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Serological, Rapid Molecular Characterization and Antibiotic Resistance for Field Isolates of *Mycoplasma Gallisepticum* in Chicken in Saudi Arabia

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ABSTRACT

Mycoplasma Gallisepticum (MG) is considered as one of the most economically important mycoplasmal pathogens among poultry industry worldwide. This pathogen has various strains, therefore their detection using culture method is not sufficient. From this point of view, this study was designed to develop a novel TaqMan® real-time polymerase chain reaction (TaqMan RT-PCR) assay for direct detection of MG using cytadhesin to encode a surface protein (*mgc2*) containing a TaqMan FAM-labeled minor groove binder probe that targets this gene and to compare it with conventional polymerase chain reaction and immunological methods. Using serial broth dilution methods against identified MG isolates, minimum inhibitory concentrations of various antimicrobial agents were detected. Throughout Al-Qassim region, Kingdom of Saudi Arabia, 208 specimens were collected from 18 commercial chicken broiler farms where respiratory diseases were present. Furthermore, 180 blood serum samples were investigated for serological diagnosis of MG. Serum plate agglutination and enzyme-linked immunosorbent assay techniques detected positive serological identification in 83 (46.11%) and 97 (53.88%) isolates, respectively. The sensitivity recorded for TaqMan RT-PCR was 10^{-3} CFU/ml for MG template DNA. Results of TaqMan RT-PCR revealed 169 positive samples (81.25%), while 108 samples (51.92%) were identified as positive through conventional polymerase chain reaction assay. The results of MIC for 11 antimicrobial agents against 60 identified MG isolates indicated that all groups of MG exhibited a higher degree of sensitivity to tiamulin (93.33%) at low level of MIC_{50} and MIC_{90} (≤0.032 µg/ml) followed by tylosin (85%) and doxycycline (81.66%) with MIC_{50} and MIC_{90} ranged from ≤0.032 to 2 µg/ml; In contrast, gentamycin, tilmicosin, erythromycin, ciprofloxacin, oxytetracylcine and enrofloxacin did not show a higher activity at low concentrations. In conclusion, the developed TaqMan RT-PCR exhibited higher sensitivity and applicable accuracy than other molecular and serological techniques and thus could be highly recommended for the management of MG susceptibility and to facilitate implementation of effective treatment and prophylactic measures.

Key words: *Mycoplasma Gallisepticum*, TaqMan RT-PCR assay, Antimicrobial resistance

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1. INTRODUCTION

Avian mycoplasmosis was firstly identified in turkeys in 1926 and in chickens in 1936 (Charlton et al., 1996). *Mycoplasma Gallisepticum* (MG) is considered to be a highly pathogenic and economically important bacterial respiratory pathogen of chickens. Throughout the world, it is one of the most significant problems in the poultry industry. The infection is frequently known as chronic respiratory disease (CRD) in chickens and as infectious sinusitis in turkeys (Levisohn and Kleven, 2000). MG infections cause great economic losses in the poultry industry due to decreased hatchability and egg production, reduced quality of day-old chicks, reduced growth rate, increased costs of eradication measures involving site cleaning and depopulation, and increased drug and vaccination costs (Kaboli et al., 2013; Khalifa et al., 2013).

Traditionally, diagnosis of MG infection in the poultry industry depended upon serological screening such as serum plate agglutination (SPA) and enzyme-linked immunosorbent assay (ELISA) hemagglutination inhibition (HI), which historically are considered the most reliable tools for identifying subclinical infection in the flock (Barua et al., 2006; Purswell et al., 2012; Kaboli et al., 2013); however, they sometimes lack the required specificity and sensitivity (Carli, and Eyigor, 2003; Kaboli et al.,

