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Genetic analysis of Iranian *Mycoplasma gallisepticum* isolates using gene-targeted sequencing of complete *pvpA* gene

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Abstract: This study aims to evaluate Mycoplasma gallisepticum (MG) genetic diversity through the complete sequencing of the pvpA gene among six MG isolates present in industrial poultry from 2015 to 2016. The genetic comparison of these six isolates showed similarities of over 94% and 92% in terms of nucleotide and amino acid sequences, respectively. The size of the PvpA protein in the studied isolates included three different sizes with 361, 364, and 366 amino acids. The carboxy-terminus of the studied PvpA proteins contained 11 tetrapeptides of P-R-P-M/Q/N motifs with truncation in the proline-rich region. Based on the BLAST analysis, the pvpA genes of six MGs were closely related to isolates from house finch in the USA and the S6 strain. According to the phylogenetic analysis, two isolates were located along with the S6 strain that showed an outgroup association with MG isolates from Germany, China, and the USA. Other isolates of this study were placed in a separate cluster with MGs isolated from Iran during 2006–2013. The results of this study are useful to recognize the circulating genotypes in Iran and their differences from other MGs from different parts of the world. In addition, continuous monitoring of poultry flocks could be significant for obtaining valuable information related to different MG strains and designing effective control strategies.

Key words: Mycoplasma gallisepticum, MG, pvpA gene, sequencing, chicken, Iran

1. Introduction

Mycoplasma gallisepticum (MG) is one of the well-known pathogenic avian mycoplasmas affecting chickens and turkeys. MG infections are commonly known as chronic respiratory disease in chickens and infectious sinusitis in turkeys. Primary clinical manifestations of MG include tracheal rales, coughing, sneezing, nasal discharges, and conjunctivitis, while the common symptom of infected turkeys is swelling of the infraorbital sinuses [1]. MG infection usually progresses slowly and remains unidentified as long as stress conditions lead to an outbreak of the disease. The synchronization of this infection with other respiratory pathogens, such as Newcastle disease virus, infectious bronchitis virus, and *Escherichia coli*, may cause some complications in the treatment and diagnosis. This infection causes severe economic losses in the poultry industry. In broiler chickens, MG is responsible for reduction in weight gain and increased feed conversion ratio, carcass condemnation rate, and mortality. In commercial breeding flocks, it may reduce egg production and increase mortality [1–3].

To control mycoplasma infection effectively, three main approaches are used: management, antibiotic treatment, and vaccination. The most important control strategy in most countries, especially Iran, is to maintain mycoplasma-free breeding flocks. Control of MG is generally possible based on the eradication of this organism from breeders and their progeny by adopting various biosecurity measures. Although antibiotic therapy is an effective procedure to reduce the transmission of mycoplasma vertically as well as clinical signs and lesions, this method does not wholly eliminate the infection from the flock and may cause the emergence of antibiotic-resistant mycoplasmas [2]. Vaccination is recommended only in areas where exposure to MGs is unavoidable. The three live vaccines commercially available in the world include strain F, 6/85, and ts-11, which induce vaccine strain replacement in multiage poultry farms [1,2,4].

The protein banding patterns in sodium dodecyl sulfate-polyacrylamide gel electrophoresis and restriction fragment length polymorphism have been used to differentiate MG isolates [5–7]. In addition, MG strains

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have been characterized using DNA and ribosomal RNA gene probes and physical chromosomal mapping [8–11]. The disadvantages of these methods include being time-consuming and having high costs and complexity. DNA fingerprinting techniques such as arbitrary primed PCR and random amplified polymorphic DNA (RAPD)-PCR are useful in vaccine and strain analysis as well as in epidemiological studies. However, standardization of these technique makes the reproducibility difficult, and also creates different interpretations for banding patterns of RAPD-PCR results [12–16]. Furthermore, pure culture of MG is one of the initial needs for performing fingerprinting techniques, which is hardly achieved in field situations. The targeted sequencing of single or multiple genome loci (*mgc2*, *pvpA*, *gapA*, MGA_0319, and 16S/23S rRNA intergenic spacer region) is the preferred method for genotyping of MG strains [17,18].

