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GENETIC DIVERSITY OF *MYCOPLASMA GALLISEPTICUM* FIELD ISOLATES USING PARTIAL SEQUENCING OF THE SECOND CYTADHESIN-LIKE PROTEIN ENCODING GENE FRAGMENT IN IRAN

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ABSTRACT

Mycoplasma gallisepticum (MG) is an economically important pathogen of poultry worldwide, causing respiratory diseases in chickens and turkeys. Differentiation and characterization of MG isolates is critical for epidemiological studies. The aim of this study was the partial sequencing of the second cytadhesin-like protein encoding gene (mgc2) of Iranian MG isolates for detection of nucleotide variation and genetic diversity. For this purpose polymerase chain reaction was conducted on DNA extracts from tracheal swab samples of broiler breeder flocks in Tehran province, Iran. Ten MG isolates were identified and bidirectional sequencing was performed on targeted region. Based on Blast and aligning the DNA sequences were arranged in two main groups and wasn't found any significantly or unique large nucleotide insertion or deletion in the targeted region of detected isolates in comparison with pathogenic reference strain Rlow (97-98.67 percent homogeneity). Accordingly, it appears that the different MG isolates of Tehran province, Iran are not new or diverged MG strains.

Keywords: Mycoplasma gallisepticum, Genetic diversity, Second cytadhesin-like protein encoding gene, Iran

INTRODUCTION

Mycoplasma gallisepticum (MG) is an important avian pathogen that causes respiratory diseases in chickens and turkeys imposing severe commercial losses on the poultry industry worldwide **[1]**. The experience with eradication campaigns in commercial poultry suggests that MG will continue to be a disease requiring ongoing control measures **[2]**. Morever, epidemiological studies show that a diverse range of wild bird species may carry or have been exposed to MG in the USA as well as in Europe and Asia **[3]**.

In Iran, the incidence of MG infection has decreased during the past decade because of control programs on broiler breeder flocks, but extensive epidemiological data is needed for eradication of disease.

MG strains differ markedly in their pathogenicity for chickens depending on the genotypic and phenotypic characteristics **[1]** and the effects of MG on the performance and physiology of the commercial poultry have been shown to vary in relation to strain **[4]**. Accordingly, reliable methods for the differentiation of MG strains are fundamental to epidemiologic investigations of outbreaks, trace the point sources of infection and design relevant controlling strategies **[5]**.

MG has a prominent polar tip organelle or bleb structure that mediates attachment to the host target cells. This tip structure is hemispherical and composed of surface-exposed proteins, called adhesins or cytadhesion proteins. These adhesins are considered to be the most dominant antigens and responsible for successful colonization **[6, 7]**. Nucleotide sequence analysis of genes encoding antigenic surface proteins has been proven useful in the surveillance of pathogenic bacteria **[8]**. Thus, identification and sequencing of genes encoding MG surface-exposed components with adhesive properties have been performed in recent years **[5, 9]** and a large collection of data has underlined the versatility of the MG surface architecture, which is mediated via spontaneous high-frequency variation in the expression and structure of surface proteins **[6]**.

The second putative cytadhesion protein (Mgc2) has been proved to be located at the tip organelle in MG through immunogold labeling and its anti-serum is able to prevent attachment of MG to chicken embryo fibroblast (CEF) cells **[10, 11, 12]**.

Presently, the circulating pool of Iranian field MG has not been genotyped. The main goal of this study was to investigate the molecular

characteristics of Iranian MG isolates and compare them with a well-known reference strain (R_{low}).

MATERIALS AND METHODS

Sampling

Respiratory samples from choanal cleft and trachea of ten broiler breeder flocks with respiratory problems and positive RSAT suspected to be infected with MG in Tehran Province were collected on cotton swabs by vigorously rubbing the mucosa with the tip.

DNA extraction

DNA extraction was accomplished using phenol-chloroform method **[13].** 0/5 ml of each sample was transferred to Eppendorf tube and centrifuged for 15 min at 13000 rpm. The supernatant fluid was discarded and added lysis buffer to the tube equal volume of the tube containing and incubated for at least 4 hrs at 56 ºC. Equal volume of the material in the tube added phenol and mixed well by vortex, Centrifuged at 13000 rpm for 15 min. Removed all aqueous layer (top layer) and transfered in a new tube. Added phenol: chloroform (1:1) in tube equal volume of the tube containing, Centrifuged at 13000 rpm for 15 min and transfered all aqueous layer in a new tube. Added chloroform in the tube, equal volume of the tube containing mix them well by vortex and centrifuged at 13000 rpm for 15 min. Transfered all aqueous layer in a new tube and sodium acetate (3M) was added 1:10 volume of the tube containing and mixed well. Added to them ethanol (ETOH) two fold of material in tube. This solution was placed on -20 °C for 20 min and centrifuged for 15 min at 13000 rpm. Discarded liquid containing of tube softly and Added 200 µl of 70% ETOH, centrifuged for 5 min at 13000 rpm. The DNA was dried and resuspended in 50 µl distilled water at 4ºC and used for PCR.

