Research Journal of
POULTRY
SCIENCES
SINCE 2000
MEDWELL PUBLICATIONS

Multiplex-PCR for 2 Mycoplasmal Agents of Chicken Breeder Flocks and MG Vaccine Strain Differentiation by mgc2-PCR RFLP

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Key words: *Mycoplasma gallisepticum*, *Mycoplasma synoviae*, mPCR, mgc-2PCR RFLP

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Page No.: 15-21
Volume: 13, Issue 3, 2020
ISSN: 1993-5285
Research Journal of Poultry Sciences

Abstract: Due to the economic impact of Mycoplasma infection in poultry, it is essential to have a fast, reliable and accurate diagnostic test to diagnose the infection. Multiplex-PCR (mPCR) is advantageous in that a single swab can be used to identify the presence of either *Mycoplasma gallisepticum* (MG) or *Mycoplasma synoviae* (MS), testing can be completed in half the time, using fewer materials resulting in lower expense. The objectives of this study are two-fold: to optimize a mPCR for the detection of MG and MS from a single tracheal swab in order to investigate the presence of MG and MS in breeder flocks in Turkey and to differentiate the MG vaccine strains, ts-11 and 6/85 from field infection. Sensitivity of the mPCR was determined to be 6 colony forming units (CFU) mL^{-1} and 10 CFU mL^{-1}, respectively, from pure MG S6 and MS WVU1853 cultures. In artificially spiked samples with pure MG S6 and MS WVU1853 cultures, sensitivity decreased to 60 and 100 CFU mL^{-1}, respectively. A total of 900 tracheal swab samples were collected from nine chicken breeder flocks, three flocks each from Ankara, Bolu and Eskisehir provinces. Swabs were pooled into groups of 5, (180 pools) and were examined for the presence of MG and MS by mPCR and bacteriology. Testing revealed, 1/180 (0.55%) was found MS positive by both mPCR and culture. While 6/180 (3.88%) were determined MS positive, solely by mPCR. Differentiation of 6/85 and ts11 MG vaccine strains from field strains was achieved by mgc2 PCR-RFLP using HaeII restriction endonuclease enzyme.

INTRODUCTION

Mycoplasma gallisepticum (MG) and *Mycoplasma synoviae* (MS) are important avian respiratory pathogens[1, 2]. MG causes chronic respiratory disease accompanied by catarrhal inflammation of the respiratory tract mucosa[2]. MS causes synovitis and airsacculitis[1]. Both Mycoplasmal agents lead to economic losses in terms of reduced egg production and carcass quality. Breeder flocks are monitored to retain Mycoplasma-free status. Diagnosis of avian mycoplasmosis was performed by primary screening tests such as, Rapid Plate Agglutination test (RPA), Haemagglutination-Inhibition (HI), Enzyme-Linked Immunosorbent Assay (ELISA) and confirmation tests such as Polymerase Chain Reaction (PCR) and culture[3-5]. Serological tests are the primary screening tests for flock examination, however they sometimes lack the required specificity and sensitivity due to cross-reactions with other pathogenic mycoplasmas, false positive results due to oil-emulsion vaccines against other avian pathogens and antigenic variation among Mycoplasma strains resulting in false negative HI tests. Delayed antibody response makes early diagnosis difficult. For instance, RPA detects IgM antibodies found 7-10 days post-infection and IgG antibodies are detected by haemagglutination-inhibition test and ELISA 3-4 weeks post-infection[6-8]. Culture is laborious, expensive and requires serial passages. Moreover, a conclusive negative result confirming a Mycoplasma free flock takes up to 30 days. Furthermore, overgrowth by other contaminating bacteria and/or inhibitory effects of antibiotic therapy may make culture unsuccessful[9-11]. Recently, PCR was proposed as a reliable confirmatory test for the detection of MG and MS infection by the Office International Epizootie (OIE) and National Poultry Improvement Plan (NPIP)[3].

Various molecular techniques such as nucleic acid probes[12], recombinant DNA probes[13, 14], conventional PCR [15-18] and real-time PCR[11, 19-21] were developed in order to detect MG and MS. The primers used in PCR target the 16S rRNA gene [22], the genes encoding surface adhesion (pvpA, gapA, mgc-2, LP) in MG[22-24] and haemagglutinin proteins (pMGA, vlhA) in MS[25].

