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Phylogenetic analysis of mgc2 gene of *Mycoplasma gallisepticum* isolates from broiler breeder flocks in Tehran province, Iran

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ABSTRACT

Mycoplasma gallisepticum (MG) is an economically important pathogen of poultry worldwide. Genetic characteristics, Infectivity, tissue tropism and pathogenicity differ significantly between MG strains. The aim of this study was to analyze the partial DNA sequence of mgc2, a cytadhesin-encoding gene of MG isolates from broiler breeder flocks in Tehran province, Iran and compare them with MG isolates from other countries. For this purpose, a total of 320 samples were collected from choanal cleft and trachea of broiler breeder flocks with positive RSAT. 38 out of the 320 samples were positive in culture and reacted with specific antisera. The 15 positive samples were subsequently selected for molecular study. The PCR products containing mgc2-cytadhesine gene were generated with specific primers and then sequenced. Alignment and comparison of the sequences in DNASIS program revealed that 3 isolates were quite similar and 12 other isolates were different from first group. Based on clustal-W alignment and phylogenetic analysis of 15 MG feild isolates and 25 MG isolates and strains from other countries (*available in GenBank*), *the feild isolates were arranged in two distinct groups with 97/6-98/3 percent homogeneity and each one of two groups had higher homogeneity (Up to 99/6 percent) with some of the isolates and strains of MG from other countries. Our study revealed heterogeneity between MG isolates from broiler breeder flocks in Tehran province, Iran and high mgc2 gene- homology between MG isolates of Tehran province origin and some of the MG strains and isolates from other countries. This results can represent the precence of different MG isolates due to the existence of multiple sources of pollution and epidemiological relationships or MG mutagenesis for adaptation to environmental conditions.*

Keywords: *Mycoplasma gallisepticum*, phylogenetic analysis, mgc2, broiler breeder, Tehran

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INTRODUCTION

Mycoplasma gallisepticum (MG) is the most pathogenic and economically significant mycoplasma of poultry. Airsacculitis in chickens or turkeys resulting from MG infections, causes increased condemnations at processing. Economic losses from downgrading of carcasses, reduced feed and egg production efficiency and increased medication costs are additional factors that make this one of the costliest disease problems confronting commercial poultry production worldwide [17]. Eradication is an ideal control measure for MG infections in poultry farms [5]; but rapid expansion of poultry production in restricted geographic areas, with the resulting high concentrations of birds of different ages and different poultry sectors in close proximity, makes it more difficult to maintain MG-free flocks and a reevaluation of the strategies utilized to control MG infections of poultry is needed [5, 13, 18].

MG infection mainly is transmitted through ovaries and the MG infected chicks that derived from infected breeder flocks have low quality and cost; hence, according to Iranian Veterinary Organization rules MG-infected broiler breeder flocks should be depopulated in order not to produce infected chicks. A decreasing incidence of MG has been observed, at least in breeding stock, in countries with control and eradication programmes for MG [8, 16] including Iran. Based on the relative succsess in control and prevention of MG infection on broiler breeder flocks in Iran, the prevalence of MG is lower in comparison with previous years and the use of a MG vaccine for these flocks has not been approved by Iranian Veterinary Organization yet, but extensive epidemiological data is needed for eradication of disease. Phenotypic and genotypic heterogeneity in MG strains has been demonstrated by serological assays, electrophoretic analysis of cell proteins and molecular techniques and these strains may differ markedly in their antigenic profiles and their virulence-related surface properties [15, 17]. Recognition of MG strains heterogeneity has become more sensitive and discriminatory with the application of molecular techniques [17]. Recently analysis of selected regions of the targeted genes has been introduced as a useful tool for investigate sequence variability and molecular characterization and as a new approach for studying the molecular epidemiology of MG field isolates [6, 18]. While MG has been characterized in some countries, very little information is documented on Iran isolates.

The purpose of this study was to investigate partial sequencing of the second cytadhesin- encoding gene (mgc2) of MG isolates from broiler breeder flocks in Tehran province, Iran and compare them with MG from other countries .

