Standardization and Evaluation of Random Application of Polymorphic DNA-Polymerase Chain Reaction in Subspecies Typing of *Mycoplasma gallisepticum*

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ABSTRACT

This study evaluated and standardized the sensitivity, specificity, and reproducibility of Random Amplification of Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) for subspecies typing of *Mycoplasma gallisepticum* (MG). The RAPD-PCR revealed amplicons with a maximum number of clear bands of 912, 854, and 416 banding pattern at a standardized level of 2mM of $MgCl_2$, 0.8 mM of each deoxynucleoside triphosphate (dNTP), 0.025 nmole of each primer (M16 SPCR, M13 F, and S1OLIGO), and 750 ng of MG-DNA in a 50-µL reaction mixture. The standardized method showed a high sensitivity resulting in amplification of field MG-DNA at low levels between 50 ng and 100 ng. The specificity of the method in differentiating the DNA of MG from that of *Salmonella enteritidis* (SE) and *Escherichia coli* was 100%, resulting in highest amplicon predominance of 912, 854, and 416 bp for MG; amplicons 935, 769, 680, and 353 bp for SE, and amplicons of 1084, 914, 517, 465, 384, and 303 bp for *E coli*. The reproducibility in obtaining the same banded amplicons of ts-11 MG vaccine strain and of field MG isolates was 87.5% and 100%, respectively. The findings are an important step toward global standardization of this method and for its application in future epidemiologic investigations to trace and control pleomorphic subspecies of MG involved in economic poultry diseases, as well as in differentiating such subspecies from live vaccine strains of ts-11.

INTRODUCTION

Mycoplasmas are small prokaryotic bacteria without cell walls, which colonize mucosal surfaces. *Mycoplasma gallisepticum* (MG) is commonly involved in chronic respiratory diseases in chickens and infectious sinusitis in turkeys. MG infections are often complicated by *Escherichia coli* and/or respiratory

viruses.[1] The economic consequences of MG are highly significant because of decreased egg production, reduced growth and hatchability rate, and significant downgrading of infected carcass at slaughter.[2] *M gallisepticum* can also cause upper respiratory tract disease in pet and game birds.[3,4]

Identification and control of MG infections require a combination of practices including identification of the etiologic agent, followed by expensive eradication of infected birds, and high biosecurity management practices to eliminate the introduction of or contacts with infected birds, thus protecting the MG-free commercial breeder flocks. Moreover, continual monitoring is necessary to identify MG-infected flocks, even under the most stringent biosecurity practices.[5]

The continuous monitoring of poultry flocks requires rapid and sensitive methods to detect and differentiate MG field isolates from live vaccine strains, a critical approach for successful epidemiology as well as for evaluation of control programs based on live vaccine implementation.[6]

Molecular methods such as DNA probes and polymerase chain reaction (PCR) techniques have been used for rapid and sensitive detection of MG isolates.[5,7] Compared with sodium dodecyl sulfate-polyacrylamide gel electrophoreses[8,9] and restriction endonuclease analysis of MG-DNA,[10-12] Random Amplification of Polymorphic DNA-PCR (RAPD-PCR) seems to result in greater detection of MG-DNA polymorphism and to be technically much simpler and faster.[3,7]

The purpose of this study was to standardize and evaluate the sensitivity, specificity, and reproducibility of RAPD-PCR for subspecies typing of field MG isolates and the possible differentiation from live temperature-sensitive mutant MG vaccine strains, namely, ts-11.

MATERIALS AND METHODS

RAPD-PCR Standardization

A reference MG ts-11 live vaccine strain (Merial Company, Lyon, France) was used to standardize the RAPD-PCR technique. The strain was grown for 4 days at 32˚C in Frey's broth prepared as previously described.[13] In brief, the method used for MG-DNA extraction[14] is as follows: the MG culture in Frey's broth was centrifuged at 15557 x g for 15 minutes. Lyses of pelleted MG cells was accomplished by 10% sodium dodecyl sulfate (SDS). Denatured proteins and polysaccharides were removed by the addition of 5M sodium chloride followed by a mixture of chloroform/isoamyl alcohol (volume/volume, 0.7/0.8). The nucleic acid in the supernatant was precipitated by isopropanol, washed by 70% ethanol, and the DNA was dried under vacuum in a lyophilizer at -10˚C for 15 minutes. The dried pellet of MG-DNA was reconstituted in 100 µL tri-ethylene-diamine-tetraacetic acid (TE) buffer. The reconstituted DNA was diluted 1 in 100 using RNAase-DNAase-free water thus allowing a spectrophotometric determination of DNA concentration at a wavelength of 260/280 nm (BioMate 3 Series Spectrophotometry, ThermoSpectronic, Rochester, NY, USA). Checkerboard RAPD-PCR was performed with reaction mixtures in a total volume of 50 µL in each reaction. Three experiments were concluded in the checkerboard:

Experiment 1: Extracted MG-DNA was fixed at a level of 1000 ng and each of the 3 primers used was set at 0.006 nmole per 50-µL reaction mixture, while each deoxynucleoside triphosphate (dNTP) concentration (guanine, cytidine, thymidine, and adenine) was included in the reaction at 0.05, 0.2, and 0.8 mM concentration. The inclusion of each dNTP concentration was in the presence of $MgCl_2$ at either 1-mM or 2-mM levels.