Control of MG and MS infection is a major problem in the poultry industry. Increasing poultry production in small geographic areas, rearing multiage poultry together and poor biosecurity render MG and MS free flocks impossible[5]. In order to monitor outbreaks efficiently, track infection and develop effective control strategies, rapid and specific diagnosis of MG and MS infection is essential[26, 27]. Due to increased use of live MG vaccines, discrimination of MG vaccine strains from field strains is important. Various molecular typing methods such as Random Amplified Polymorphic DNA (RAPD)[28], Amplified Fragment Length Polymorphism (AFLP)[29], Restriction Fragment Length Polymorphism (RFLP)[30, 31] and sequencing[32, 33] are available to differentiate MG vaccine from field strains.

The primary goal of this study is to optimize a multiplex-PCR (mPCR) by using formerly validated primers to detect MG and MS from a single tracheal swab and investigate the presence of MG and MS in breeder flocks located in Ankara, Bolu and Eskisehir provinces, Turkey. An additional goal of this study is to successfully differentiate MG vaccine strains ts-11and 6/85 and R challenge strain by mgc-2 PCR-RFLP.

MATERIALS AND METHODS

Standard MG and MS strains: MG S6 and MS WVU1853 were kindly provided by National Mycoplasma Reference Laboratory, Pendik Veterinary Control Institute, Istanbul, Turkey. MG A5969, K180, 6/85, ts11, F and R isolates on FTA cards and other Mycoplasma strains were kindly provided by Department of Population Health, College of Veterinary Medicine, University of Georgia, Athens, GA, USA (Table 1). All MG and MS isolates were used in multiplex-PCR or mgc-2 PCR optimizations and as positive controls with clinical samples for mPCR and mgc-2 PCR Table 1.

Clinical samples: Nine randomly selected flocks were sampled from three breeder flocks from each of the provinces of Ankara, Bolu and Eskisehir. These flocks are regularly controlled by the Veterinary Control Central Research Institute (VCCRI), Ankara, Turkey. A total of 900 tracheal swab samples (100 swabs/flock) were collected and pooled. Pools of 5 tracheal swabs were inoculated into tubes containing 5 mL Frey's broth (BBL,

Table 1: *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and other related strains used in this study

Strain No.	Strain name
1	*Mycoplasma gallisepticum* S6
2	*Mycoplasma gallisepticum* K180 (F)
3	*Mycoplasma gallisepticum* A2569
4	*Mycoplasma gallisepticum* A5969
5	*Mycoplasma gallisepticum* K3254 (6/85)
6	*Mycoplasma gallisepticum* ts-11
7	*Mycoplasma gallisepticum* K781(R)
8	*Mycoplasma gallisepticum* F vaccine
9	*Mycoplasma synoviae* WVU1853
10	*Mycoplasma synoviae* 4927C
11	*Mycoplasma gallinarum* K285-'B'-LPG16
12	*Mycoplasma meleagridis* E-2
13	*Mycoplasma gallopavonis* SA
14	*Mycoplasma iowae* K3761(1)
15	*Mycoplasma lipofaciens* Bx101 (40)
16	*Mycoplasma pullorum* D2403 (396)
17	*Mycoplasma columbinasale* Bx63 (15)
18	*Mycoplasma cloacale* Bx101 (48)
19	*Acholeplasma laidlawii* Bx250

Table 2: Primers, sequence, target gene, amplicon size

Primers	Sequence	Target gene	PCR product size (bp)
MG-14F	5'-GAGCTAATCTGTAAAGTTGGTC-3'	16 S rRNA	183
MG-13R	5'-GCTTCCTTGCCCTTAGCAAC-3'		
Mspcl4	5'-TCA TTC AGC GCC AGC TGG TTC-3'	Membrane protein	422
Mspcl5	5'-GCTTGAGTCTCCATTAACTTGTTGTTC-3'		
mgc2-F	5'-CGCAATTTGGTCCTAATCCCCAACA-3	Adhesine encoding gene	237-303
mgc2-R	5'-TAAACCCACCTCCAGCTTTATTTCC-3'		

