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# Random amplified polymorphic DNA (RAPD) fingerprinting of *Mycoplasma gallisepticum* isolates from chickens

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## ABSTRACT

Ten field isolates of *Mycoplasma gallisepticum* (MG) from different geographical areas of Iran were analyzed by random amplification of polymorphic DNA (RAPD). RAPD analysis produced reproducible banding patterns on the basis of which various distinct amplification patterns could be detected. MG isolates compared with reference strains (S6 and Mg SS) and vaccine strain (ts-11) and demonstrated distinct RAPD profiles. The results indicated genotypic diversity and heterogeneity among MG isolated from field, which can be used for epidemiological studies and for differentiation between vaccine strain, and field isolates.

***Keywords:*** Chicken, *Mycoplasma gallisepticum*, RAPD, AP-PCR

## INTRODUCTION

*Mycoplasma gallisepticum* (MG) is one of the most economically significant pathogens of poultry and the cause of chronic respiratory disease of chickens and infectious sinusitis of turkeys with a wide diversity of clinical manifestations of which chronic and asymptomatic infections are the most common (Levisohn & Kleven 2000, Ley 2003). Additionally, MG has recently known as an emerging infection, which causes conjunctivitis in free-ranging house finches (Ley *et al* 1996). As MG infection mainly is transmitted through trans-ovarian, the MG-infected breeder flocks should be depopulated; hence, the preferred method for MG control is to maintain MG-free flocks (Stipkovits & Kempf 1996). However, in some situations such as multi-age production farms, maintaining the flocks free of MG may be difficult or impossible. Vaccination may be an alternative choice (Levisohn & Kleven 2000) in these situations. If a live vaccine is applied, however, differentiation between field isolates and vaccines strains is of a major concern. Phenotypic and genotypic heterogeneity in MG strains has been demonstrated by serological assays, electrophoretic analysis of cell

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proteins and molecular techniques (Kleven *et al* 1988, Ley 2003). The later techniques have proven very useful to detect similarities and differences among MG strains and isolates. Random amplification of polymorphic DNA (RAPD) or arbitrary primed PCR (AP-PCR) is a powerful molecular technique used for epidemiological studies and for identification and differentiation of vaccine strains and field isolates (Fan *et al* 1995, Geary *et al* 1994, Ley *et al* 1997, Kleven *et al* 2004b).

The purposes of this study were (a) to distinguish diversity among MG isolates from different geographical areas of Iran, and (b) to compare the field isolates with a number of reference strains and ts-11 vaccine strain using RAPD fingerprinting technique.

## MATERIALS AND METHODS

**Bacterial strains.** Reference MG strains used in this study included: S6 strain (University of Liverpool), Mg SS strain (GD Animal Health Service Ltd., Netherlands) and commercial vaccine ts-11 strain (Merial, Italy).

**Sampling.** Ten farms located in three major poultry producing provinces (Tehran, Qazvin & Markazi) were sampled during 2005-2006. From each farm 30-50 individual birds were sampled. Different kinds of production: broiler, broiler breeder, commercial layer and layer breeder were included in this study.

**Bacteriological culture.** Trachea and choanal cleft samples from live birds were collected using sterile cotton swabs and were each inoculated into 3 ml broth media (Mycoplasma broth base, Frey, BBL, US) containing 15% inactivated horse serum. Inoculated broth media were shipped by overnight carrier to laboratory and incubated at 37° C as soon as possible. Samples were observed 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 media agar and incubated in a very moist atmosphere containing 5% $CO_2$ (Kleven 1998). Mycoplasma colonies were detected by microscope, and an isolated colony was picked and inoculated onto broth. The growth was filtered by 0.45-µm-syringe filter (Nunk, Denmark) and re-plated on Frey's media agar. This procedure was repeated three times in order to obtain a pure culture of each MG isolates. The presence of MG was confirmed with PCR amplification of a segment in 16S rRNA specific for *Mycoplasma gallisepticum* as described later (Kleven *et al* 2004a). One isolate from each farm was included in RAPD analysis.

**DNA extraction.** A 2-ml quantity of broth culture at lag phase was used for DNA extraction. The suspension was centrifuged at 13,000 x *g* for 30 min at 4° C, washed two times with phosphate-buffered saline (PBS), and the pellet was resuspended in 25 µl PBS. The samples were boiled for 10 min, cooled on ice for 10 min, and centrifuged at 13,000 x *g* for 5 min. The supernatant was removed and stored at 4° C (Kleven *et al* 2004a, Ley *et al* 1997).