Experiment 2: Based on the results of banded amplicons in experiment 1, DNA was fixed at 1000 ng, while each dNTP was set at 0.8mM, and the $MgCl_2$ level was set at 2 mM/50-µL reaction mixture. The variable in the checkerboard of experiment 2 was the level of each of the three primers used at either 0.006 nmole, 0.012 nmole, or 0.025 nmole. The three oligonucleotide

primers used were: M16SPCR5', M13F, and S1OLIGO3' (Amersham, Glyfada, Greece).[15] The sequence, base pair size, guanine plus cytosine (G + C) content, and melting temperature of each primer were as follows:

The M16SPCR5' primer had a base pair size of 17, a G+C of 52.9%, a melting point of 43.7˚C, and a base sequence of 5'AGGCAGCAGTAGGGAAT3'.

The M13F primer had a base pair size of 13, A G+C of 53.8%, a melting point of 30.5˚C, and a base sequence of 5'GTAAAACGACGGC3'.

The S1OLIGO3' primer had a base pair size of 20, a G+C of 35%, a melting point of 42.6˚C, and a base sequence of 5'CATAACTAACATAAGGGCAA3'.

Experiment 3: Based on the results of banded amplicons from experiments 1 and 2, each dNTP, $MgCl_2$, and each primer were fixed at 0.8mM, 2mM, and 0.025 nmole, respectively. The variable in the checkerboard of experiment 3 was the level of MG-DNA that was used at 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, and 1000 ng/50-µL reaction mixture. It is worth noting that in each of the 3 experiments described here, the level of *Taq* polymerase included in the 50-µL reaction mixture of the RAPD-PCR was 1.25 units. The amplification was performed in a Perkin-Elmer Cetus GeneAmp PCR System 2400 (Perkin-Elmer Cetus, Norwalk, CT), programmed for 3 cycles of 94˚C for 15 seconds, 28˚C for 2 minutes, and 74˚C for 3 minutes, and 35 cycles of 94 for 15 seconds, 45˚C for 2 minutes, 74˚C for 3 minutes. At the end of the cycles the amplicons were set at 4˚C until subjected to banding by agarose gel electrophoresis.

Banding of Amplicons by Agarose Gel Electrophoresis

Banding of PCR products (amplicons) was carried out by agarose gel electrophoresis, as indicated previously.[16] Briefly, a 2% agarose gel was used with amplicon load per lane of 9 µL mixed with 4.5 µL of a loading dye, prepared by dissolving 2 g of sucrose plus 12.5 mg of bromophenol blue dissolved in 30 µL of sterile, nuclease-free water. Ten lanes existed on each gel, with one lane reserved for loading with a 100 base-pairs ladder (Amersham, Glyfada, Greece). The loaded amplicons were electrophoresed at 120 V for 90 minutes and the gel was then stained with ethidium bromide (30 µL of 1% ethidium bromide in 300 µL of distilled water) by shaking at 600 rpm for 30 minutes. The banded amplicons and the base pairs ladder were visualized under UV light and then photographed using a GelDoc 2000 System (BioRad Laboratories, Hercules, CA).

Based on the results of the three experiments described, revealing maximum numbers of clear-banded amplicons, the method was standardized at the following levels: 2 mM of $MgCl_2$, 0.8 mM of each dNTP, 0.025 nmole of each primer (M16SPCR, M13F, and S1OLIGO), and at 750 ng of MG-DNA. These standardized levels were respected in the following method's evaluation of the sensitivity, specificity, reproducibility, and applicability to field MG subspeciation.

Sensitivity of the Standardized RAPD-PCR

The DNA of each of two field MG-isolates was included in the standardized RAPD-PCR procedure at the following varied levels of 400, 350, 300, 250, 200, 150, 100, 50, and 25 ng. The produced amplicons from each DNA level of each of the two MG isolates were banded by agarose gel electrophoresis and photographed as described above.

Specificity of the Standardized RAPD-PCR

The standardized RAPD-PCR was applied to 9 replicates each of SE and *E coli*, and on 15 replicates of ts-11 MG strain. The percent predominance of banded amplicon patterns produced from each of the three bacteria, and the specificity of RAPD-PCR in differentiation of MG genetic material from SE and *E coli* was determined.