MG is classified as a member of the genus *Mycoplasma* within the family Mycoplasmataceae, order Mycoplasmatales, and class Mollicutes [19]. This organism is one of several genera with a special ending structure, which attaches to the respiratory epithelium. The PvpA protein localizes at the tip structure, showing high size frequency and phase variations [20,21]. Phenotypic changes in the MGs are related to the expression of this protein, which helps these bacteria escape the host immune system and survive in infected flocks for a long period of time [20,22]. This protein is a nonlipid integral membrane protein in which the carboxy-terminus is located at the surface of the MG organism. The carboxy-terminus of this protein is rich in proline and contains direct repeat (DR) sequences of DR-1 and DR-2 with 52 amino acids each. The size variation of this gene among different strains of MG results from truncation of the PvpA protein within the proline-rich region (PRR) at the C-terminal ends [20].

In the past decades, adoption of a comprehensive monitoring and control plan for commercial poultry farms and optional removal of MG-positive flocks have reduced the incidence of MG in Iran. However, MG has occasionally caused outbreaks, mortalities, and significant economic losses in the poultry industry. To our knowledge, genetic variation of MG isolates based on the *pvpA* gene from industrial poultry in Iran has not been published yet. Some studies have been limited to the identification and serology detection of MG, or the survey of other genes in Iran [23–25]. Therefore, prevention strategies and effective monitoring of infection are pivotal in the surveillance of MG in industrial poultry.

2. Materials and methods

2.1. Isolation and cultivation of MG isolates

All samples were isolated from tracheal and palatine cleft swabs of broiler breeders and layer chickens in 2015–2016 according to the method described previously [26,27]. Briefly, the swabs were placed in 5 mL of modified Frey's broth as a transport medium and transferred to the *Mycoplasma* reference laboratory (Razi Vaccine and Research Institute, Karaj, Iran) at 4 °C within less than 24 h. The medium containing samples was incubated immediately at 37 °C in an incubator maintaining 5% CO_2 for about 8 h, and then passed through a 0.45-μM filter. Subsequently, samples were subcultured in fresh broth (1:10 v/v) and examined daily for 7–10 days. Growth was indicated by acid color change from red to orange or yellow in the broth. In some instances, several passages were needed to isolate MG from field materials. A positive broth culture containing MG was confirmed by PCR reaction according to OIE methods [27].

2.2. Cloning and transformation

Total genomic DNAs of all studied isolates were extracted from broth cultures in Frey's medium according to the method described by Markham et al. [28]. The mycoplasma DNA was subjected to PCR to amplify the full length of the *pvpA* gene using a specific primer set (primer sequences available upon request), which were designed to amplify a 1735-bp fragment. PCR products were purified with a High Pure PCR Product Purification Kit (Roche Life Science, Mannheim, Germany). PCR-purified products were cloned using a ClonJET PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, the cloned products were selected randomly and plasmid DNAs were extracted by High Pure Plasmid Isolation Kit (Roche Life Science). The expected *pvpA* gene insert was confirmed using either the BglII restriction enzyme (Thermo Fisher Scientific) or PCR with vector-specific primers.

2.3. DNA sequencing and analysis

Cloned products were sequenced by a DNA service company (Faza Pajooh Company, Tehran, Iran) with two vector sequencing primers (forward and reverse) and two internal primers (primer sequences available upon request). All sequence data were compiled and analyzed using BioEdit 7.0.5 software [29]. Nucleotide and deduced amino acid sequences were aligned with the Clustal W method in MEGA software version 7.0.26 [30]. BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was performed to retrieve sequences identical to our queries. A phylogenetic tree was generated by neighbor-joining method with maximum composite likelihood model in MEGA 7.0.26 software [30]. The robustness of the phylogenies was determined by bootstrap analysis of 1000 replicates.

2.4. Nucleotide sequence accession numbers

The six *pvpA* gene sequences of MG isolates obtained in this study are available from GenBank under accession numbers MK984191–MK984195 and MK984197.