2013). Therefore, in the last decade, rapid polymerase chain reaction (PCR) methods with high specificity and sensitivity for MG have been very useful for laboratory diagnosis of infected birds (Ehtisham-ul-Haque et al., 2015; Raviv and Kleven, 2009). Polymerase chain reaction based on identification of the *mgc2*-cytadhesin encoding surface protein gene for MG is the most widely used methods for the detection, typing and determination of the source of infection (Gerchman et al., 2009). Nevertheless, some problems are associated with PCR, including the existence of inhibiting substances in the samples, the danger of environmental contamination and the probability of the recognition of nonviable Mycoplasma (Kempf, 1998; Kleven, 1997; Carli and Eyigor, 2003).The progress in diagnostic PCR technology has greatly resolved the problems with the time, specificity, and sample size. Thus, the TaqMan RT-PCR avoids the need for post amplification processing, which saves time and labor compared to traditional PCR methods (Glew et al., 2000; Kawahara et al., 2008; Ehtisham-ul-Haque et al., 2015). The TaqMan RT-PCR is a simpler technique on which to base a multiplex PCR assay in order to detect mixed infections in a single PCR reaction. Until now, only a limited number of TaqMan RT-PCR-based diagnostic techniques have been reported that target the *mgc2* and *gapA* genes for the detection of MG (Grodio et al., 2008; Raviv and Kleven, 2009; Sprygin et al., 2010; Ehtisham-ul-Haque et al., 2015). To improve the specificity of TaqMan RT-PCR procedures, TaqMan minor groove binder (TM-MGB) probes are preferred because their small size enables binding in the minor groove of double-stranded DNA, thus stabilizing the duplex, which results in high melting temperature (Tm) values (Kutyavin et al., 2000; Guo et al., 2009; Ehtisham-ul-Haque et al., 2015) and greater accuracy in measurements because of low background fluorescence (Chiu and Ou, 1996; Farkas et al., 2009).

The chronic nature of Mycoplasma infections illustrates a failure of the host immune system to deal efficiently with these pathogens. The antigenic dissimilarity of surface proteins permits MG to get away from the host immune system through the generation of escape variants (Papazisi et al., 2003; Ehtisham-ul-Haque et al., 2015). In addition, intracellular attack by MG and its continued existence within eukaryotic cells may contribute to this organism's resistance to the host's immune response and to various antimicrobial agents (Winner et al., 2000; Papazisi et al., 2003). Consequently, prevention and control of MG have been considered the leading significant methods of management among poultry farms. One of the major practical methods of reducing the economic losses of MG infection is the wide use of antimicrobial drugs. Several groups of antibiotics including tetracyclines, macrolides, pleuromutilin, and fluoroquinolones are considered to be effective against MG (Bradbury et al., 1994; Hannan et al., 1997; Gerchman et al., 2008; Pakpinyo and Sasipreeyajan, 2007). Moreover, it is recognized that the usage of antimicrobial agents for a long period can lead to resistance to the MG organisms (Gautier-Bouchardon et al., 2002; Pakpinyo and Sasipreeyajan, 2007). The wide use of antimicrobial agents has led to selected resistance in many bacterial species, and this has become a major public health problem all over the world (Freire-Moran et al., 2011; Card et al., 2012). Consequently, in infected poultry farms, antimicrobial susceptibility tests must be conducted before initiating a treatment program.

The objective of our study was to compare the various diagnostic methods, including cultural, serological and conventional PCR with TaqMan RT-PCR for MG isolated from various broiler chicken farms. A further main objective was to demonstrate the sensitivity of the identified MG to various groups of antibiotics. The results of this study should be supported for the prevention and control of MG isolated from poultry farms in Saudi Arabia.

2. MATERIALS AND METHODS

2.1. Samples

A total of 208 samples were collected from eighteen chicken commercial breeding farms reporting the presence of respiratory diseases. Samples from the trachea (102), lungs (53) and air sacs (53) were collected from each bird at necropsy and divided in two parts, one for direct on the spot inoculation in Fray's broth and the other retained for DNA extraction. Furthermore, for serological diagnosis of MG, 180 blood serum samples (10 samples from each flock) were collected via wing vein puncture of each investigated bird. All samples were collected during the period from December 2013 to June 2015 in districts throughout Al-Qassim region, Kingdom of Saudi Arabia. All birds were selected on the basis of respiratory problems like sneezing, coughing,

lacrimation and respiratory sounds. All samples were transported in a refrigerated container to the microbiology laboratory at the College of Public Health and Health Informatics, Qassim University. Samples collected for DNA extraction were stored at -20°C to await further investigation.