Reproducibility of the Standardized RAPD-PCR

The standardized RAPD-PCR was applied on 3 replicates of each of 3 field MG strains, and 15 replicates of ts-11 MG strain. The percent reproducibility in obtaining a certain banding pattern of the amplicons of these isolates was determined.

Standardized RAPD-PCR in Subspeciation of Field MG Strains

Eleven field MG isolates were recovered from the tracheas of birds from four poultry flocks, and were speciated by colonial morphology, direct immunofluorescence on suspected colonial cells, and by Western immunoblotting.[8] The standardized RAPD-PCR was used to produce amplicons for each of the 11 field MG isolates to investigate the possibility of having different amplicon banding patterns, an indication of MG subspeciation.

Standardized RAPD-PCR in Sequencing of Specific MG Gene(s)

A major clear and conserved band of 912 bp was revealed from RAPD-PCR of ts-11 MG. It was not present in any field MG strain and was cut from the 2% agarose gel under UV light, using a Gel Cutter (BioRad Laboratories, Hercules, CA, USA). Briefly, the gel was dissolved at 55˚C for 10 minutes, and the DNA was precipitated with Silica gel. The precipitate was washed in buffer and the primer M16SPCR was used for extension products. Amplification was performed in a thermocycler system (PX2, Thermo Hybaid, Franklin, MA, USA) programmed for 40 cycles of 96˚C for 10 seconds, 50˚C for 10 seconds, and 60˚C for 4 minutes, and then set at 4˚C until use. The product was loaded in 20-µL volume in an automated sequencer (3100 Avant Genetic Analyzer-ABI PRISM, Applied Biosystems, Hitachi, Japan). The sequenced band was then compared for a part or complete gene sequence identity using the multiple sequence alignment program available at www.ncbi.nlm.nih.gov/BLAST. Nucleotides in the revealed sequence were labeled C, G, T, and A, respectively for guanine, cytidine, thymidine, and adenine. Non-identified nucleotides were reported as N.

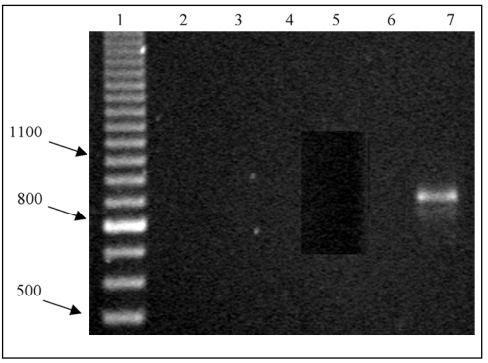

Figure 1. Electrophoretic analysis (2% agarose gel) of RAPD-PCR patterns of ts-11 DNA amplified with different dNTP and $MgCl_2$ concentrations in 50-µL PCR reaction mixtures. Lane 1 = molecular size marker in base pairs (100 bp); lane 2 = mixture, 1 containing 0.05 mM dNTP and 1mM $MgCl_2$; lane 3 = mixture 2, containing 0.05 mM dNTP and 2mM $MgCl_2$; lane 4 = mixture 3, containing 0.2 mM dNTP and 1 mM $MgCl_2$; lane 5 = mixture 4, containing 0.2 mM dNTP and 2 mM $MgCl_2$; lane 6 = mixture 5, containing 0.8 mM dNTP and 1 mM $MgCl_2$; lane 7 = mixture 6, containing 0.8 mM dNTP and 2 mM $MgCl_2$. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; dNTP, deoxynucleoside triphosphate; bp, base pairs.

RESULTS

The results of the checkerboard in experiment 1 for standardization of the dNTP and $MgCl_2$ concentration are shown in Figure 1 and Table 1. The amplification of the DNA of ts-11 MG was successful at dNTP and $MgCl_2$ concentration of 0.8mM and 2mM, respectively (Figure 1, lane 7; Table 1), but not at any of the lower concentrations used in the checkerboard. However, the results of the checkerboard in experiment 2 for standardization of each of the three primers resulted in maximum number of four clear bands of amplicons at each primer concentration of 0.025 nmole /50-µL reaction mixture of the RAPD-PCR (Figure 2, lane 2; Table 2). In addition, the results of the checkerboard in experiment 3 for the stan-

Table 1. Number and Length of Different Bands Resulting from Amplification of ts-11 DNA with Different dNTPs and $MgCl_2$ Concentrations in the Reaction Mixtures

Mixtures*	Each dNTP† concentration (mM)	MgCl² concentration (mM)	Number of bands (amplicons)	Band lengths (base pairs)
1	0.05	1	0	–
2	0.05	2	0	–
3	0.2	1	0	–
4	0.2	2	0	–
5	0.8	1	0	–
6	0.8	2	2	915, 853

*Each mixture had a volume of 50 μL and contained, in addition to varied dNTPs and $MgCl_2$ concentrations, constant amounts of 0.25 μL of 0.025 nmole/μL of each primer (M16SPCR, M13F and S1OLIGO), 1.25 units of *Taq* polymerase, and 1 μL containing 1,000 ng of ts-11 DNA. The volume was completed to 50 μL with nuclease-free water.
†Individual dNTPs in the mixture were: cytidine, guanine, thymidine, and adenine.dNTP indicates deoxynucleoside triphosphate.