3. Results

3.1. Analysis of nucleotide and amino acid sequences of *pvpA* gene

The *pvpA* gene of six MG isolates was completely sequenced from 2015 to 2016. The amino acid and nucleotide sequences of the *pvpA* genes were then compared with other MG isolates in GenBank. The peptide chain of the PvpA protein was composed of three different length sizes with 361, 364, and 366 amino acids, which shows the size variation in the studied MGs. The number of amino acids truncated within both DR-1 and DR-2 regions were 17 and 20 in the analyzed isolates (Figure 1). The genetic comparisons of these six isolates in terms of nucleotide and amino acid sequences revealed that there were 94.5%–99.9% and 92.3%–100% homologies between these six strains, respectively (Table 1). The results of BLAST analysis showed the most similarity to MGs from house finch isolated from the USA in 1994–2008 [31] and strain S6 (Tables 2 and 3).

The amino acid sequence of six isolates at the carboxy-terminus of the PvpA protein is shown in Figure 1. In

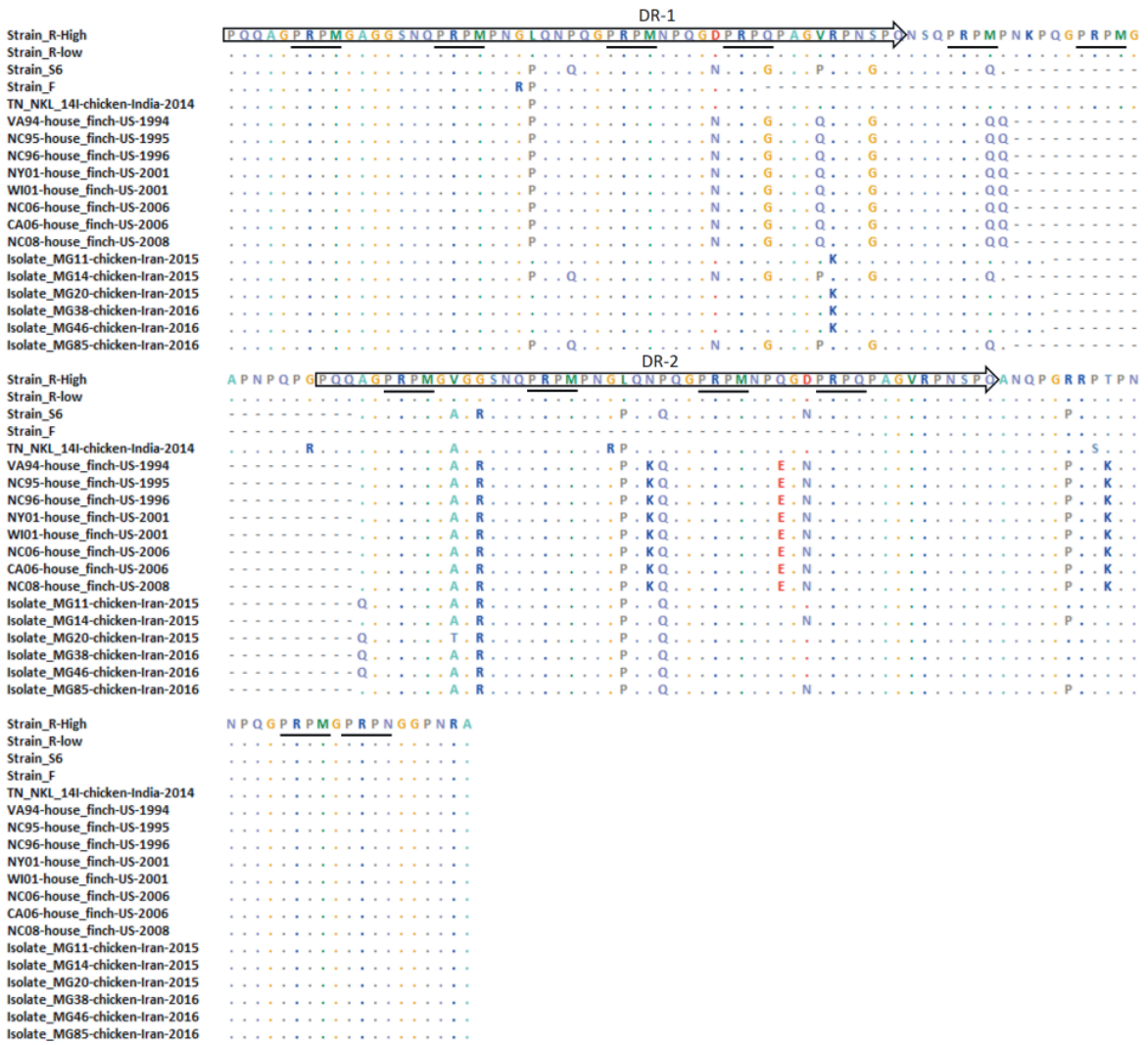

Figure 1. Amino acid substitutions in the carboxy-terminus of the PvpA protein of the Iranian MG isolates. Direct repeated (DR) regions are shown with right arrows. The repeated tetrapeptide motifs are underlined. Dots indicate no change from Strain R (High). Dashes represent deletions in comparison to Strain R (High).

Table 1. Nucleotide (nt) and amino acid (aa) sequence identities of Iranian MG isolates.

Isolate	Isolate MG11-chicken-Iran-2015		Isolate MG14-chicken-Iran-2015		Isolate MG20-chicken-Iran-2015		Isolate MG38-chicken-Iran-2016		Isolate MG46-chicken-Iran-2016		Isolate MG85-chicken-Iran-2016	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Isolate MG11-chicken-Iran-2015			94.7	92.6	99.8	99.7	**99.9**	**100.0**	97.9	96.7	**94.5**	92.6