2.2. Isolation and identification of MG

The collected tissue samples (trachea, lungs and air sacs) were minced under aseptic conditions and diluted in Mycoplasma broth (about 5 grams of tissue sample per 25 ml broth). Using a cotton swab, each sample was transferred and inoculated into 3 ml of broth of pleuropneumonia-like organisms (PPLOs) and incubated at 37 ºC for 3 days, after which, 20 µl of the incubated broth culture was transferred and streaked on PPLO agar. The agar plate was incubated at 37ºC in a moist candle jar under reduced oxygen tension (Sabry and Ahmed, 1975). Using an inverted microscope, the cultures were then examined at 5, 10, 15 and 30 days of incubation in the presence of identical fried-egg colonies of MG. Suspected MG isolates were confirmed through growth inhibition test (Lauerman et al., 1995) by moving a single colony on Fray's agar containing hyper immune serum raised in rabbits against reference strain (ts-11 vaccine strain, Merial international). All agar plates were incubated and observations documented.

2.3. Purification and maintenance of the isolates

According to the previous study (Sabry, 1968), A single colony with fried egg shape was picked up with an agar- block and transferred into a broth medium to obtain a pure culture of the isolates. The purified isolates were maintained at 20ºC in a form of agar blocks.

2.4. Serological diagnosis of MG/

2.4.1. Rapid serum plate agglutination (SPA)

The 180 serum samples were examined against the MG antigen (Nobilis®, Intervet Philippines, INC. Schering-Plough Animal Health, 8767 Paseo de Roxas, Makati City), according to the manufacturer's instructions. Briefly, a drop of serum was added to an equal drop of the well- mixed antigen and then the two drops were mixed with a stirring-rod and spread to a circular area of approximately 1.5 cm diameter. By rocking the plate at room temperature, a positive agglutination was stimulated within 2 min.

2.4.2. Enzyme-linked immunosorbent assay (ELISA)

All serum samples were tested against MG antibody (Biochek Smart Veterinary Diagnostics, USA). The sensitivity was determined to some extent by the manufacturer's instructions.

2.5. Molecular diagnosis of MG/ DNA extraction

Extraction of the Mycoplasma DNA from the organs (trachea, lungs and air sacs) of the infected birds was carried out according to the previously described method (Santha et al., 1990). The physical method for extracting DNA from tissue swabs was adopted by applying heat (95 °C in a water bath for 10 min) and cold (-20°C for 10 min) shocks. The swabs were then dipped in phosphate buffer saline (PBS) and centrifuged at 13 000 x g for 20 min. The pellet was then washed twice in PBS and the supernatant was collected in 1.5 ml screw-capped Eppendorf tubes for DNA amplification.

2.5.1. DNA amplification

The primer set was designed and used according to the previously described method (García *et al*., 2005) to amplify the *mgc2* gene. The sequences of the oligonucleotide primers used in this study were 5`- AACACCAGAGGCGAAGGCGAGG -3` and 5`- ACGGATTTGCAACTGTTTGTATTGG -3`. The DNA samples were amplified with an automated T100 Thermal Cylcer (BioRad, USA) to give an amplicon of 300 bp.

2.5.2. Electrophoresis

The amplified PCR products were analyzed by gel electrophoresis in 1.5% agarose gel containing ethidium bromide (García et al., 2005) and the results were photographed with PhotoDoc-ItTM 65 Imaging system (Cambridge, UK).