Table 2. Number and Length of Different Bands Resulting from Amplification of ts-11 DNA with Different Primers Concentrations in the Reaction Mixtures

	Primers							
	M16SPCR		M13F		S1OLIGO			
Mixture*	Conc. (nmole/μL)	Vol. (μL)	Conc. (nmole/μL)	Vol. (μL)	Conc. (nmole/μL)	Vol. (μL)	Band numbers	Band length (base pairs)
1	0.025	1	0.025	1	0.025	1	4	912, 854, 705, 416
2	0.025	0.5	0.025	0.5	0.025	0.5	2	912, 416
3	0.025	0.25	0.025	0.25	0.025	0.25	1	912

*Each mixture had a volume of 50 mL and contained, in addition to the varied primer levels, 0.8mM of each deoxynucleoside triphosphate (dNTP), 2mM of $MgCl_2$, 1.25 units of Taq polymerase, and 1 mL containing 1,000 ng of ts-11 DNA. The volume was completed to 50 mL with nuclease-free water.
†Conc. indicates concentration; Vol, indicates volume.

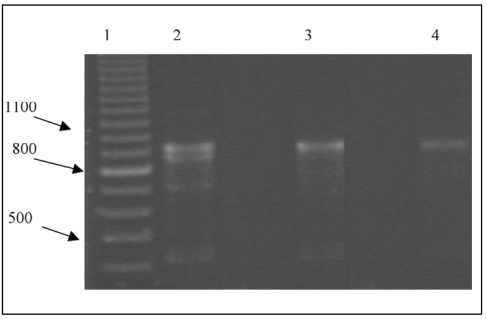

Figure 2. Electrophoretic analysis (2% agarose gel) of RAPD-PCR patterns of ts-11 DNA amplified with different primer concentrations in 50-μL PCR reaction mixtures while keeping each dNTP concentration at 0.8 mM and $MgCl_2$ concentration at 2 mM. Lane 1 = molecular size marker in base pairs (100 bp); lane 2 = mixture 1, containing 1 μL of 0.025 nmole/μL of each primer (M16SPCR, M13F, and S1OLIGO); lane 3 = mixture 2, containing 0.5 μL of 0.025 nmole/μL of each primer; lane 4 = mixture 3, containing 0.25 μL of 0.025 nmole/μL of each primer. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; dNTP, deoxynucleoside triphosphate; bp, base pairs.

dardization of DNA concentration of MG revealed three bands at a DNA concentration ≥ 500 ng. However, the clarity of the three bands was more visually obvious at ≥ 750 ng (Figures 3a and 3b; Table 3).

The sensitivity of the standardized RAPD-PCR in amplification of the DNA of two field MG isolates is shown in Figures 4 and 5. The method was able to amplify a minimum of 50 ng of DNA from the first field MG isolate (Figure 4, lane 8), and a minimum of 100 ng of DNA from the second field MG isolate (Figure 5, lane 9).

In the 9 replicates of each SE and *E coli* and the 15 replicates of ts-11 MG, the band patterns of amplicons from the 3 bacteria were totally different, with no presence of a common band—a conclusion of 100% specificity of the standardized RAPD-PCR to MG (Table 4). In addition, the percent predominance of amplicon banding patterns of the three bacteria were as follows: MG

Table 3. Number and Length of Different Bands Resulting from Amplification of Different Concentrations of ts-11 DNA in the Reaction Mixtures

Mixture*	Amount of ts-11 DNA in the reaction mixture (ng)	Band numbers	Band length (base pairs)
1	150	1	912
2	200	1	912
3	250	1	912
4	300	1	912
5	350	1	912
6	400	1	912
7	450	1	912
8	500	3	912, 416, 854
9	550	3	912, 416, 854
10	600	3	912, 416, 854
11	650	3	912, 416, 854
12	700	3	912, 416, 854
13	750	3	912, 416, 854
14	800	3	912, 416, 854
15	850	3	912, 416, 854
16	900	3	912, 416, 854
17	950	3	912, 416, 854
18	1000	3	912, 416, 854

*Each mixture in a volume of 50 μL contained, in addition to the ts-11 DNA, 0.8mM of each deoxynucleoside triphosphate (dNTP), 2mM of $MgCl_2$, 1.25 units of *Taq* polymerase, and 1 μL of 0.025 nmole/μL of each primer (M16SPCR, M13F and S1OLIGO). The volume was completed to 50μL with nuclease-free water.