Isolate MG14-chicken-Iran-2015	94.7	92.6			94.7	**92.3**	94.8	92.6	96.4	95.8	99.8	**100.0**
Isolate MG20-chicken-Iran-2015	99.8	99.7	94.7	**92.3**			**99.9**	99.7	97.9	96.4	**94.5**	**92.3**
Isolate MG38-chicken-Iran-2016	**99.9**	**100.0**	94.8	92.6	**99.9**	99.7			98.0	96.7	94.6	92.6
Isolate MG46-chicken-Iran-2016	97.9	96.7	96.4	95.8	97.9	96.4	98.0	96.7			96.4	95.8
Isolate MG85-chicken-Iran-2016	**94.5**	92.6	99.8	**100.0**	**94.5**	**92.3**	94.6	92.6	96.4	95.8		

The highest and lowest percentages of homology are shown in bold.

Table 2. Phylogenetic affiliation of studied isolates based on BLAST comparison to other strains in GenBank database.

Isolate	Most affiliated to	Identity (%)
Isolate MG11-chicken-Iran-2015	House finch/US/2008.031-4-3P/2008 House finch/US/2001.043-13-2P/2001	95 95
Isolate MG14-chicken-Iran-2015	Strain S6 House finch/US/2008.031-4-3P/2008	99 98
Isolate MG20-chicken-Iran-2015	House finch/US/2008.031-4-3P/2008 House finch/US/2001.043-13-2P/2001	95 95
Isolate MG38-chicken-Iran-2016	House finch/US/2008.031-4-3P/2008 House finch/US/2001.043-13-2P/2001	96 96
Isolate MG46-chicken-Iran-2016	Strain S6 House finch/US/2008.031-4-3P/2008	97 97
Isolate MG85-chicken-Iran-2016	Strain S6 House finch/US/2008.031-4-3P/2008	99 98

this region, the number of prolines in the studied strains was 29, 31, 32, 33, and 37. Each of six isolates had 11 tetrapeptide motifs with the P-R-P-X sequence (amino acid X can be methionine, glutamine, and asparagine) at the carboxy-terminus of the PvpA protein. There were four tetrapeptide motifs in each DR region.