2.5.3. TaqMan RT-PCR

The TaqMan RT-PCR for MG was then carried out (7500 Fast Real-Time PCR System (Applied Biosystems, USA). Genesig standard kits (Primer design Ltd, Southampton, United Kingdom) for detection of the MG *mgc2* gene were used for this reaction according to the manufacturer's instructions. Briefly, the reaction mix was prepared with the total volume of 15 µl (10 µl of oasig™ or PrecisionPLUS™ 2× qPCR Master Mix, 1 µl of MG primer/probe mix and 4 µl of RNAse/DNAse-free

water (Table 1). Subsequently, 15 µl of this mixture was transferred into each well, and then 5 µl of DNA template was added to the well, while for the negative control wells, 5 µl of RNAse/DNase free water was used; thus, the final volume in each well was 20 µl. Preparation of a standard curve dilution series was done by adding 90 µl of template preparation buffer to five tubes and labeling them. The positive control template (MG strain ATCC 15302) was considered as tube 1**,** and 10 µl was transferred from this tube to tube 2 and mixed thoroughly. Then, 10 µl from tube 2 was transferred into tube 3, continuing in the same manner up to the 9^{th} tube. Finally, for the standard curve, 5 µl of the standard template was transferred to each well, making the final volume in each well 20 µl. Amplifications were then performed on an 7500 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal cycling consisted of 37 °C for 15 min for AmpErase® uracil-N-glycosylase (UNG) to prevent PCR carryover contamination and 95 °C for 2 min for enzyme activation followed by 40 cycles with every denaturation step at 95 °C for 10 seconds. Annealing and extension was performed at 60 °C for 60 s. Data were analyzed using sequence detection system (SDS) software. Amplification results were expressed by plotting Delta Rn (ΔRn) versus cycle number for the interpretation of infection (Brittain-Long et al., 2008).

Table 1. Primers and TaqMan minor groove binder (MGB) probes used in the duplex real-time PCR technique for MG detection

Target gene	Primers/TaqMan MGB probes sequence	GenBank accession number
mgc2-F	5'- GCTGGGTTGATTGTTGTTTCTT -3'	
mgc2-R	5'- TCTTCACGTTCTTGG ATCATCAT-3'	AY556229
mgc2-probe	Cy5: 5'-CTCTT(G/C)GGTTTAGGGATTGGG ATTCCG-3'	

2.6. Antibiotic susceptibility for MG

In the current study, a total of 60 identified field isolates of MG were isolated from birds suffering from respiratory distress and tested against 11 antimicrobial agents. The isolates were divided into three groups: Group A (n = 40) was collected from tracheal swabs, Group B (n = 11) was collected from air sacs and Group C (n = 9) was collected from the lungs. Six antibiotic groups were included: Macrolides (tylosin, tilmicosin and erythromycin); Pleuromutilins (tiamulin); Aminoglycosides (gentamicin); Aminocyclitols (spectinomycin); Quinolones (ciprofloxacin and enrofloxacin) and Tetracyclines (doxycycline and oxytetracycline and). All antibiotics were purchased from the Mobedco-Vet company (El-Hassan Industrial Estate, Irbid, Jordan). The MICs were determined according to the previously designated method (Hannan, 2000). Due to the absence of the cell wall which protects Mycoplasma from the influence of beta lactam antibiotics, penicillin G was used in this study as a negative control. The final volume of 0.1 ml of twofold serially-diluted antimicrobial in concentrations ranging from 0.0312 to 64 µg/ml was prepared in 96-well plates, and 0.1 ml of each inoculum containing 10^3–10^5 color change unit (CCU) /ml was added to each well. Each plate contained un-inoculated medium as a sterility control, a color-changed medium as an endpoint. A viable count row for each isolate was performed concurrently in order to confirm that the inoculum was within the accepted range. All plates were potted and incubated at 37°C until the color in the growth-control well visually matched that of the endpoint control. The lowest concentrations capable of inhibiting the growth of 50% and 90% of the isolates were determined, respectively, as the MIC_{50} and MIC_{90} values (Hannan, 2000; Hannan et al., 1997; Gerchman et al., 2009). The test was carried out two times. To ensure the validity of the agar dilution technique for determining antibiotic susceptibility, the standard MG strain ATCC 15302 (American type culture collection, USA) was used as a positive control.