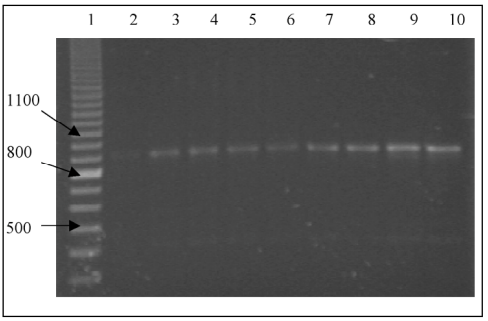

Figure 3a. Electrophoretic analysis (2% agarose gel) of RAPD-PCR patterns of different concentrations of ts-11 DNA in 50-μL PCR reaction mixtures. Lane 1 = molecular size marker in base pairs (100 bp); lanes 2-10 contain ts-11 DNA at the following levels: lane 2 = mixture 1, containing 150 ng ts-11 DNA; lane 3 = mixture 2, containing 200 ng; lane 4 = mixture 3, containing 250 ng; lane 5 = mixture 4, containing 300 ng; lane 6 = mixture 5, containing 350 ng; lane 7 = mixture 6, containing 400 ng; lane 8 = mixture 7, containing 450 ng; lane 9 = mixture 8, containing 500 ng; lane 10 = mixture 9, containing 550 ng. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; bp, base pairs.

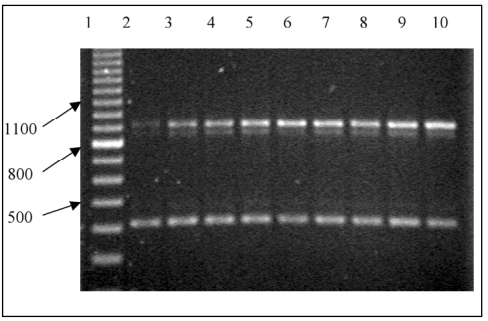

Figure 3b. Electrophoretic analysis (2% agarose gel) of RAPD-PCR patterns of different concentrations of ts-11 DNA in 50-μL PCR reaction mixtures (continued). Lane 1 = molecular size marker in base pairs (100 bp); lanes 2–10 contain ts-11 DNA at the following levels: lane 2 = mixture 10, containing 600 ng of ts-11 DNA; lane 3 = mixture 11, containing 650 ng; lane 4 = mixture 12, containing 700 ng; lane 5 = mixture 13, containing 750 ng; lane 6 = mixture 14, containing 800 ng; lane 7 = mixture 15, containing 850 ng; lane 8 = mixture 16, containing 900 ng; lane 9 = mixture 17, containing 950 ng; lane 10 = mixture 18, containing 100 ng. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; bp, base pairs.

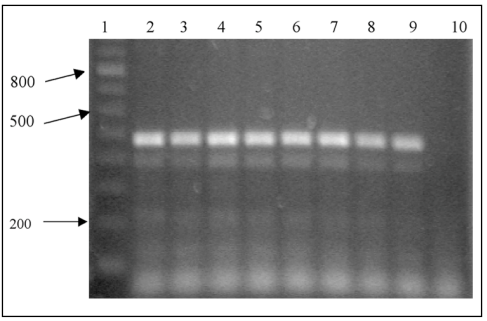

Figure 4. Electrophoretic analysis (2% agarose gel) of RAPD-PCR amplicons produced from different amounts of DNA of the first field MG isolate in 50-µL PCR reaction mixtures. Lane 1 = molecular size marker in base pairs (100 bp); lanes 2-10 contain s-11 DNA at the following levels: lane 2 = 400 ng; lane 3 = 350 ng; lane 4 = 300 ng; lane 5 = 250 ng; lane 6 = 200 ng; lane 7 = 150 ng; lane 8 = 100 ng; lane 9 = 50 ng; lane 10 = 25 ng. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; MG, *Mycoplasma gallisepticum*; bp, base pairs.

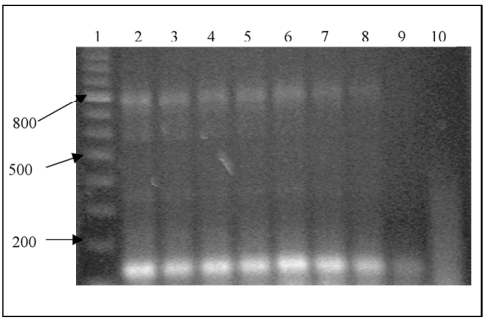

Figure 5. Electrophoretic analysis (2% agarose gel) of RAPD-PCR amplicons produced from different amounts of DNA of the second field MG sample in 50-µL PCR reaction mixtures. Lane 1 = molecular size marker in base pairs (100 bp); lanes 2-10 contain tw-11 DNA at the following levels: lane 2 = 400 ng; lane 3 = 350 ng; lane 4 = 300 ng; lane 5 = 250 ng; lane 6 = 200 ng; lane 7 = 150 ng; lane 8 = 100 ng; lane 9 = 50 ng; lane 10 = 25 ng. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; MG,*Mycoplasma gallisepticum*; bp, base pairs.