3.2. Analysis of nucleotide and amino acid sequences of *pvpA* gene

The phylogenetic tree of the nucleotide sequences of the *pvpA* gene from MG isolates is shown in Figures 2 and 3. Considering the limitations of complete *pvpA* gene sequences in GenBank, two phylogenetic trees of MG isolates were designed based on the complete open reading frame (ORF) and partial sequence of this gene. In both phylogenetic trees, MG14 and MG85 isolates were placed alongside the S6 strain and formed an outgroup association with MGs isolated from Germany, China, and the USA. These results indicate that these strains probably originated from the same genetic pool associated with MGs. Other isolates of this study formed a separate cluster, along with MGs isolates obtained from Iran in 2006–2013 (Figures 2 and 3).

4. Discussion

Thirty years after its first detection in 1926, MG was isolated and identified in Iran by Sohrab et al. [1,32]. Subsequently, MG-induced mycoplasmosis outbreaks have been reported in most major poultry producers worldwide [2,3]. In recent years, the incidence of MG has been reduced in commercial poultry due to the implementation of effective biosecurity programs and removal of MG-infected flocks from breeder farms. However, MG infection still occurs in production farms, including broilers and layers. This infection is endemic in most multiage poultry farms, especially in layers [1,2]. In addition, there is some evidence of subclinical infection of backyard poultry by MG and their role in the transmission of infection to commercial flocks [33,34]. Although several studies have been performed on molecular and

Table 3. Nucleotide sequence identity (%) of the complete *pvpA* gene of the Iranian MG isolates with other representative MGs.

Isolate	Isolate MG11-chicken-Iran-2015	Isolate MG14-chicken-Iran-2015	Isolate MG20-chicken-Iran-2015	Isolate MG38-chicken-Iran-2016	Isolate MG46-chicken-Iran-2016	Isolate MG85-chicken-Iran-2016
Strain R-High (NC017502)	92.9	91.3	92.9	93.0	93.2	91.3
Strain S6 (NC023030)	94.7	**99.8**	94.7	94.8	**96.6**	**99.8**
Strain F (NC017503)	78.2	75.1	78.2	78.2	76.8	75.1
Isolate TN_NKL_14I-chicken-India-2014 (KY745775)	93.3	90.4	93.3	93.4	92.1	90.4
Isolate VA94-house finch-US-1994 (NC018406)	94.9	97.8	94.9	95.0	95.5	97.8
Isolate NC95-house finch-US-1995 (NC018407)	94.9	97.8	94.9	95.0	95.5	97.8
Isolate NC96-house finch-US-1996 (NC018408)	94.9	97.8	94.9	95.0	95.5	97.8
Isolate NY01-house finch-US-2001 (NC018409)	**95.1**	98.1	**95.1**	**95.2**	95.8	98.1
Isolate WI01-house finch-US-2001 (NC018410)	**95.1**	98.1	**95.1**	**95.2**	95.8	98.1
Isolate NC06-house finch-US-2006 (NC018411)	94.8	97.8	94.8	94.9	95.6	97.8
Isolate CA06-house finch-US-2006 (NC018412)	94.9	97.8	94.9	95.0	95.5	97.8
Isolate NC08-house finch-US-2008 (NC018413)	**95.1**	98.1	**95.1**	**95.2**	95.8	98.1

The highest and lowest percentages of homology are shown in bold.

serological diagnosis of MG infection in Iran [23–25], sufficient information related to circulating genotypes in Iran is scarce. Given that the differentiation index of gene-targeted sequencing of the *pvpA* gene is 0.92 [17], a pair of primers was designed to sequence the whole gene in this study in order to better understand the variability and epidemiology of MG in the Iranian poultry industry.