MATERIALS AND METHODS

Sampling and bacteriology

During 2010 to 2013, a total of 320 respiratory Samples from choanal cleft and trachea of broiler breeder flocks with respiratory problems suspected to be infected with MG in Tehran province of Iran were collected with sterile cotton swabs and inoculated into 3 ml specific broth media (PPLO broth). Inoculated broth media were shipped by overnight carrier to laboratory and incubated at 37 °C for a short period (5 to 6 h) and then filtered by 0.45 µl syringe filter (Nunk, Denmark) and inoculated in new broth media and incubated at 37 °C. Samples were observed 3 to 5 days for the evidence of growth and kept in incubator for one month before being discarded as negative culture. Any suspected growth was subcultured on Frey's medium agar, using microbiological loop and incubated in very moist atmosphere at 37 ^{o}C with 5% CO_2 for 5-7 days. Mycoplasma colonies were detected by microscope, and an isolated colony was picked and inoculated into broth. Finally, the positive broth media were confirmed with specific MG antisera (SPAFAS, CANADA).

DNA extraction

DNA was extracted from the samples according to phenol-chloroform method [1]**.** 0/5 ml of each sample was transferred to Eppendorf tubes and centrifuged for 15 min at 13000 rpm. The supernatant fluid was discarded and added lysis buffer (Tris-HCL 50 mM pH=8, SDS 1%, Nacl 100 mM, EDTA 50 mM, proteinase K 20 µl to 200 µl) to the tubes equal volume of the tubes containing and incubated for at least 4 hrs at 56 ºC. Equal volume of the material in the tubes 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 tubes. Added phenol: chloroform (1:1) in tubes equal volume of the tubes containing, Centrifuged at 13000 rpm for 15 min and transfered all aqueous layer in a new tubes. Added chloroform in the tubes, equal volume of the tubes containing mix them well by vortex and centrifuged at 13000 rpm for 15min. Transfered all aqueous layer in a new tubes and sodium acetate (3M) was added 1:10 volume of the tubes containing and mixed well. Added to them ethanol (ETOH) two fold of material in tube. This solution was placed on -20 ºC for 20 min and centrifuged for 15 min at 13000 rpm. Discarded liquid containing of tubes 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 in 4 ºC and used for PCR.

Primers and PCR procedure

In this study two primers mgc2-2F (5'- CGC AAT TTG GTC CTA ATC CCC AAC A-3') and mgc2-2R (5'-TAA ACC CAC CTC CAG CTT TAT TTC C-3') were used [7, 18]. They flank and amplify a 300 bp region of the mgc2 gene of MG. The PCR was performed in 25 µl PCR reaction mix consisting of 2.5 µl 10 x PCR buffer (CinnaGen Inc.), 2 µl MgCL2 (50 mM) (CinnaGen Inc.), 0.2 µl 10 mM dNTP (CinnaGen Inc.), 0.1 µl of each primers, 0.1 µl Taq DNA polymerase (5 u/µl) (CinnaGen Inc.), 19 µl of deionized distilled water and 1 µl of template DNA. All amplification reactions were performed in a Gradient Mastercycler (Eppendorf, Germany) using the following temperatures and times. After denaturation at 95 °C for 1 min the first reaction was performed in 40 cycles with denaturation (95 °C for 20 s), annealing (60 °C for 40 s), and primary extension (72 °C for 10 s), and a final extension at 72 °C for 5 min [18].

Gel electrophoresis

A 10 µl aliquot of each PCR products was mixed with 2 µl loading buffer and seperated by electrophoresis in an 2% agarose gel with 0.25 µl/ml ethidium bromide (70 volts for 1/5 h) following U.V. transillumination (Visi-Doc_It system, UVP, UK).

Purification, DNA sequencing and bioinformatic methods

The purification and bidirectional sequencing (with the forward and reverse PCR primers) of PCR products were performed by Bioneer Company (South Korean branch). Assembly of sequences and sequence managing were performed with DNASIS and MEGA5 bioinformatic programs. Comparison and multiple sequence alignment of the sequences was performed with Clustal W method. Finally, the phylogenetic tree was planned.