Table 4. Predominance of Each Amplicon Banding Pattern Resulting from RAPD-PCR Amplification of 9 Replicates Each of *Salmonella enteritidis* and *Escherichia coli* and 15 Replicates of *Mycoplasma gallisepticum* ts-11*

Bacterium	Amplicons banding pattern (base pairs)	Predominance of pattern
Salmonella enteritidis	353, 680,769, 935	55.6%
	680, 769	11.1%
	Absence of pattern	33.3%
Escherichia coli	303, 384, 465, 517, 914, 1084	77.8%
	Absence of pattern	22.2%
MG ts-11 strain	912, 854, 416 + any other band.	87.5%
	912, 854, 729, 416	44.0%
	1158, 912, 854,729, 416	31.0%
	Absence of pattern	12.5%

*MG indicates *Mycoplasma gallisepticum*.

had a 87.5% predominance of banding pattern 912, 854, 416, plus any other band, whereas the *E coli* had a 77.8% predominance of 1084, 914, 517, 465, 384, and 303, and the *S enteritidis* had a 55.6% predominance of 935, 769, 680, and 353 bp.

The reproducibility of the standardized RAPD-PCR in obtaining the same banded amplicon pattern from each of the 3 field MG isolates was 100% (Figure 6); however, the frequency in obtaining 3 different amplicon patterns from ts-11 MG has varied, with a maximum reproducibility of 87.5% in obtaining one pattern, namely the 912, 854, 416 plus any other band (Table 5).

Eleven field chicken tracheal isolates were suspected as MG infected based on the fried-egg morphology of the colonies on Frey's agar. All of the 11 isolates were positive for MG by direct immunofluorescence, and were confirmed by Western immunoblot as *Mycoplasma gallisepticum* species isolates, showing typical banding patterns of their polypeptides. Figure 7 demonstrates four field MG isolates applied respectively in lanes 3, 4, 5, and 6, and confirmed to the species level as MG, based on specificity of the reaction of polyclonal antibodies to major polypeptides of MG.

The standardized RAPD-PCR revealed apparent differences in amplicon patterns of the 11 field MG isolates (Table 6). Isolate

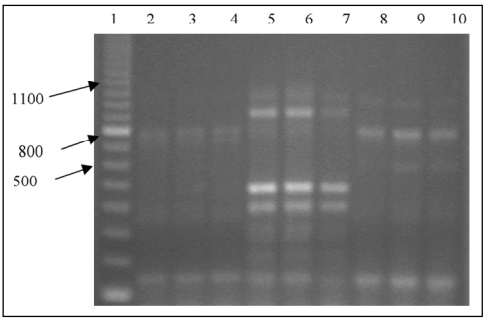

Figure 6. Reproducibility of RAPD-PCR. Each of 3 field MG isolates was used in triplicate to assess the repeatability of the banding patterns. Lane 1 = molecular size marker in base pairs (100 bp); lanes 2 through 4 = first MG isolate; lanes 5 through 7 = second MG isolate; lane 8 through 10 = third MG isolate. RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; MG, *Mycoplasma gallisepticum*.

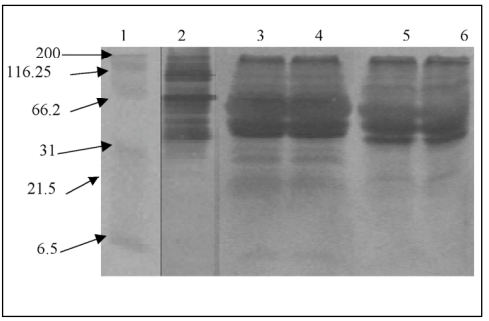

Figure 7. Immunoblots of 4 field MG and a control positive ts-11 isolate. Lane 1 = molecular weight marker (kD), lane 2 = ts-11; lanes 3–6 each contain polypeptides of individual field MG strain. MG indicates *Mycoplasma gallisepticum*.

number 1 from flock 1 showed a complete difference from all other 10 field isolates. The isolates from different birds in the same flock had higher similarity, for example, isolates 2-6 from flock 2, isolates 7 and 8 from flock 3, and isolates 9-11 from flock 4, an indication of the predominance of similar subspecies of MG in the same flock. However, the RAPD-PCR was also able to detect subspecies differences in MG isolates recovered from the same flock, for example, isolate 4 versus isolate 2 from flock 2.