The PvpA protein, which is located at the surface of the MG membrane, shows size and phase variations. This variability causes MG to enter the epithelial cells of the respiratory tract, thereby surviving and escaping the immune system [20,21]. Size polymorphisms in different strains of MG are mainly caused by deletions in the carboxy-terminus within the DR-1 and DR-2 regions, resulting in the removal of 17 and 20 amino acids in these regions [20]. In addition, the variations in the PvpA protein indicate that the region is exposed to selective pressure in the host [20]. The size variations of *mgc2* and *pvpA* have been evaluated to be due to the presence of different nucleotide insertions/deletions as a method for typing of different MG isolates. Since agarose gel electrophoresis is not able to differentiate between two fragments of just a dozen of base pair differences, it is not considered a reliable method for genotyping. Thus, it is highly required to sequence the aforementioned genes [17].

PRRs in prokaryotes and eukaryotes are often found as tandem repeats, which play a role in the conformation of proteins and immunogenic surface antigens [35,36]. These regions are found in the surface-exposed carboxy-terminus of PvpA protein, indicating their roles in binding these proteins to specific ligands and in pathogenicity [20,37]. In addition, there are two identical and repetitive sequences of 52 amino acids interspersed by 25 amino acids and tetrapeptide motifs of 4 P-R-P-M/Q/N in this region [20]. Most amino acid repeats are involved in host-pathogen interactions and binding to cell receptors. In the studied MGs, PRRs with tetrapeptide motifs were observed at the carboxy-terminus of the PvpA protein, as in other representative isolates.

The nucleotide similarities of the *pvpA* gene in two and four isolates were more than 99% and 97%, respectively, indicating that these isolates are likely to be derived from the same ancestor in commercial poultry populations. Furthermore, BLAST analysis was used to investigate the similarities of the isolates to other MGs submitted to GenBank. The results showed 95% similarity to MG strains isolated from house finch and the S6 strain. The latter is one of the acute mycoplasma strains isolated from the turkey brain with encephalitis symptoms, which has been reported to cause salpingitis and decline in the quality and quantity of eggs in experimental studies [38,39]. Since early 1994, MG infection has been diagnosed with clinical symptoms of conjunctivitis in house finches in the Mid-