3. RESULTS

3.1. Culture, isolation, serological and PCR tests for MG

The cultural isolation of MG from birds suffering from respiratory distress revealed only 37.01% as positive samples (Table 2). The highest detection

was shown in samples isolated from the trachea (55.88%), followed by the air sacs (20.75%), and the lowest in the lungs (16.98%). The current results were based on visualization of individual colonies with typical fried-egg appearance through observing agar plates under the 10× objective lens of a stereo microscope. After 10 days, the primary color of the colonies was yellow and light brown. Antibodies to SPA were detected in 83 serum samples representing a rate of 46.11% of the total isolates. With the ELISA technique, positive reactors were found in 97 (53.88%) serum samples (Table 3). The PCR product was observed in 108 isolates (51.92%) of the extracted DNA and appeared as a single DNA band close to that of the 300-bp in size and similar to the positive control when visualized electrophorically (Fig. 1). Repeat testing of some of the strains that were examined showed the same results. Identification of MG isolated from the trachea was relatively higher (79.41%) with PCR than in the case of the lungs and air sacs, which were 22.64% and 28.30%, respectively (Table 2). These results indicate that the PCR technique may be seen as a reliable tool for initial detection of infectious agents in field cases (Fig. 1). Repeat testing of some of the strains that were examined showed the same results.

3.2 TaqMan RT-PCR for identification of MG

Out of 208 suspected samples, 169 (81.25%) were positive using TaqMan RT-PCR, while 77 (37.01%), 83 (46.11%), 97 (53.88%) and 108 (51.92%) samples, were positive through culture, SPA, ELISA and conventional PCR assay, respectively (Table 2 & 3). As can be seen from Table 4, the reproducibility results are demonstrated in the form of cut-off cycle threshold (CT) values obtained from 10-fold serial dilutions in duplicates for MG starting with a titer of 1×10^4 CFU/ml. For TaqMan RT-PCR, the sensitivity recorded was 10^{-3} CFU/ml MG template DNA. These DNA quantifications were made on the lowest dilutions with readable CT values of 37.90 for MG (Table 4). The results obtained from the present experiment indicated that a lower CT value (19.57) was detected earlier in the real-time reaction in samples containing a higher initial concentration compared with samples with a lower initial concentration (Fig. 2).

Table 2. Culturing, PCR and TaqMan RT-PCR techniques for identification of MG isolates isolated from trachea, lung and air sacs

Organ	No. of Samples	Culture		PCR		TaqMan RT-PCR	
		Positive samples	Percentage (%)	Positive samples	Percentage (%)	Positive samples	Percentage (%)
Trachea	102	57	55.88%	81	79.41%	92	90.19%
Lung	53	9	16.98%	12	22.64%	34	64.15%
Air sac	53	11	20.75%	15	28.30%	43	81.13%
Total	208	77	37.01	108	51.92%	169	81.25%

Table 3. SPA and ELISA techniques for identification of MG isolates isolated from serum samples

Sample	**No. of Samples**	**SPA**		**ELISA**	
		Positive samples	Percentage (%)	Positive samples	Percentage (%)
Serum	180	83	46.11%	97	53.88%

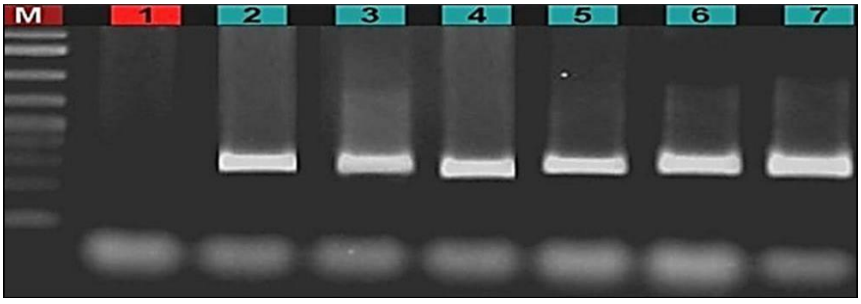

Fig. (1): Electrophoresis profile of MG DNA obtained from 5 samples by PCR (300 bp) on 1.5% Agarose Gel. Lane M: DNA Ladder; Lane 1: negative control; Lane 2: positive control; 3-7: positive samples.