Becton-Dickinson, No. 212346) and transferred to the laboratory on ice within 5 h. All the tracheal swab samples were examined for MG and MS by m-PCR and culture. Each pool was considered as one sample for multiplex-PCR and culture as follows: After vortexing 5 min, the swabs were discarded from the tube. One ml of Frey's broth was transferred into sterile eppendorf tubes and used for DNA extraction. One ml and 100 µL of remaining inoculated Frey's broth was transferred into another tube of Frey's broth and onto Frey's agar (BBL, Beckton-Dickonson, No.211456) respectively, for culture as described in Mycoplasma isolation section[34].

DNA extraction: DNA extraction of MG isolates: A5969, K180, 6/85, ts11, F and R from FTA cards (Whatman; FTA cards, WB120305, Germany) followed manufacturer's guidelines and used commercial FTA purification reagent (Whatman; FTA purification reagent, WB120204) and TE-1 buffer. Cultures including standard strains MG S6, MS WVU1853 and other Mycoplasma strains and clinical samples were extracted using a commercial DNA isolation kit (Roche; High Pure Template Preparation Kit, 11796828001, Germany) according to the manufacturer's instructions. All extracted DNA was stored at -20°C.

Primers: 16S rRNA (MG-14F and MG-13R) primers for MG and Mspcl4 and Mspcl5 primers for MS were used for the detection of both Mycoplasmal agents by multiplex-PCR. Primers as previously described by Garcia *et al*.[35], complementary to the 16S rRNA gene consisted of the following sequences: MG-13R; 5'-GCT TCC TTG CCC TTA GCA AC-3' and MG-14F; 5'-GAG CTA ATC TGT AAA GTT GGT C-3'. Expected amplicon size was 185 bp. Mspcl4 and Mspcl5 primers detecting MS mRNA coding for a membrane protein, previously described by Marois *et al*.[22] had a nucleotide sequence as follows: Mspcl4; 5'-TCA TTC AGC GCC AGC TGG TTC-3' and Mspcl5; 5'-GCT TGA GTC TCC ATT AAC TTG TTG TTC-3'. Expected amplicon size was 422 bp. For differentiation of MG vaccine and field strains, the primers detecting adhesin-encoding gene of MG previously described by Garcia *et al*. (2005) were used and the nucleotide sequence as follows: mgc2-F; 5'-CGC AAT TTG GTC CTA ATC CCC AAC A-3'and mgc2-R: 5'-TAA ACC CAC CTC CAG CTT TAT TTC C-3. The approximate PCR product for those primers was 237-303 bp (Table 2).

mPCR: Each reaction volume of 25 µL included 2 µL template DNA and 23 µL reaction mixture containing: 2.5 µL 10×PCR buffer (without $MgCl_2$), 0.5 µL dNTP (10 mM), 3 µL $MgCl_2$ (25 mM), a 1 µL of each (10 pmol μL^{-1}) primer (MG-14F, MG-13R, Mspcl4, Mspcl5) (Table 2), 0.25 µL Taq DNA polymerase (5 U μL^{-1}) (Fermentas; EP402), 12.75 µL nuclease free water. Cycling parameters were as follows: initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 40 sec, extension 72°C for 30 sec and final extension 72°C for 5 min. using Thermal Cycler (Techne 5000, Bibby Sci. Techne, China).

mgc2-PCR: Each 23 µL reaction mixture containing 2.5 µL 10×PCR buffer (without $MgCl_2$), 0.5 µl dNTP (10 mM), 3 µL $MgCl_2$ (25 mM), 1 µL of each (10 pmol μL^{-1}) primer (mgc-2F, mgc-2R) (Table 2), 0.25 µL Taq DNA polymerase (5 U μL^{-1}) (Fermentas ; EP402), 12.75 µL nuclease free water and 1 punch (3 mm size) of FTA filter template. Cycling parameters were as follows: initial denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 40 sec, extension 72°C for 30 sec and final extension 7224°C for 5 min. PCR was performed using Thermal Cycler (Techne 5000, Bibby Sci. Techne, China). The expected amplicon size was 237-303 bp (Table 2).

mgc2 PCR-RFLP: Restriction endonuclease enzymes SfaN1(Vivantis, SfaN1, RE1376) and HaeII (Vivantis, HaeII, RE1222) were used to cut mgc-2 PCR products, according to the manufacturer's instructions.

