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Full Length Research Paper

Molecular study and phylogenetic analysis of *Mycoplasma synoviae* isolated from poultry flocks from Mazandran Province of Iran

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***Mycoplasma synoviae* (MS) is one of the important pathogens in chicken and turkey which cause great economic losses in poultry industry. *M. synoviae* has one serotype but there is heterogeneity among MS strains. The aim of this study was to analyze the DNA sequence of Mycoplasma *synoviae* isolates from Mazandran province poultry flocks of Iran and compare them with MS from other countries. For this purpose, 32 samples were collected from choanal cleft, trachea and air sac of flocks with respiratory symptom and cultured in specific PPLO medium. Ten out of the 32 samples were positive in culture and reacted with specific antisera. The 10 positive samples were subsequently used for molecular study. The PCR products containing 16s rRNA genome were generated with specific primers and then sequenced. Multiple alignment and phylogenic analysis of DNA sequences was performed with DNASTAR program. Alignment and comparison of the sequences in DANSTAR program revealed that 7 of 10 isolate are quite similar and 3 other isolate are different from group one. Phylogenic analysis of *M. synoviae* isolated from Mazandran province and 21 *M. synoviae* from the entire world revealed low homology among most of them. However, some of the Mazandran isolates showed high homology with *M. synoviae* isolated from Brasilia. Overall, these results revealed the different molecular structure and heterogeneity among *M. synoviae* isolated from Mazandran province in this study.**

Key words: *Mycoplasma sinoviae*, sequence, phylogenetic, poultry, Mazandran.

INTRODUCTION

Avian mycoplasmas occur in a variety of bird species. The most important mycoplasmas for chickens and turkeys are *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS) and *Mycoplasma meleagridis*. Besides, *Mycoplasma* iowe (MI) is an emerging pathogen in turkeys, but of little concern for chickens. Mycoplasmas are bacteria that lack cell wall and belong to the class Mollicutes. Although, they have been considered as extracellular agents, scientists admit nowadays that some of them are obligatory intracellular microorganisms, whereas all other mycoplasmas are considered facultative intracellular organisms (Kleven, 2003; Nasimento et al., 2005).

M. synoviae (MS) infection most frequently occurs as a subclinical upper respiratory infection. It may cause air sac lesions when combined with Newcastle disease (ND), infectious bronchitis (IB) or both. At other times, MS becomes systemic and results in infectious synovitis, an acute to chronic infectious disease of chickens and turkeys, involving primarily the synovial membranes of

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joints and tendon sheaths producing an exudative synovitis, tenovaginitis or bursitis (Kleven, 2003).

The clinical manifestations of MG are coughing, sneezing, snicks, rales, ocular and nasal discharge, decrease in feed consumption and egg production, increased mortality, poor hatchability, and primarily in turkeys, swelling of the infraorbital sinuses. In chickens, turkeys and other birds, a milder form of some of these symptoms can be seen in MS infections, besides lameness, pale comb and head, swollen hocks and foot pad. Acutely affected birds may show green feces, but respiratory infection caused by MS is usually asymptomatic (Nascimento et al., 2005).

M. synoviae has a broader host range than other Mycoplasmas. Chickens, turkeys, and guinea fowl are the natural hosts. Several other species have been naturally infected, and others have been infected by artificial inoculation. Mycoplasmas may be transmitted horizontally, through infectious aerosols coughed and sneezed by infected birds and through contaminated feed, water, personal contact and communication with animals, mainly birds. Transmission occurs vertically from parents to their offspring, through contamination of laid eggs (transovarian transmission). Mycoplasma is commonly transmitted within species and/or between closely related species, that is, they are host-specific, with rare exceptions (Nascimento et al., 2005; Stipkovits, 2006).

MG and MS infections occur mostly in chickens and turkeys. However, they have been frequently isolated from quails and several avian species. Transmission of MS is similar to other mycoplasmas, except that MS spreads more rapidly (Nascimento et al., 1998).

The aim of this study was to analyze the DNA sequence analysis of *M. synoviae* isolated from Mazandran province poultry flocks of Iran and compared them with MS from another country obtained from GenBank.