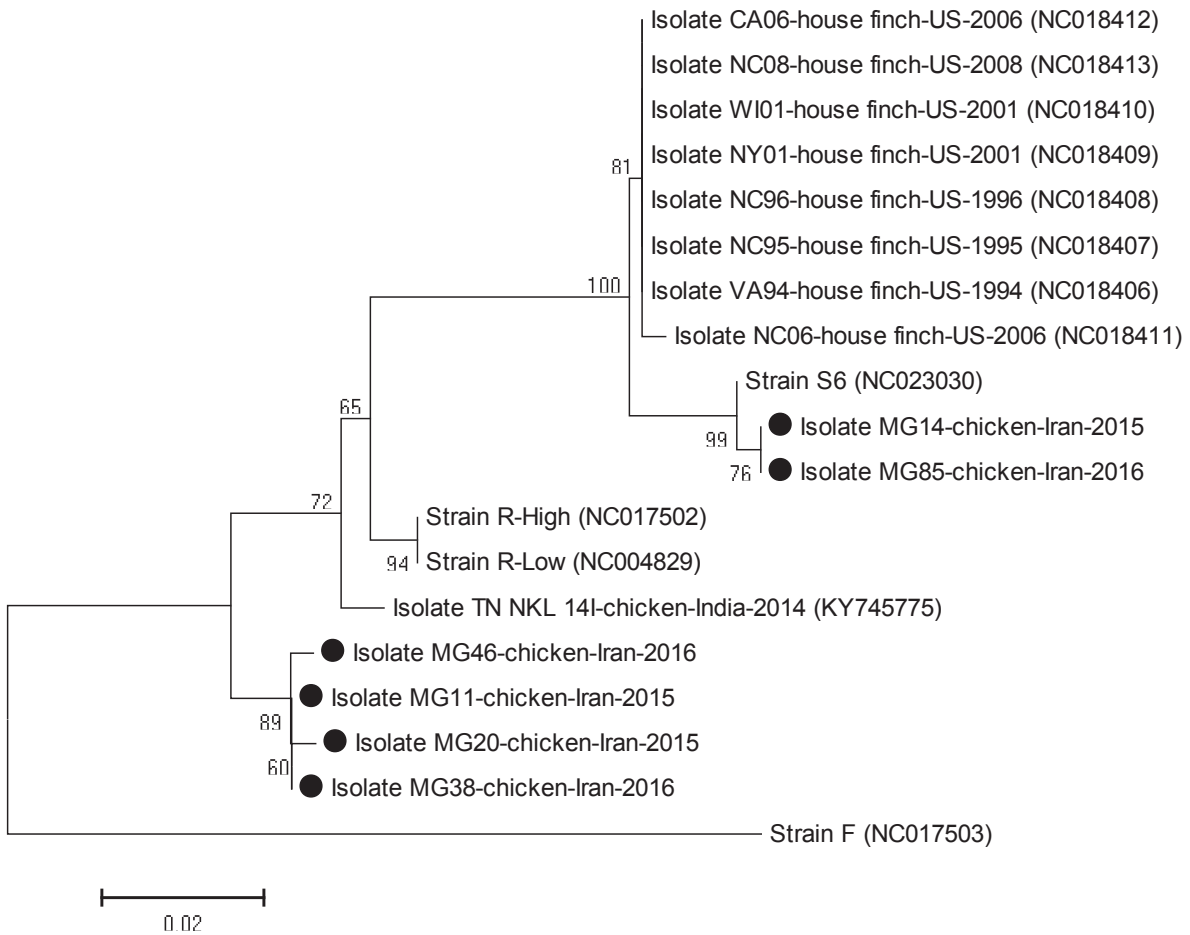

Figure 2. Phylogenetic analysis of the complete *pvpA* gene of the Iranian MG isolates based on complete ORF. The phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replications. The black circle indicates the studied MG isolates.

Atlantic states, and it has spread rapidly to other eastern regions of the USA [40,41]. A genetic comparison of songbird isolates using RAPD-PCR showed the similarity of RAPD banding patterns of these isolates to each other, and differences from other MGs isolated from commercial poultry and vaccine strains. These results revealed that the outbreak of MG in these birds was associated with a strain and a single source [42]. However, MGs isolated from commercial turkeys had a RAPD type similar to that of house finch isolates, and the sequencing results showed that commercial poultry might have been contaminated by a songbird-like MG strain. Given the similarity of the isolates to other MGs from other birds, further subsequent epidemiological studies are required to confirm this similarity.

A previous study partially investigated MG sequences from Russia, the USA, Australia, China, and Iran. According to the phylogenetic tree of the *pvpA* genes, three clusters of these isolates were shown, including groups I, II, and III. Most Russian isolates with less than 8% difference were placed in group I. Group II contained only Iranian MG isolates along with the US isolates and the R strain. Other Russian MGs with strains isolated from the USA, 6/85, S6, ts-11, and F, created group III [43]. Another study revealed that the US MGs isolated from poultry outbreaks and house finches along with vaccine strains formed a distinct cluster. Other isolates from the US and Australia fell into another group, while the Israeli strains were entirely in a separate group [17]. In the present study, MG14 and MG85 were placed in a separate cluster with strain S6, which had a similar ancestor to the other isolates from Germany, China, and the USA, and the other four isolates clustered with other MGs isolated in Iran from 2006 to 2013.

Different genotyping methods are useful for differentiating MG strains in terms of diagnostic and epidemiological purposes for tracing the source of infections. Therefore, identification of new genotypes in