Agarose gel glectrophoresis: Electrophoreses was performed on a 1.5% agarose gel in TBE buffer containing 0.5 µL mL^{-1} of ethidium bromide at 100 V for 45 min and visualized under an ultra-violet light transilluminator.

Detection limit of mPCR with pure MG-MS culture: Initial concentrations of MG S6 and MS WVU1853 strain cultures were determined, respectively, of 6×106 colony forming units (CFU) mL^{-1} and 1×106 CFU mL^{-1}. The CFU's were determined by preparing serial ten-fold dilutions in Frey's broth and triplicate plating 100 µL onto Frey's agar. DNA was extracted from 1 ml of each of the

serial tenfold dilutions of MG S6 and MS WVU1853 Frey's broth cultures (10-1-10-7) and was used as template in mPCR.

Detection limit of mPCR with artificially spiked samples: Tracheal swabs of sero-negative chickens spiked artificially with the MG S6 and MS WVU1853 strains were used for this purpose. Briefly, 1 mL of 10-fold Frey's broth dilutions from 6×106-6 CFU mL^{-1} MG S6 and 1×106-1 CFU mL^{-1} MS WVU1853 strain mixed with individual tracheal swabs from MG and MS negative chickens determined by rapid agglutination test, hemagglutination inhibition test, ELISA and culture as indicated[3]. After vortexing approximately 5 min, culture-inoculated tracheal swabs were discarded. DNA was extracted from 1 mL of each of the 10-fold Frey's broth dilutions. One µL of each extract was used as a template in mPCR.

Specifity of mPCR: For this purpose, optimized mPCR was applied to the templates prepared from the cultures of selected Mycoplasma strains listed in Table 1. These Mycoplasma strains were specifically selected for testing the specificity of mPCR because they can normally be found in chicken trachea (Table 1).

Mycoplasma isolation: Tracheal swabbing was performed as described by Zain and Bradbury[36] and the isolation of MG and MS from these tracheal swabs was done following the standard culture method as described by Kleven[34]. Briefly, 5 tracheal swabs were placed into Frey's broth, vortexed 5 minutes and discarded. One ml of inoculated Frey's broth was transferred into another Frey's broth and 100 µL from each Frey's broth was streaked onto Frey's agar and incubated at 37°C with 5% CO_2 in high humidity. Color change in Frey's broth was observed daily and color change from pink to orange-yellow was considered as positive and were streaked onto Frey's agar and incubated at 37°C with 5% CO_2 in high humidity. One week post-incubation if still no color change, sub-culture into new Frey's broth, followed by one more passage if the color was unchanged after 1-week incubation. Plates were checked for typical colonies under an inverted microscope for at least 2 weeks. MG S6 and MS WVU1853 standard cultures were used as positive controls to assure the testing efficacy of the media used.

RESULTS AND DISCUSSION

Sensitivity of mPCR: The sensitivity of mPCR was determined as 6 and 10 CFU mL^{-1}, respectively with pure MG S6 and MS WVU1853 cultures. The limits of detection decreased in artificially spiked samples to 60 and 100 CFU mL^{-1}, respectively in MG S6 and MS WVU1853 cultures.