RESULTS

Among the 320 samples collected from choanal cleft and trachea of broiler breeder flocks with positive Rapid Serum Agglutination Test (RSAT), 38 were positive in culture and reacted with specific antisera. The 15 positive samples were subsequently selected for molecular study. The PCR products containing mgc2-gene were generated with mgc2 primers and produced a specific 300 base pair (bp) band in Gel electrophoresis (Figure 1). Alignment and comparison of the sequences revealed that 3 isolates were quite similar and 12 other isolates were different from first group with 97/6-98/3 percent homogeneity. Based on multiple sequence alignment and phylogenetic analysis of DNA sequences of MG isolates from Tehran province and 25 MG isolates and strains from other countries obtained from GenBank (Table 1) the Tehran isolates were arranged in two distinct groups (Figure 2). Each one of two groups showed higher homogeneity with the some of MG strains and isolates from other countries (Up to 99/6 percent with a well-known reference strain S6 and Up to 99/3 percent with some isolates of America and one from south Africa). Also, the detected isolates showed 97/3-99/3 percent homogeneity with ts-11 vaccinal strain and 97/3-98/3 percent with one isolate of MG from Iran obtained from GenBank.

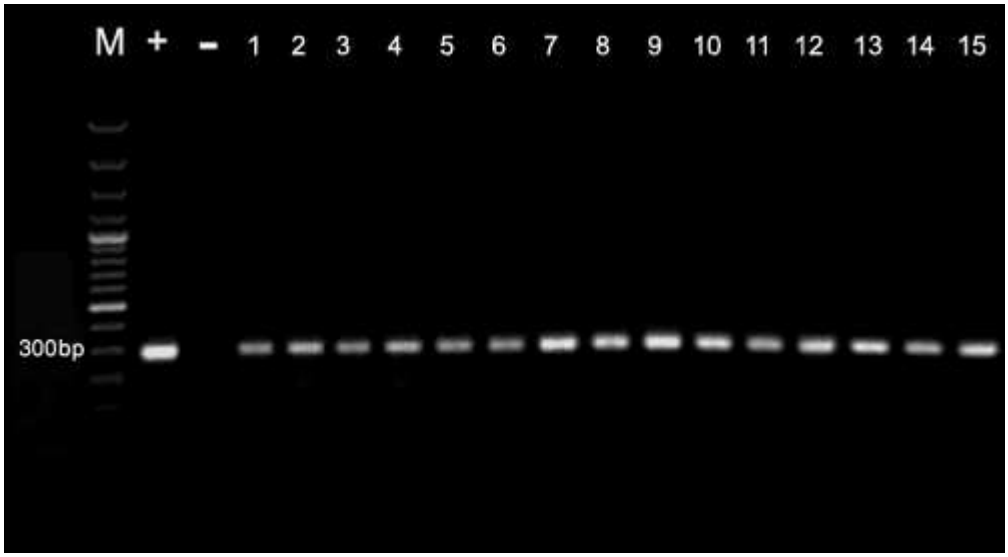

Figure 1. Electrophoresis of *M.gallisepticum* mgc2- PCR amplicons. M: ladder, +: positive control, - : negative control, 1 to 15: isolates from broiler breeder flocks of Tehran province