PCR

In this study two published primer sets were used for the specific detection of genus and species of MG. For genus Mycoplasma as follow, MYF: 5'-GCTGCGGTGAATACGTTCT-3', MYR: 5'-TCCCCACGTTCTCGTAGGG-3' **[13]**. The 163 bp fragments were amplified. The PCR mix was performed in a total volume of 25 µl per sample, containing 2.5 µl of 10X PCR buffer (CinnaGen, Iran), 4 µl of 25 mM MgCl2, 0.5 µl of 10 mM dNTPs, 0.1 µl of each primer, 0.5 U Taq DNA polymerase (5 U/µl). Consequently 15.7 µl of deionized distilled water and 2 µl of extracted DNA as template was carried out. After denaturation at 94 °C for 7.5 min the reaction was performed in 30 cycles with denaturation (94 °C for 30 s), annealing (56 °C for 30 s), primary extension (72°C for 1 min) and a final extension at 72°C for 5 min.

For Species: Amplification of a target sequence in the mgc2 gene was performed using primers mgc2-2F: 5'-CGCAATTTGGTCCTAATCCCCAACA-3' and mgc2-2R: 5'-TAAACCCACCTC CAGCTTTATTTCC-3' **[14]**. They flank and amplify a 300 bp region of the mgc2 gene of MG. The PCR was performed in 25 µl reaction volume consisting of 2.5 µl 10X PCR buffer (CinnaGen, Iran), 2 µl of 50 mM MgCL2 (CinnaGen, Iran), 0.2 µl 10 mM dNTPs (CinnaGen, Iran), 0.1 µl of each primer, 0.1 µl Taq DNA polymerase (5U/µl), 19 µl of deionized distilled water and 1 µl of template DNA. After denaturation at 95°C for 1 min the reaction was performed in 40 cycles with denaturation (95°C for 20 s), annealing (60°C for 40 s), primary extension (72°C for 10 s) and a final extension at 72°C for 5 min.

All amplification reactions were performed in a Gradient Mastercycler (Eppendorf, Germany). Visualization of amplified products was done by UV transillumination after ethidium bromide staining and electrophoresis (1% agarose gel in 1X Tris–acetic acid–EDTA buffer).

Sequencing and nucleotide sequence analysis of PCR amplicons

To evaluate sequence variations among MG isolates from Tehran province of Iran, partial mgc2 gene from 10 isolates were sequenced and sequences were determined for both strands of DNA. The R_{low} strain of MG was chosen as a reference pathogenic strain and it's mgc2 gene-nucleotide sequence was obtained from GenBank. Assembly of sequences and sequence managing was performed with DNAsis Max 3.0 software. Afterward BioEdit 7.0 program was used for aligning all the sequences.

RESULTS

The PCR products containing the mgc2-gene targeted region were generated with mgc2-2F/2R primers and produced a specific 300 base pair (bp) band in Gel electrophoresis (data not shown). Based on Blast and aligning, the detected isolates were arranged in two main groups and showed 97-98.67 percent homogeneity with the pathogenic R_{low} strain. There were some bases changes at nucleotide positions but any large or unique nucleotide insertion or deletion was not found **(Figure1)**.

DISCUSSION

In Iran, Mycoplasma gallisepticum has been detected by serological and cultural methods for many years. Also, PCR methods and molecular studies have been performed for detection of MG isolates **[15]**. The genotypic diversity and heterogeneity among MG isolates from different geographical areas of Iran have been shown by RFLP-PCR **[16]**.

Furthermore, RAPD-PCR have been used for epidemiological study and differentiation of various field isolates **[17]**. However, sequencing of Iranian MG isolates has been less done and the characteristics of existing isolates are not completely clear yet.

In this study, Single locus typing using partial mgc2 gene target was be employed for studying MG isolates**.** The mgc2-2F/2R primers which have been used react only with MG and do not react with DNA prepared from 22 other Mycoplasma and two Acholeplasma species originating in domestic poultry and other fowl or with nine non-Mycoplasma bacterial species that may be present in chickens **[18]**.

Tehran MG isolates showed both of homology and heterology and were arranged in two main groups in present study. Based on Blast and aligning results, any significantly or unique large nucleotide insertion or deletion in the mgc2 gene-targeted region of seperated isolates was not found.

The MG genome evolves rapidly and persistence of MG is probably due to high frequency of phenotypic variation of major surface antigens **[4, 19]**, resulting in genetic variants(strains) with marked differences in antigenicity, pathogenicity and transmissibility **[20]**. The Mgc2 antigen, encoded by mgc2 gene is one of cytadhesins that have been reported to undergo changes **[21]** and the genetic variability of the mgc2 gene has been previously documented by means of mgc2-1F/1R primers **[5]**.

The advantage of using the mgc2 gene-based molecular method by means of mgc2-2F/2R primers is the ability to differentiate between pathogenic field strains and vaccine strains by combining it with RFLP or sequencing of the DNA amplicons, which is important particularly in countries that poultry flocks are vaccinated with the live vaccine strains. There is really no way to predict the applicability of the mgc2-PCR-RFLP assay for this purpose in a region or country, unless the mgc2 gene sequence is analyzed in a variety of isolates **[14]**. Although, the use of a MG vaccine for broiler breeder flocks has not been approved by Iranian Veterinary Organization yet.