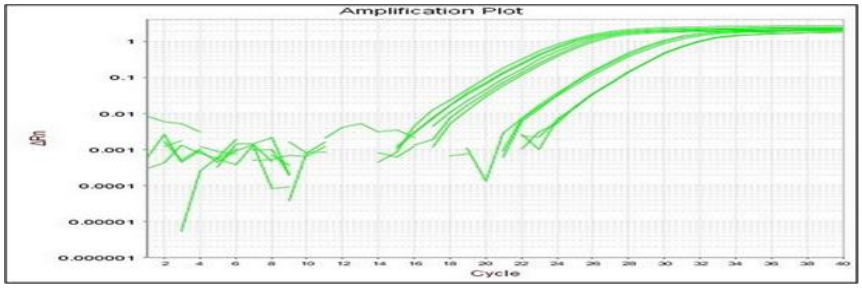

Fig. (2): TaqMan RT-PCR demonstrating amplification curves (linear view) for detection of MG. Only MG positive samples are revealed in the figure. Mean Cycle threshold (CT) values shown are 24.74 for MG.

Table 4. Demonstration of CT values obtained from TaqMan RT-PCR for detection of MG

Template copies	Average CT	Dilution of genomic DNA	Sensitivity (CFU/ml)
10^8	19.57	10^{-1}	10^4
10^7	23.19	10^{-2}	10^3
10^6	24.59	10^{-3}	10^2
10^5	26.76	10^{-4}	10^1
10^4	27.66	10^{-5}	10^0
10^3	29.87	10^{-6}	10^{-1}
10^2	33.15	10^{-7}	10^{-2}
10^1	37.90	10^{-8}	10^{-3}
10^0	no	10^{-9}	10^{-4}

3.3. Antimicrobial susceptibility of MG

The frequency distribution of MIC_{50}, MIC_{90} and the sensitivity percentage of the 11 antimicrobial agents against groups A, B and C of MG isolates are detailed in Table 5 & 6. In this study, tiamulin, gave the lowest level of MIC_{50} and MIC_{90} (≤ 0.032 µg/ml) for all groups of MG which exhibited a higher sensitivity percentage (93.33%), followed by tylosin, doxycycline and spectinomycin (85%, 81.66%, 81.66%), at MIC_{50} and MIC_{90} ranged from ≤ 0.032 µg/ml to 2 µg/ml for the total isolates. However, a high percentage of MG isolates exhibited the maximum degree of resistance (100%, 91.67% and 86.67%) to penicillin G, gentamycin and tilmicosin, respectively. In addition, erythromycin ciprofloxacin, oxytetracycline and enrofloxacin, were resisted by 81.67%, 80.00%, 78.34% and 76.67% of the all three groups MG isolates, correspondingly. These results indicate that there was an apparent increase in the resistance for all groups of MG isolates to most of the tested antimicrobial agents. As illustrated here, in general, when an isolate demonstrated an increase in resistance to one antibiotic, there was an increase in resistance to other antimicrobial agents, particularly those of the same class.