MATERIALS AND METHODS

Sampling and microbial culture

Samples from choanal cleft, trachea and air sac of flocks with respiratory problem suspected to be *M. synoviae* from Mazandran province of Iran were collected with sterile cotton swabs and inoculated into 3 ml specific broth media. 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 with 5% CO_2. 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 and incubated in very moist atmosphere containing 5% CO_2. Mycoplasma colonies were detected by microscope, and an isolated colony was picked and inoculated onto broth. Finally, the positive broth media were confirmed with specific MS antisera (SPAFAS.CANADA).

PCR procedure and Gel electrophoresis

The primers for amplification of 16S rRNA were MS-1 (5'-GAAGCAAAATAGTGATATCA-3') and MS-2 (5'-GTCGTCTCCGAAGTTAACAA-3') (Lauerman et al., 1993). The PCR was performed with a DNA engine thermocycler in 25 µl PCR reaction mix consisting of 2.5 µl 10 x PCR buffer, 4 µl 1.5 mM $MgCL_2$, 0.5 µl 200 mM dNTP, 0.1 µl of each of ms-1 and ms-2 primers (20 µM), 0.25 µl Taq DNA polymerase (2.5 u/µl), 16.55 µl of deionized distilled water and 1 µl of template DNA. All amplification reaction were performed in a Gradient Mastercycler (Eppendorf, Germany) using the following temperatures and times. After denaturation at 95°C for 5 min the first reaction was performed in 35 cycles with denaturation (94°C for 30 s), annealing (57°C for 1 min), and primary extension (72°C for 2 min), and a final extension at 72°C for 7 min.

The PCR products were detected by electrophoresis (Apelex, France) in 2% agarose (Agarose MP, Roche) gel in TAE buffer. Gels were run for 1.5 h at 60 V, stained with ethidium bromide, destained with distilled water, observed using ultraviolet trans illumination and then photographed (Visi-Doc_It system, UVP, UK).

Purification, DNA sequencing and bioinformatics method

The purification of PCR products were performed with purification kit (High pure PCR product purification kit-Roche). The amplified DNA fragment was sequenced bidirectional with the forward (MSF) and reverse (MSR) PCR primers by MWG BIOTECH CO. (Germany), and for each samples forward and reverse sequences were obtained. Assembly of sequences and sequence managing were performed with sequencing project management and alignment program Segman (DNASTAR, Inc., Madison, WI). Comparison of the sequences was performed with Megalign program and multiple alignment performed by Clustal V. Finally, the phylogenic tree was planned.

RESULTS

Among the 32 samples collected from choanal cleft, trachea and air sac of flocks with respiratory problem and cultured in specific medium, 10 were positive in culture and reacted with specific antisera. The 10 positive samples were selected for molecular study. The PCR product containing 16s rRNA genome that was generated with specific primers and produced a specific 207 base pair (bp) band was sequenced (Figure 1) Alignment and comparison of the sequences in DNASTAR program revealed that 7 out of 10 isolates are quite similar and 3 other isolates are different from group one. Identity plot (IP) of nucleic acids showed only 30% homology among two groups and phylogenic analysis arranged them in two distinct lineages (Figure 2).

Phylogenetic analysis of DNA sequences of *M. synoviae* isolated from Mazandran province of Iran and 21 *M. synoviae* from the entire world (Table 1) revealed low homology among most of them. However, some of the Mazandran isolates showed high homology with MS isolated from Brasilia (Figure 3).