Overall, due to the slight nucleotide modifications observed in the targeted region in comparition with strain R_{low}, it seems that the detected isolates are not new or diverged pathogenic strains. However, additional and geographically broader investigations are needed in order to determine the genetic variability of the mgc2 gene among the Iranian MG isolates. On the other hand, the discriminatory power of nucleotide

sequencing using multiple genes will be higher for evaluation of genetic diversity **[5]**.

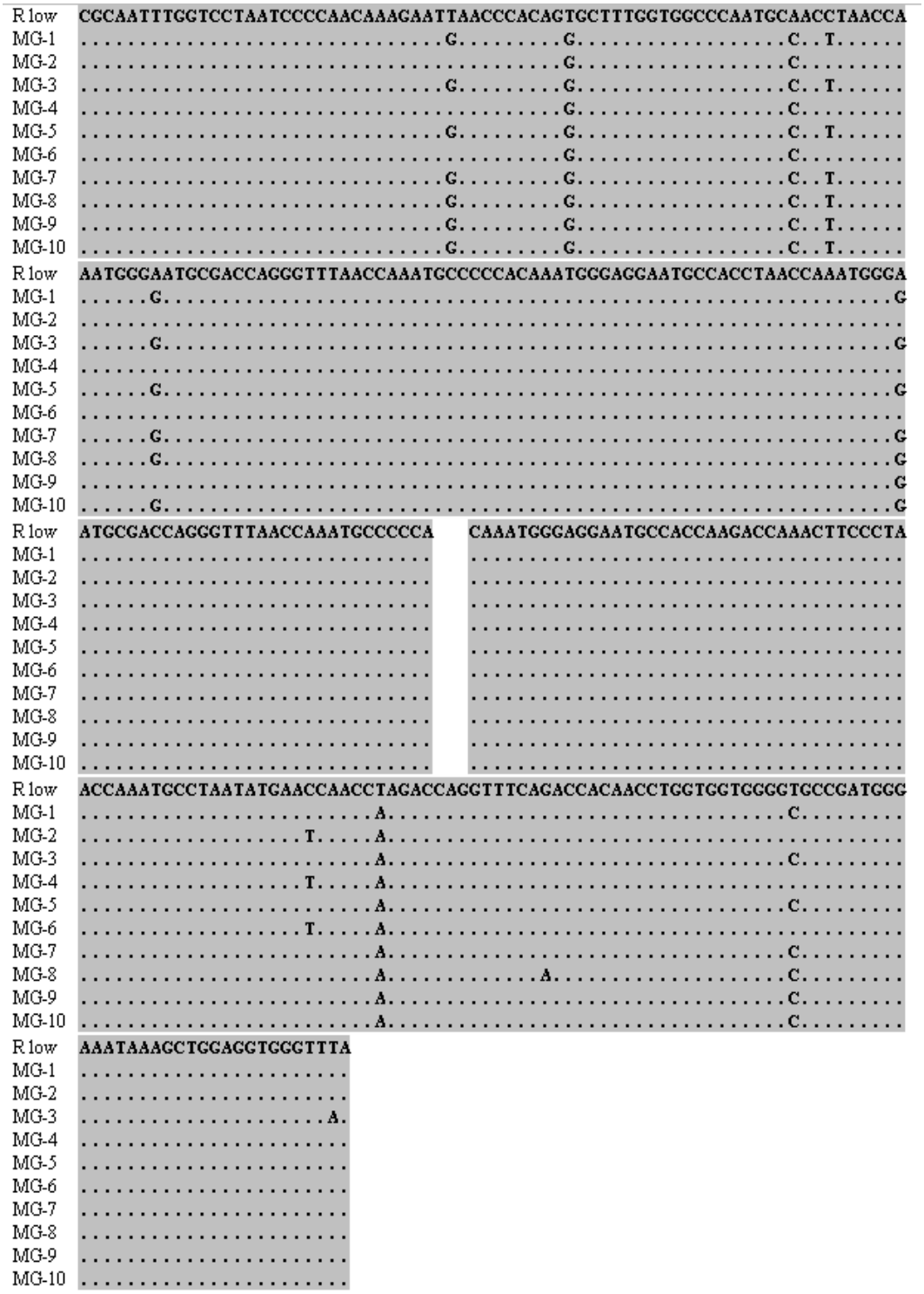

Figure1: Comparison of the partial mgc2 gene-nucleotide sequences of 10 MG isolates from Tehran province with R_{low} strain (GenBank accession number AY556228.1)

CONCLUSION

It appears that the different MG isolates of Tehran province, Iran are not new MG strains. Further work and biological study would however be needed to conclusively prove this speculation. It is unlikely that MG will be eradicated from the commercial poultry industry in the coming years in Iran. However, epidemiological studies of MG isolates from different provinces will help to improve the understanding of the relationship among MG isolates from Iran and other countries. This is central to the management of infection and may contribute to the optimization of control strategies and perhaps the use of a unique vaccine strain.

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