The developed RAPD-PCR is able to sequence bands of amplicons that are of significance in differentiating vaccine from field MG strains. The vaccine ts-11 strain had a conserved band that doesn't get produced from the amplification of any of the field MG strains—namely, the 912 bp. The nucleotide sequence of the band is as follows:

GGGTAANAGNGCNTNGCTGCTG-
GTTNGTGGNTGNAGAGAGANGNT-
TATTNCTNNTTCGANANAGNAAAAG
AACAANGGAGTGCNCGTGTAAC-
TANNNCCCCGAGNTAACTNNTAN-
NTAGAGNACCGNNTTAANANCGGTT
TGGTCNAATTTAGATATTGTGAAAC-
CGGTAACTANNCGCNATTTGTTTAT-
CACNCCNCAGATCTCTAACNNGATGA
TCTTCGNGNTTATTGCTGGGA-
CAAGCCGATTGCTTANTTTAATGAG-
TAGTCCGATCTNTTTANAGCT-
TAATCAGCGTTTAGATAAGATCAGCT
TACTTCGTTTATTTGGCGTATCAN-
NTANCGTTAGCTTAGGGTTTTATGGN-
GATTAATTGCGTACTAGTAAATANAG
GTTGGACAACGTTCTTATATT-
TAATTGGGATCATCGTGATGATC-
GATTCGNTTGCTTATATTTATGGNNA
ACGGNTTGGNAAGATCAAGATG-
GCTCCNGAGATCAGNCNCATGA-
GACTTGNGCTGGAGCAATCTATGGGA
TTATTACCACGATCNNNAT-
NATGGGTGGTTGTTAGTATTCTT-
TAAGCGNGCCCNGNTAGNATCGGGA
AGGGTACCANAANGTATCACCCCTG-
GAAGNAATGGACCNCNTAATT-
TATAGTCATATNTGTACATCNCTTTT
NCANANAAAATANNTCCTAAC-
CTCTGGAGGGATTTGGGGGTTGAN-
NCTAGTTAGCCCATGCATNTAGGGAC
CGNATTTCTAGTGAAGCGGATTAA.

Table 5. Reproducibility of Each Amplicon Banding Pattern Obtained from the Amplification of ts-11 DNA using RAPD-PCR*

Banding pattern (base pairs)	Reproducibility (%)
912, 854, 416 + any other band	87.5
912, 854, 729, 416	44.0
1158, 912, 854,729, 416	31.0
No bands	12.5

*RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction.

Table 6. Standardized RAPD-PCR in Subspeciation of 11 Field MG Isolates Recovered from Trachea of 4 Different Poultry Flocks Based on Amplicon Banding Patterns*

Amplicon band (bp)	Presence of the band (+) in each of 11 field MG isolates											Total
	Flock 1	Flock 2					Flock 3		Flock 4			
	1	2	3	4	5	6	7	8	9	10	11	
142									+	+	+	3
217										+		1
294		+										1
307										+		1
353		+	+	+	+	+	+					6
361								+				1
371				+								1
372									+			1
382											+	1
397										+		1
504			+	+			+	+		+		5
528		+										1
559							+					1
583		+		+	+	+						4
598									+		+	2
627				+								1
680	+											1
705				+			+	+				3
720		+										1
748			+	+	+	+			+	+	+	7
767		+										1
799									+	+	+	3
862				+								1
949										+		1
976				+								1
1037									+		+	2
1115										+		1

*RAPD-PCR indicates Random Application of Polymorphic DNA-Polymerase Chain Reaction; MG, *Mycoplasma gallisepticum.*

The revealed sequence showed 86% identity with a part of the cytidine diphosphate-diglyceride synthetase (cDSA) gene of the MG-R strain.

DISCUSSION

The optimal concentrations of $MgCl_2$, each dNTP, template MG-DNA, and each of the three primers used in RAPD-PCR was found to be 2.0 mM, 0.8 mM, 750 ng, and 0.025 nmole (283 ng)/50 µL of the reaction mixture, respectively (Figures 1, 2, 3a, and 3b; Tables 1–3). The standardized concentrations of $MgCl_2$ and dNTPs were near, but not typical, to those reached by previous investigators,[7] who used 1.5 mM of $MgCl_2$, 0.25 mM of each dNTP, and 250 ng of each primer in a 50-µL reaction mixture. In addition, in 1995 Fan et al.[15] standardized the RAPD-PCR at 2 mM of magnesium chloride, 0.2mM of each dNTP, and 125 ng of each primer in a 50-µL reaction mixture. In this study, although the optimal concentration of template DNA was 750 ng /50-µL

reaction mixture, complete banding patterns were weakly obtained when the DNA template ranged between 300 ng and 500 ng/50-µL reaction mixture. These results agree with those obtained by previous researchers,[7] who used lesser amounts of template MG-DNA (250 ng/50-µL reaction mixture). Others[15] have reported using a template in volume units of 0.5 µL/100-µL reaction mixture, without indicating the amount in weight units, thus impeding the chance of comparison.