DISCUSSION

Our study showed the genotypic diversity and heterogeneity among MS isolated from poultry flocks of

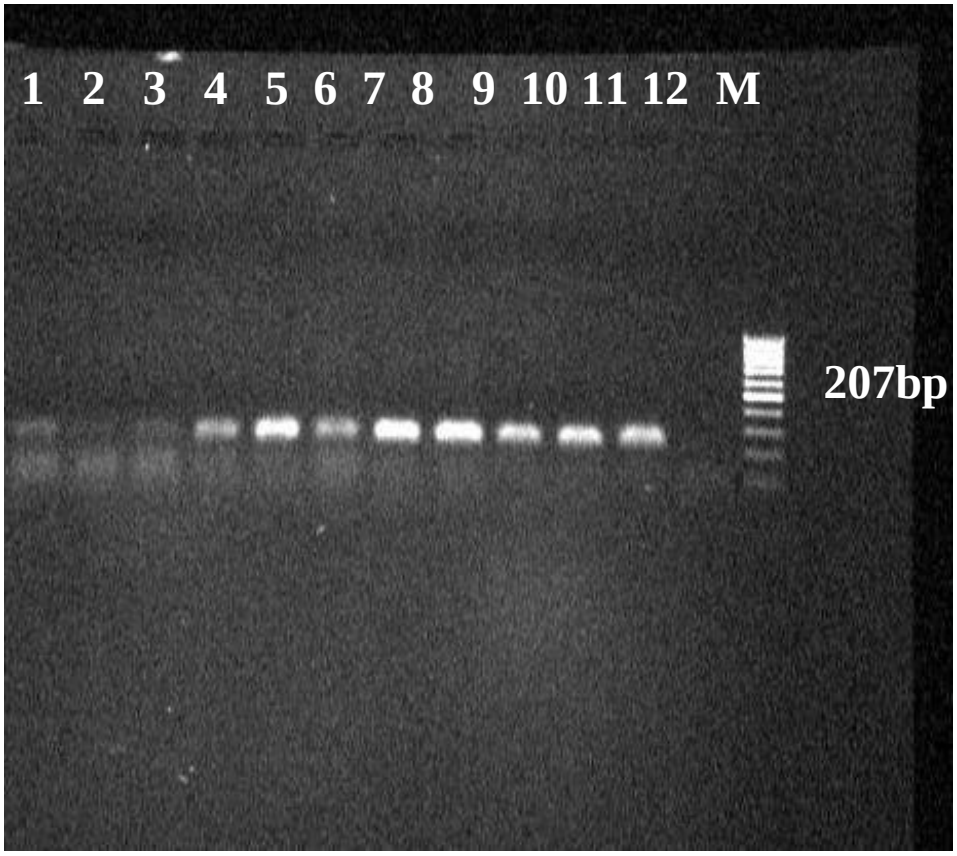

Figure 1. Electrophotogram of *M. synoviae* PCR amplicon. M = DNA marker, 12 = negative control, 11 = positive control
1 to 10 = samples.