**Amplification of rRNA**. The primers for amplification of 16S rRNA were MG-14F (5'-GAG CTA ATC TGTAAA GTT GGT C-3') and MG-13R (5'-GCT TCC TTG CGG TTA GCA AC-3') as described earlier (Kleven *et al* 2004a). The amplification was carried out in 50 µl reaction volume consisting of 5 µl 10 x PCR buffer, 1 µl 10 mM dNTP, 0.5 µl of each primer (20 µM), 0.25 µl Taq DNA polymerase (5U/µl), 2 µl 50 mM $MgCl_2$, 39.75 µl of deionized distilled water and 1 µl of template DNA. All amplification reaction were performed in a Gradient Mastercycler (Eppendorff, Germany) as follows: 94° C for 3 min, followed by 40 cycles of 94° C for 30 sec, 55° C for 30 sec, 72° C for 60 sec, and a final extension at 72° C for 5 min.

**RAPD fingerprinting.** RAPD method was performed as described previously (Geary *et al* 1994,

Ley *et al* 1997). The amplification was carried out in a total reaction volume of 50µl: 1.25 units Taq polymerase (Cinnagen, Iran), 3 mM $MgCl_2$, 250 µM of each dNTP, 200 ng of primer 1254 (5'-CCG CAG CCA A-3') and 200-300 ng of template DNA. The amplification conditions were included: four cycles of 94° C for 5 min, 36° C for 5 min, and 72° C for 5 min, followed by 30 cycles of 94° C for 1 min, 36° C for 1 min, and 72° C for 1 min. These were followed by one cycle of 72° C for 10 min.

**Gel electrophoresis.** The PCR products were detected by gel electrophoresis (Apelex, France) in 2% agarose (Agarose MP, Roche) gel in TAE buffer. Gels were run for 1.5 hr at 60 V, stained with ethidium bromide, destained with distilled water, exposed to ultraviolet light and photographed (Visi-Doc-It system, UVP, UK). Commercial DNA ladders were used as molecular-weight markers in each gel running.

## RESULTS

**16S rRNA.** Amplification with diagnostic primers MG-14F/ MG-13R yielded a PCR product of 185 base pairs (bp) from all MG strains and isolates (Figure 1).

**RAPD fingerprinting.** The optimal concentration of primer, DNA template and $MgCl_2$ used in RAPD were first determined (Tyler *et al* 1997).A range of 50-200 ng of primer, 1-4 mM $MgCl_2$ and 50-300 ng of DNA template were used. While the primer concentration at >125 ng produced the same banding pattern, lower primer concentration (<100ng) resulted in relatively poor or no amplification. No amplification was obtained at 1mM $MgCl_2$. Four mM $MgCl_2$ concentration resulted in a smear background. The DNA template concentration against a fixed primer concentration was tittered to obtain ideal condition, and finally rang of 200-300 ng was chosen. Figure 2 and 3 show RAPD banding patterns of strains: S6, Mg SS, ts-11, and field isolates. Each of MG strains produces unique binding patterns, and can distinguish from one another and MG isolates. RAPD profile of MG isolates show both of homology and heterology (five RAPD type). Isolated with similar RAPD banding patterns are interpreted as being the same or a closely related strain. Difference in RAPD profile indicate genomic variability among the ten fields isolated tested. The reproducibility of RAPD fingerprinting was assessed by comparing


**Figure 1.** Amplification of 16S rRNA**.** *M:* O'Range Ruler 50bp DNA Ladder, (Fermentas). *Lanes 1 to 10:* Field isolated; *Lane 11:* S6; *Lane 12:* ts-11.

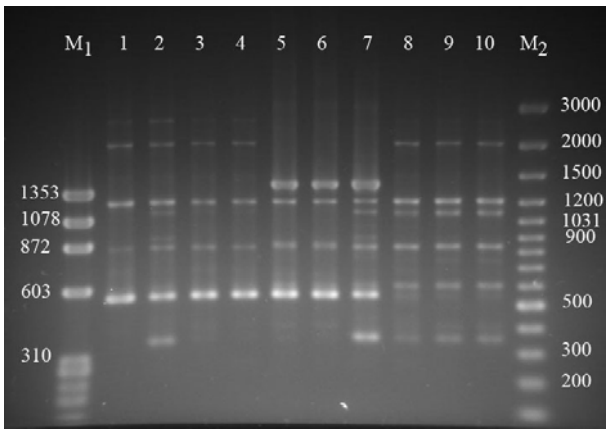


**Figure 2.** RAPD analysis of *M. gallisepticum* isolates from chickens. *$M_1$:* DNA Molecular Weight Marker IX (Roche); *M2:* Gene Ruler 100bp (Fermentas); *Lanes 1 to 10:* Field.

banding patterns obtained from all samples tested on different dates and repeated at least three times. DNA extraction was repeated and quantified for all samples to ensure reproducibility of banding patterns.