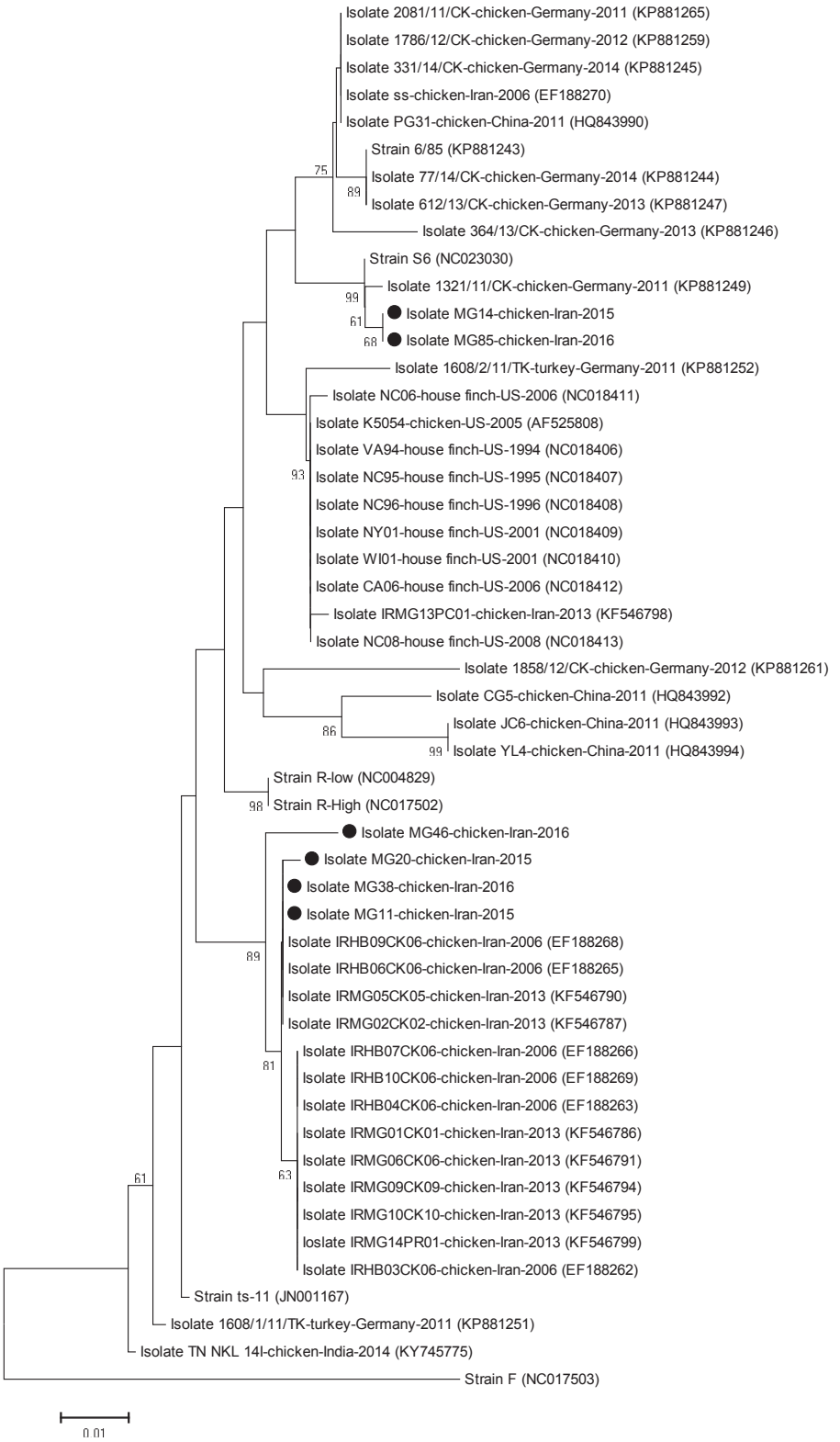

Figure 3. Phylogenetic analysis of the partial *pvpA* gene of the Iranian MG isolates based on 345 nucleotides. The phylogenetic tree was constructed by neighbor-joining method with 1000 bootstrap replications. Bootstrap values of >60 are shown above the branches. The black circle indicates the studied MG isolates.

different geographic regions is effective for developing novel strategies to better control mycoplasmosis. Considering the similarity of studied isolates to other MGs isolated from house finch, whole-genome sequencing may be helpful in determining the relationship between these genotypes and other strains. In addition, further studies are needed to obtain sufficient information about circulating genotypes and establish the relationship between MG outbreaks and geographical areas.

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