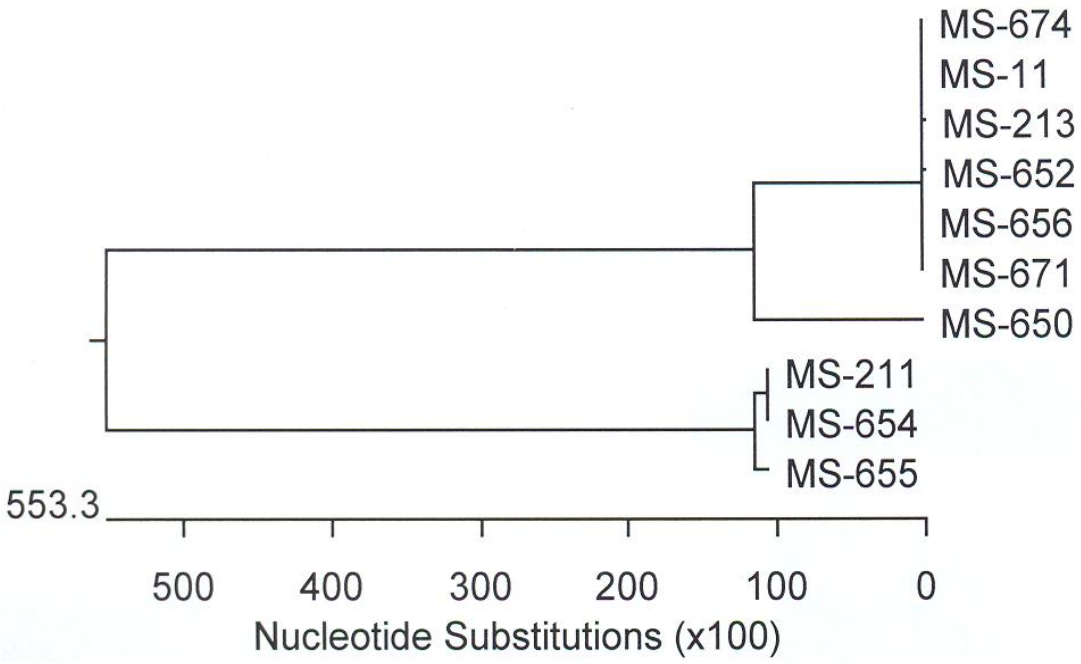

Figure 2. Phylogenetic tree of 10 MS isolates from Mazandran poultry flocks of Iran. There are two distinct lineages, 7 isolates are quite similar and 3 others are different.

Table 1. The characteristics of *M. synoviae* isolates from the entire world obtained from GenBank for phylogenetic analysis.

S/N	Access number	Strains	Country
1	X52083	WVU1853	Australia
2	Uo4645	WVU1853	Sweden
3	Aj781002	WUV1853	Great Britain
4	Amo73015	-	Vietnam
5	Ay566216	-	Northern Korea
6	Ay623914	Imso2-38	Southern Korea
7	Ay904348	Tn/427	India
8	Ay904349	Tn/781	India
9	Ay904350	Tn/815	India
10	DQ497544	KIO	Brasilia
11	DQ497545	ANA	Brasilia
12	DQ497546	B4	Brasilia
13	DQ497547	BEM	Brasilia
14	DQ497549	HIR	Brasilia
15	DQ497551	1853	Brasilia
16	DQ497552	BIO	Brasilia
17	DQ497553	COM	Brasilia
18	DQ497554	MSH	Brasilia
19	DQ497555	KAK	Brasilia
20	DQ497557	PAR	Brasilia
21	DQ497561	TER	Brasilia

Mazandran province of Iran. *M. synoviae* has one serotype but there is heterogeneity among MS strains (Kleven, 2003). Several studies were performed to survey the heterogeneity among MS strains. Intraspecies genotypic heterogeneity among strains of *M. gallisepticum* and *M. synoviae* was tested using genomic fingerprints with a ribosomal RNA (rRNA) gene probe (Yogev et al., 1998). A diverse group of *M. synoviae* strains from various hosts, pathological processes and geographic areas collected over 25 years were analyzed by restriction endonuclease analysis. The results suggest that restriction endonuclease analysis of *M. synoviae* strains may be a useful strain identification tool to study epidemiological problems (Morrow et al., 1990). Polymerase chain reaction (PCR) and DNA sequence analysis of the N-terminal end of the hemagglutinin encoding gene *vlhA* were used as an alternative for the detection and initial typing of field strains of *M. synoviae* in commercial poultry (Hong et al ., 2004). *M. synoviae* species-specific primers selected from the 16S rRNA sequence were evaluated by polymerase chain reaction (Lauerman et al., 1993).

In Iran, poultry mycoplasmas have been detected by serological and cultural methods for many years. Also, PCR method and molecular study have been performed for detection and identification of avian mycoplasmas. The RFLP-PCR was used for diagnosis of both cultural as well as field sample flocks suspected to have infection of MG (Ghaleh Golab et al., 2005). The genotypic diversity and heterogeneity among MG isolates have been showed by RFLP-PCR (Hosseini et al., 2006).