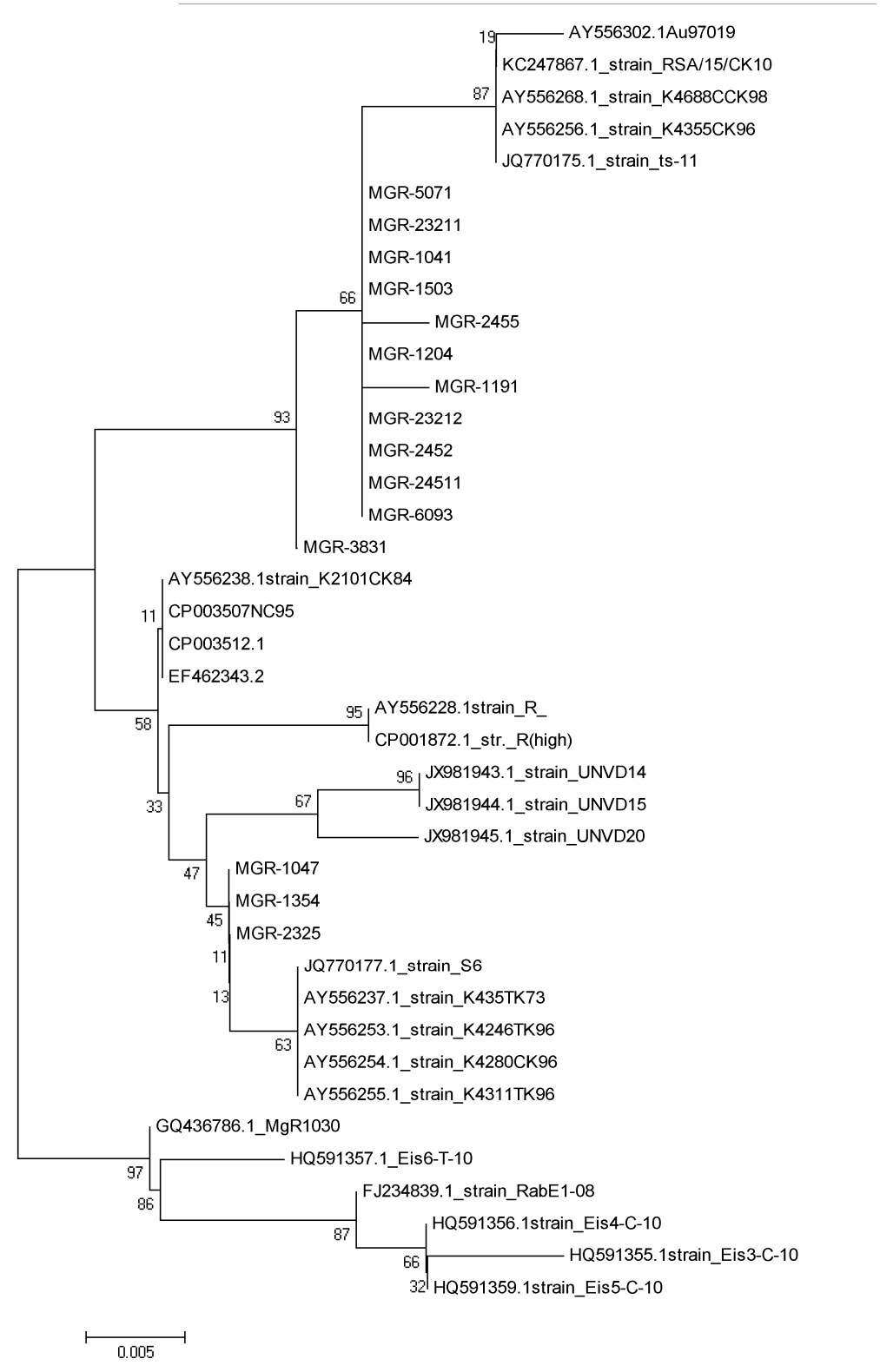

Figure 2. Phylogenetic tree of 15 M. gallisepticum isolates from broiler breeder flocks of Tehran province, Iran (marked with the MGR-prefix) and 25 MG obtained from GenBank

Table 1. The characteristics of *M.gallisepticum* strains and isolates from the entire world obtained from GenBank