Increasing the three primers mix from 0.25 to 1 µL of 0.025 nmole/µL of each primer in the 50-µL reaction mixture resulted in an increase in band number from 1 to 4. These results are in agreement with previous investigators,[17,18] who found that lower primer concentrations resulted in lower product yield. Low concentrations of $MgCl_2$, down to 1mM, affected—even inhibited—the formation of RAPD-PCR products or amplicons. These results are in agreement with other studies,[19] which mentioned that RAPD amplification is highly affected by many factors, especially the Mg^{++} concentrations.

These similarities in the presence of some variations among laboratories emphasize the need for a global RAPD-PCR standardization before any regional or international epidemiological investigation of MG-subspeciation is initiated.[19] The standardization of RAPD-PCR ensures higher reproducibility and reduces intrinsic errors,[15,20] thus allowing better comparison of results among different laboratories.

The standardized RAPD-PCR showed a high sensitivity in amplifying an amount as low as 50 ng of first field MG-DNA in the 50-µL reaction mixture (Figure 4), and as low as 100 ng of the second field MG isolate (Figure 5). These results agree with those obtained by others,[7] who found that a range of 50 µg–300 µg of template MG-DNA was sufficient for RAPD-PCR to generate a complete banding pattern of amplicons. In addition, previous workers[21] found that a concentration of 10 pg to 100 ng/µL is even adequate for amplification by RAPD-PCR.

The RAPD-PCR was also highly specific to MG resulting in banded amplicons that don't match at all with any band of amplicons produced by other bacteria (Table 4). Such specificity is in agreement with the data of Marios et al.,[3] who were able to differentiate even among 21 MG strains by RAPD. In addition, other researchers[7] were able to accomplish this differentiation among field MG isolates from songbirds in the Eastern United States in a comparison with a reference ts-11 vaccine strain of MG.

The reproducibility of the standardized RAPD-PCR in producing certain amplicon banding patterns was 100% for field MG isolates and 87.5% for the reference ts-11 MG strain, producing 3 consistent bands: the 912, 854, and 412 bp (Figure 6 and Table 5). Others obtained similar results,[3,15] in which the banding patterns were consistent for each tested MG strain. The high reproducibility of RAPD-PCR is most likely due to appropriate standardization procedures, including the use of proper concentration of template MG-DNA,[21] primers concentration,[17,18] standard gel electrophoresis protocol,[7] standardization of reagent concentrations, and reducing intrinsic errors through the use of the same thermocycler model, the same batch of primers, and other reagents—namely the $MgCl_2$, dNTPs, and buffers.[19]

The direct immunofluorescence and Western immunoblot can confirm only the identity of the field isolates to the species level, namely as MG isolates. As shown in Figure 6, the representative 4 field MG isolates revealed typical polypeptide patterns. All had a 32-KDa polypeptide, previously defined as a cytadhesin,[22] the 67-KDa polypeptide, a major hemagglutinin,[8,23] and the 44-KDa and 36-KDa polypeptides that are both common to all MG isolates.[24]

In spite of the typical banding pattern of the polypeptides of 11 MG isolates, the standardized RAPD-PCR was able to detect differences in these isolates based on the banding pattern of their amplicons (Table 6).

This allows the future use of such amplicon patterns as genetic epidemiologic markers to trace the vertical and horizontal transmission of these MG subspecies. In addition, the presence of different amplicons produced from the vaccine strain of MG (ts-11) versus those from the 11 field strains of MG (Table 4 versus Table 6) allows the differentiation of vaccine from field MG strains. This helps in future studies of immunity and protection conferred by ts-11 vaccine[25] against different subspecies of MG present in poultry around the world.

In this study, the sequence analysis of the 912 bp ampilcon from the ts-11, along with the multiple–sequence alignment program, helped in comparing the ts-11 gene locus of the enzyme cytidine diphosphate-diglyceride synthetase (cDSA) to that of the main parent field strain of MG—namely, the R-strain. It is worth noting that the cds locus partially sequenced in amplicon band 912 bp of this study is the structural gene for cDSA,[26] an essential enzyme for fatty acid and phospholipids metabolism that is responsible for the formation of CDP-diglyceride and pyrophosphate yielded from the reaction between cytidine triphosphate (CTP) and phosphatidic acid.[27] The 86% similarity in identity between the R-strain and the ts-11 at the cds locus indicates certain deletions or mutations accomplished during the development of the ts-11 mutant. This is the first report to use RAPD-PCR amplification to study mutations by sequencing certain genes of MG, thus allowing for more specific subspeciation due to mutations in future investigations.

In brief, the use of checkerboard experiments for standardization of RAPD-PCR is an indispensable prerequisite for obtaining a highly sensitive, specific, reproducible method that can be used to differentiate field MG subspecies and vaccine from field MG strains, and to sequence specific bands to uncover genomic mutations and alterations from the original field MG strain, such as the R-strain.

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