Specifity of mPCR: The expected products of 186 bp and 422 bp were observed for all MG and MS strains, respectively with no PCR product observed with other Mycoplasma strains tested (Table 1).

mPCR and bacteriology with clinical samples: Tracheal swab samples were pooled into groups of 5 and considered as 1 sample. Hence, 100 tracheal swab samples from each breeder firm were assumed as 20 samples. Consequently, 180 samples were examined by mPCR and bacteriology. Testing revealed, 1/180 (0.55%) was found MS positive by both mPCR and culture while mPCR found 6/180 (3.33%) MS positive. In summary, mPCR detected 7/180 (3.88%) MS positive (Table 3). On a flock basis, 2/9 (22.2%) flocks were found MS by mPCR and 1/9 (11.1%) flocks were MS positive by both mPCR and culture. The breeder firm B-B in Bolu was found to be MS positive by mPCR in 3/20 samples, while 1/20 was found to be MS positive by culture. Altough, 4/20 tracheal swab samples were determined MS positive from the E-C breeder firm in Eskisehir by mPCR, none of them was found to be positive by bacteriology. Of the 60 tracheal swab samples from A-A, A-B and A-C firms in Ankara, no MG or MG was found by either mPCR or bacteriology (Table 3).

mgc2-PCR and mgc2 PCR-RFLP: Since, no field isolates of MG were found, we performed mgc2-PCR with vaccine strains ts11, 6/85 and F, a low-passage F strain, K180 and other MG strains: A5969, MG-S6 and K-781(R strain). Subsequent RFLP was performed on the PCR products of ts11, 6/85 and R strains to differentiate vaccine strains from field strains. It was observed that 6/85 vaccine strain was easily differentiated from the tested strains using only mgc-2 PCR by yielding approximately 240 bp PCR product versus 300bp, for ts11 and other MG strains yielding approximately 300 bp PCR product as previously described by Lysnyansky *et al*.[31]. RFLP was performed on PCR products from mgc-2 PCR by digesting with SfaN1 and HaeII enzymes. Fragments following digestion with SfaN1 yielded the predicted sizes of 170 bp and 67 bp in 6/85 and 3 fragments of 170, 67 and 63 bp for ts11 vaccine strain. The HaeII enzyme digested 6/85 and ts11 vaccine strains at 2 sites, yielding fragments of the predicted sizes of 207, 30, 270 and 30 bp, respectively. R strain was not digested by either enzyme (Lysnyansky *et al*., 2005).

The primary purpose of this study was to differentiate and diagnose MG and MS infection in breeder flocks, from a single tracheal swab collected from one chicken by a sensitive, specific, reliable and rapid diagnostic method. For this reason, previously validated MG and MS primers were combined and used to optimize a mPCR. Detection limits of pure cultures in the mPCR were 6 CFU mL^{-1} for

Table 3: Number of MG and MS determined by bacteriology and mPCR

Province	Number of firms/ number of samples	Code of the firms/number of samples (each five pooled)	Results of bacteriology		Results of mPCR	
			MG(-/+)	MS (-/+)	MG(-/+)	MS(-/+)
Ankara	3/300 (100×3)	A-A/100 (20×5)	20-	20 -	20-	20-
		A-B/100	20 -	20 -	20 -	20 -
		A-C/100	20 -	20 -	20 -	20 -
Bolu	3/300	B-A/100	20 -	20 -	20 -	20-
		B-B/100	20 -	19-/1+	20 -	17-/3+
		B-C/100	20 -	20 -	20 -	20 -
Eskisehir	3/300	E-A/100	20-	20-	20-	20-
		E-B/100	20-	20-	20-	20-
		E-C/100	20-	20-	20-	16-/4+

MG and 10 CFU mL^{-1} for MS and 60 CFU mL^{-1} and 100 CFU mL^{-1}, respectively in artificially spiked tracheal swab samples. Garcia *et al*.[16], reported a detection limit of 100 CFU mL^{-1} for each species in a multispecies-PCR developed by using single oligonucleotide primers designed from known 16S rRNA sequences of MG, MS and Mycoplasma iowae. The same author in 2005[16], tested 4 different primer pairs for MG pure culture and declared the detection limits of the tested primers, 16S rRNA, mgc2, LP, gapA as 40, 40, 400 and 4 CFU mL^{-1}, respectively. In Turkey, Carli and Eyigor[19] reported the sensitivity of their real-time PCR as 3 CFU and 3000 CFU mL^{-1} with pure MG S6 culture and artificially spiked samples, respectively. Consequently, the differences between the detection limits could be due to variables such as primers in the studies, extraction procedures, optimization protocols and even the PCR technique used in the studies. In our study, detection limits in optimized mPCR were determined as 6 CFU mL^{-1} for MG and 10 CFU mL^{-1} for MS with pure cultures, 60 CFU mL^{-1} for MG and 100 CFU mL^{-1} for MS with artificially spiked samples which was satisfactory for conventional mPCR rather than real-time PCR. The difference in detection limit of mPCR with pure cultures and artificially spiked samples may be due to PCR inhibitors found in the trachea[19].