S/N	Country	Strain	Access number
1	Australia	ts-11	JQ770175.1
2	Australia	S6	JQ770177.1
3	Australia	Au97019	AY556302.1
4	America	R	AY556228.1
5	America	-	CP 003512.1
6	America	-	EF 462343.2
7	Egypt	Eis3-C-10	HQ591355.1
8	Egypt	Eis4-C-10	HQ591356.1
9	Egypt	Eis5-C-10	HQ591359.1
10	Egypt	Eis6-T-10	HQ591357.1
11	America	R(high)	CP001872 .1
12	Iran	MgR1030	GQ436786.1
13	America	K435TK73	AY556237.1
14	America	K2101CK84	AY556238.1
15	America	K4246TK96	AY556253.1
16	America	K4280CK96	AY556254.1
17	America	K4311TK96	AY556255.1
18	America	K4355CK96	AY556256.1
19	America	K4688CK98	AY556268.1
20	America	NC95	CP003507
21	Egypt	RabE1-08	FJ234839.1
22	South Africa	RSA/15/CK10	KC247867.1
23	Egypt	UNVD14	JX981943.1
24	Egypt	UNVD15	JX981944.1
25	Egypt	UNVD20	JX981945.1

DISCUSSION

Isolates and strains of MG vary widely in their relative pathogenicity, depending on the genotypic and phenotypic characteristics [17]. Reliable methods for the differentiation of MG strains play a pivotal role in understanding the epidemiology and spread of the disease because they generate the information necessary to identify and track new outbreaks [6]. Epidemiological studies according to molecular analysis of MG field isolates can determine the source of infection, as the first step in MG control programs.

We employed single locus typing using mgc2 gene target for studying MG isolates. As stated in literature mgc2-PCR is the most specific and the most sensitive PCR for the detection of MG [7]. The Mgc2 gene is fairly well conserved in MG, encodes a second cytadhesin protein which proved to be located at the tip organelle in MG through immunogold labeling and also known to play a role in the attachment process, adhering to mucosal membranes, hence initiate infection and stablishing chronic infection. This gene can be used as a basic reference in molecular identification of MG isolates [11, 22].

The mgc2-2F/2R primers which have been used for molecular differentiation and epidemiological studies of MG isolates [2, 18, 21] 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 [7].

In Iran, molecular methods have been performed for identification of MG isolates for recent years and the restriction fragment length polymorphism (RFLP) has been used for diagnosis of both cultured as well as field samples of suspected flocks to have infection with MG [9]. Also, heterogeneity among field isolates of MG from different geographical areas of Iran have been indicated by random amplification of polymorphic DNA (RAPD) [12]; but sequence analysis of certain genes reveal differences precisely and show better discriminatory power [14].

Based on the phylogenetic tree and genomic similarity of some detected MG isolates with some different strains that have been isolated outside Iran, presence of the different MG isolates due to the existence of multiple sources of pollution and epidemiological relationships is suggested in this study. However, these findings could't determine difference in virulence or pathogenicity.

On the other hand, MG has a defective DNA repair system. Based on comparative genomic studies, Increasing endogenous levels of mutagenesis by the DNA repair system of MG may be a mechanism of adaptation to stress conditions [10]. Multiple membrane proteins and lipoproteins have been characterized with size- or phase-variant forms that occur at high frequency and confer phenotypic or antigenic variation to MG isolates [4]. The frequent changes on surface antigens (antigenic variations) allow MG to evade the host immune system, when adhered to the

host respiratory tract [19]. The MG genome evolves rapidly [3] and the mgc2 antigen, encoded by mgc2 gene is one of cytadhesins that have been reported to undergo changes [20]. Thus, the slight nucleotide modifications observed in the targeted region in this study may indicate mutagenesis in MG for adaptation to environmental conditions.

CONCLUSION

Our study revealed genotypic diversity and herogeneity among MG feild isolates from broiler breeder flocks in Tehran province, Iran and It seems that the ongoing MG outbreaks in Tehran province, Iran to be caused by different isolates. However, further research is needed to substantiate this claim and biological study is recommended to investigate any obvious correlation between genetic closeness, virulence and biological properties. This is the first phylogenetic study of MG based on mgc2-gene sequence in Iran. Increasing sequence analysis of the mgc2 and other genes and phylogenetic studies like this will allow a better understanding on the origin of MG outbreaks and therefore will facilitate better strategies to prevent, control and manage future outbreaks.

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