 2000). PCR followed by sequencing of the amplified produce has also been described (Hong et al., 2004) for detection of \textit{M. synoviae} strain variations; however, this technique is consuming time and the results often need interpretation. The Australian isolates 93220/C-27a and 93107/5b has been revealed to be distinct from MS-H by RFLP for genomic DNA (Markham et al., 1998).

In this study 10 isolates subjected to (HRM) analysis, the melting temperature of respiratory isolates showed clear variations with melting temperature of Joint isolates. As already mentioned in the results, the melting temperature of the respiratory isolates ranged from 83.81-84.62 °C but the melting temperature of joint isolates ranged from 73.6-74.6°C. All (HRM) curve profiles created from \textit{M. synoviae} strains in this study were found to have a major peak and no shoulder peak which occurred from primer dimmers and confirmed by only one single DNA band could be noticed when subject PCR products to agarose gel electrophoresis. There was not much difference in melting temperature of Joint isolates and their sequences were 100% similar. Also there was not much difference in melting temperature of respiratory isolates and their sequences did not differ much but, there is clear variation with nucleotide sequencing of
joint isolates. The result which mentioned by (Raphela, 2012) who proved that (HRM) curve analysis could differentiate between Ms isolates, reference strain and vaccine strains which Melting temperature for the reference strain Ms9 was observed at 84.24 °C, for the other field isolates (Ms1, Ms2, Ms3, Ms4, Ms5, Ms6, Ms7, Ms8, Ms17) the melting temperature ranged from 83.81-85.01°C (lower than the vaccine strain) and 85.62 °C (higher than reference strain and field isolates) in vaccine strain Ms10.

In summary, the usage of PCR and HRM curve analysis is a fast and specific technique for the characterization of *M. synoviae* isolates. The whole process including extraction of DNA, PCR and (HRM) curve analysis can be completed within 2 h. In this study, In comparison with the result of PCR HRM with sequencing, there was a complete concordance between all respiratory isolates and complete similarity between joint *M. synoviae* isolates but, there is a clear variation between nucleotide sequence of isolates of respiratory and joint and the 5- vlhA region sequence remain unchanged. These data indicates that changes in the vlhA genetic factor sequence can be introduced into the expressed vlhA genetic factor amino acid codons and translation therefore these changes can be effective in pathogenesis rate in flocks. Also, this study showed that a variance between isolates (respiratory and joint) and live commercial vaccine strain.

Additionally, in comparison between the vlhA gene sequences of isolates and vaccinal MS-H strain that isolates did not derived from MS-H vaccine strain. DNA sequence study and phylogenetic studies based on the haemagglutinin encoding vlhA genetic factor for apperceive the true relations among 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 to the MS-H vaccine strain, verified field isolates neither had the same vlhA sequences nor were classified into identical Bercina's type or phylogenetic cluster. Ogino et al., (2011) preserved 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 *M. synoviae* field isolates and rapid molecular diversity 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; Ghafouri et al., 2011), but do not sequences analysis for comparison Iranian isolates with MS-H strain. Till now, there is no research in the available research made sequencing of the vlhA gene to compare between *M. synoviae* isolated from respiratory and joint in which the changes in the vlhA genetic factor sequence can introduce into the expressed vlhA genetic factor amino acid codons and translation therefore these changes can be effective in pathogenesis rate in flocks. In This study is the first demonstration of variety of the vlhA genetic factor in *M. synoviae* isolates from respiratory and joint compared with vaccine MS-H strain and In this study have been presented that there was complete variation between isolates but phylogenetic analysis of joint isolates with other strain in gene bank isolated from joint as (AF035624.1) were 100% also phylogenetic analysis of respiratory isolates with other strain in gene bank isolated from trachea as (GU084388.1) were 98.8%. As revealed in figure (7).

(Harada et al., 2009) supposed 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. Ogino et al. (2011), with the alignment of MS-H and the 9 representative Japanese strains isolated, presented that the some isolates in Japanese had additional matching 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 inside conserved region in all the Japanese isolates, and they established a PCR method differentiate between isolates of *M. synoviae* and live *M. synoviae* vaccine MS-H strain (Ogino et al., 2011).

This study verified that in contrast, the complete sequence identity of the conserved region of the expressed vlhA gene of isolates with the corresponding sequence of vaccine strain, the 5-vlhA region of vlhA genetic factor in all isolates, there were insertion in joint isolates and deletion from respiratory isolates at least 39nt. In contrast with MS-H, in all isolate examined in this study, some nucleotides were substituted with other nucleotides also there were insertion in joint isolates and deletion from respiratory isolates at least 17nt. Therefore, it is likely that the presence of nucleotide changes is a result of genetic rearrangement of isolates to express variable protein for pathogenesis, Which agree with the result.
mencioned by (Benčina et al., 2001) expressed that changes in the vlhA genetic factor sequence can be introduced into the expressed vlhA genetic factor 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 pathogenicity (Benčina et al., 2001). In contrast with the MSPB sequence of MS-H strain, the MSPB of respiratory isolates had some amino acid codons differences and deletional13 aminoacids (NPPTDNSQPNPG). But the MSPB of joint isolates sequencing had some amino acid codons differences and insertion 7 amino acids (GNPGTPG) this may be due to the using of vaccine strain in Egypt not in wide range, so the isolated M.synoviae not originated from vaccine strain. In this study, data showed that variations in the vlhA genetic factor sequence can influence in insertions/deletions rate and expressed amino acid codons of the vlhA genetic factor which play role in pathogenesis of microorganism. It was recommended that the addition of DNA sequence analysis studies is essential to understand the true effects of vaccine MS-H strain on the isolates. The 5- vlhA region is present in the M. synoviae genome as a single copy and does not alter 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 consequently 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 genetic factor 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 5- vlhA region might be very useful for compare isolates with MS-H vaccine, also indicate that sequence analysis of the 5- vlhA region has the potential to become a valuable means for tracing spreading of M. synoviae isolates in poultry flocks. Inconclusion, the data of the present study offer novel information about vlhA diversification in M. synoviae isolates; the vlhA genetic factor sequence changes produced nucleotide diversity. These data indicated that changes in the vlhA genetic factor sequence could introduce into the expressed vlhA genetic factor amino acid and effective in pathogenesis rate in flocks. This study using of vlhA genetic factor analysis could effectively differentiate between vaccine MS-H strain and the isolates.

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