We have observed that, mPCR detected more MS than culture in tracheal swab samples. MS was difficult to isolate by culture because of its slow growth and competition between MS and other saprophytic mycoplasmas present in tracheal swabs, therefore an optimized mPCR could overcome those and provide a more sensitive approach than culture[37, 38]. As Kempf[4] noted, PCR has the inability to discriminate between viable and nonviable microorganisms. The mPCR positive MS results from the B-B flock in Bolu is substantiated by culture positive results. The MS positive results from the E-C flock in Eskisehir is not validated by any other diagnostic method, therefore good practice would follow-up with further testing in 7-10 days.

Live MG vaccines are used worldwide in commercial layer flocks and not permitted in breeder flocks. Live MG vaccine usage is strictly forbidden in breeder flocks in Turkey, however live MG vaccine strains were officially permitted in Turkey, approximately 3 years ago. This study was also to ascertain the use of live MG ts11 or 6/85 vaccines in breeder flocks in Ankara, Bolu, Eskisehir provinces of Turkey. No MG was found by either mPCR or culture. We performed mgc2-PCR and mgc2 PCR-RFLP to differentiate field strains from that of vaccine strains. In mgc-2 PCR, it was observed that all MG strains except 6/85 vaccine strain yielded a 300 bp PCR product, due to a deletion in 6/85 yielding an approximately 240 bp product. Consequently without a RFLP analysis, we could differentiate 6/85 vaccine strain from the other tested strains emphasized in the study. However, due to the same deletion in 6/85 vaccine and some 6/85 'like' field isolates, performing only mgc-2 PCR would not be discriminative for 6/85 vaccine and field strains. Also, we performed mgc2 PCR-RFLP with R strain, 6/85 and ts11 vaccine strains. For this purpose, after mgc2 PCR, all PCR products were digested by SfaN1 and HaeII enzymes. SfaN1 enzyme, yielded fragments of the predicted sizes of 170, 67 and 170, 67, 63 bp, respectively in 6/85 and ts11 vaccine strains. Due to its high cost and yielding very similar band profiles between two vaccine strains and also the difficulty of discrimination between fragments of 67 and 63 bp in ts11 vaccine strain, HaeII enzyme was chosen for further studies. We determined that, 6/85 vaccine strain was digested at 2 sites, yielding fragments of the predicted sizes of 207 bp and 30 bp and ts11 vaccine strain was digested at 2 sites, yielding fragments of 270 and 30 bp. Due to its small size, the restriction fragment of 30 bp was not seen under standard agarose gel electrophoresis. However, the longer restriction fragments, 207 bp for 6/85 and 270 bp for ts11 vaccine strains, were distinguishable. Similar to our study, Lysnyansky *et al*.[31] reported that, HaeII enzyme was superior to SfaN1 enzyme to differentiate MG ts-11 and 6/85 vaccine strains. R strain was not cleaved by either enzyme.

CONCLUSION

The optimized mPCR can be used reliably because of its high specificity and sensitivity, as a confirmatory test,

surpassing culture for timeliness and false negatives when primary screening tests are positive. mPCR may be another diagnostic tool in screening breeder flocks for MG and MS. Additional advantages are that it can detect the presence of MG and MS from a single tracheal swab, simultaneously and with fewer reagents making it cost effective. The differentiation of MG vaccine strains, by mgc2 PCR-RFLP, using HaeII restriction endonuclease enzyme would be a useful diagnostic tool for commercial layer flocks.

ACKNOWLEDGEMENT

We would like to thank Laboratory Technician Nusret ALTUN and Laborant Nurullah Çelik for their kind assistance. This study was supported by General Directorate of Agricultural Research and Policies (TAGEM/HS/10/13/01/166).

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