This is the first molecular and phylogenic study of MS in Iran. Our study revealed the different molecular structure and heterogeneity among *M. synoviae* isolated from Mazandran province of Iran and phylogenic analysis arranged them in two distinct lineages. Phylogenic

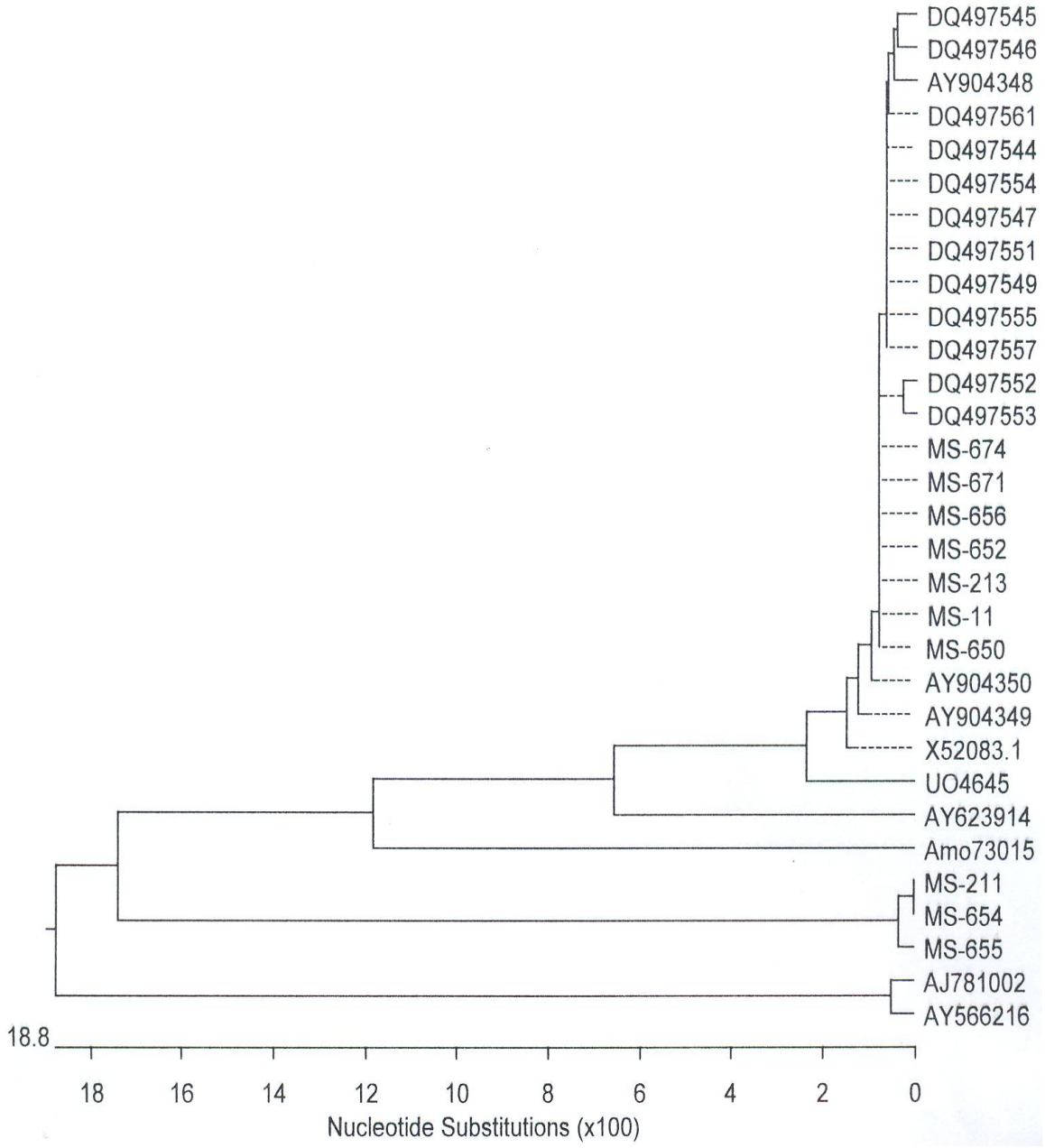

Figure 3. Phylogenetic tree of 10 *M. synoviae* isolates from Mazandran province poultry flocks of Iran and 21 *M. synoviae* isolates obtained from GenBank.

analysis of DNA sequences of *M. synoviae* isolated from Mazandran province of Iran and 21 *M. synoviae* from the entire world revealed low homology among most of them. However, some of the Mazandran isolates showed high homology with MS isolated from Brasilia (Figure 3).

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