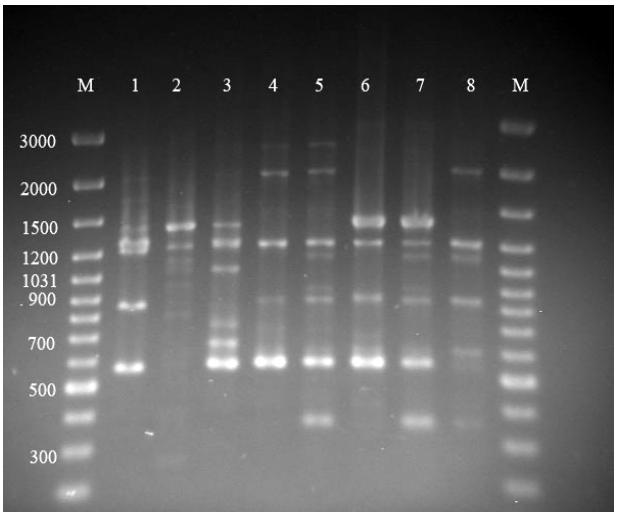


**Figure 3.** RAPD analysis of *M. gallisepticum* isolates from chickens. *M:* Gene Ruler 100bp (Fermentas). *Lane 1:* S6; *Lane 2:* Mg SS; *Lane 3:* ts-11; *Lanes 3 to 8:* selective Field isolated.

## DISCUSSION

Our results showed that the random amplification of polymorphic DNA (RAPD) can differentiate known strains of MG as described previously. A panel consists of 77 strains and isolates produce 36 RAPD type pattern. Songbird isolates had identical patterns and different from chicken and turkey isolates and vaccine strains. (Fan *et al* 1995, Geary *et al* 1994 and Ley *et al* 1997, Kleven *et al* 2004b).

RAPD fingerprinting of field isolates produced various banding patterns, which differed from reference strains and ts-11 vaccine strain. MG isolated with the same binding pattern indicates possibility of single source of infection or closely related strains. DNA polymorphisms were revealed by RAPD among MG isolates shows genetic diversity among MG infecting poultry farms. Anyway, RAPD couldn't determine difference in virulence or pathogenicity.

MG strains vary in antigenicity (Ley 2003, Levisohn & Kleven2000, Stipkovits & Kempf 1996). Antigenic variation among MG could affect the sensitivity of serological tests, depending on the strain infecting the flock and the strain used to produce antigen (Kleven *et al* 1988). Thus it appears necessary to assess the impact of the genetic diversity among MG field isolates on the sensitivity of serological tests commonly used.

RAPD method has been used for the inter- and intraspecies discrimination of bacterial isolates (Tyler *et al* 1997, Williams *et al* 1990). A single primer targets a non-specific sequence to produce genetic map. The primer will generate consistent pattern of amplification for strain and any polymorphism is due to rearrangement or deletion at or between oligonucleotide primer binding sites in the genome (Williams *et al* 1990). However, the pattern may be affected by some factors mainly $MgCl_2$, primer, DNA concentration and extraction method (Tyler *et al* 1997). In this work, all reagents for amplifications namely, $MgCl_2$, dNTP, Taq DNA polymerase, primer as well as DNA template were optimized to obtain standardized reaction conditions for RAPD analysis. After optimization of the entire reagent, RAPD amplification was performed in a total. RAPD analysis was repeated at least three times to ensure reproducibility and consistency of banding patterns for each isolates. The results showed that the banding profiles were consistently repeated with any MG strains and isolates tested. RAPD technique is a simple, rapid, powerful, and reproducible method for strains and isolates differentiation. However, the binding patterns may be different from one laboratory to another and it limits comparison of results between different

laboratories. Sequence analyses of certain genes reveal differences precisely and show better discriminatory power (Kleven *et al* 2004b).

The findings of this study showed that RAPD fingerprinting of MG strains, ts-11 vaccine strain and field isolates could be used for epidemiological studies, trace the source of infection and improve the control strategies. As the MG ts-11 vaccine strain is a potential candidate to enter Iran's poultry industry market to control MG infection, our poultry industry may benefit the findings of this study.

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