Table 5. Comparison of MIC50 and MIC90 (µg/ml) between group A, B and C of MG isolates

Group	Group A		Group B		Group C	
Antimicrobial	MIC50	MIC90	MIC50	MIC90	MIC50	MIC90
Tylosin	≤0.032	1	≤0.062	1	≤0.032	1
Tilmicosin	1	16	1	16	1	16
Erythromycin	≤8	≥64	≤8	≥32	≤8	≥64
Tiamulin	≤0.032	≤0.032	≤0.032	≤0.032	≤0.032	≤0.032
Gentamicin	4	16	2	16	2	16
Spectinomycin	0.125	2	0.125	2	0.5	2
Ciprofloxacin	2	8	2	8	1	8
Enrofloxacin	2	4	2	4	2	4
Doxycycline	≤0.032	≤0.125	≤0.062	≤0.062	≤0.062	≤0.125
Oxytetracycline	4	≥16	1	8	2	≥16

MIC_{50}: The lowest concentration of antibiotic that inhibits at least 50% of the isolates
MIC_{90}: The lowest concentration of antibiotic that inhibits at least 90% of the isolates

Table 6. Sensitivity and resistance percentage of 60 MG isolates isolated from group A, B and C against different types of antimicrobial agents.

Antibiotic	Susceptable		Resistant	
	No. of isolates	Percentage (%)	No. of isolates	Percentage (%)
Tylosin	51	85.00	9	15.00
Tilmicosin	8	13.33	52	86.67
Erythromycin	11	18.33	49	81.67
Tiamulin	56	93.33	4	6.67
Gentamycin	5	8.33	55	91.67
Spectinomycin	49	81.66	11	18.34
Ciprofloxacin	12	20.00	48	80.00
Enrofloxacin	14	23.33	46	76.67
Doxycycline	49	81.66	11	18.34
Oxytetracycline	13	21.66	47	78.34

4. DISCUSSION

In Saudi Arabia, MG is considered as one of the most economically significant poultry Mycoplasma. Generally, control of chronic respiratory disease has been based on the elimination of the microorganism from flocks and the preservation of Mycoplasma-free status in the poultry and their offspring via biosecurity practices. The success of MG control programs depends on accurate and fast diagnostic techniques. Diagnosis of MG has been performed using numerous serological techniques such as SPA and ELISA to determine the immune response (Raviv et al., 2008; Hu and Li, 2014). The SPA test is fast, sensitive and cheap, but may generate nonspecific results. The ELISA technique is more responsive and precise than the SPA test. Nevertheless, numerous studies have confirmed several disadvantages of serological methods (Sprygin et al., 2010). Regarding the results with ELISA, 97 positive serum samples were detected, an incidence of 53.88%. This finding approximated that of another study (Kempf et al., 1994) which examined serum samples for detection of MG antibodies via the ELISA test and found a positive rate of 61%. The serological test results demonstrated that the ELISA test gave better results and exhibited higher sensitivity than the SPA test for the detection of specific MG antibodies. These findings also agreed with those recorded in another study (Reda et al., 2012). One of the main objectives of this work was to facilitate the rapid detection of MG isolated from tracheal, lung and air sac swabs of clinically infected chicken. In the present study, the detection of MG isolated from the trachea was relatively higher (79.41%) using PCR than in the case of the lungs and air sacs, at 22.64% and 28.30%, respectively. Other research has obtained similar findings (Carli and Eyigor, 2003). In addition, it was noticed that PCR did not provide 100% sensitivity (Table 2), although this assay is supposed to be more sensitive than a culture (Raviv and Kleven, 2009; Nouzha et al., 2013). On the other hand, some problems are associated with PCR, some examples being the existence of an inhibiting substance in the samples, the danger of environmental contamination and the probability of the recognition of nonviable Mycoplasma (Kleven, 1997; Kempf, 1998; Carli and Eyigor, 2003). Therefore, in this study, the ABI-7500 fast real-time PCR assay using TaqMan polymerases was also developed and validated for the direct detection and quantification of MG DNA. A higher sensitivity detection (lower CT values) was achieved with the TaqMan FAM^{TM} reporter dye in this study. Others (Ehtisham-ul-Haque et al., 2015) have reported the results for FAM to be stronger that those obtained with the NED^{TM} reporter dye. Additionally, the low detection value of NED as a reporter dye is well corroborated in past experience. With MG-specific probe, detection using FAM labeling in a single target (monoplex) TaqMan MGB real-time PCR reaction revealed lower CT values (high levels of detection) for the same probe (Sambrook and Russel, 2001; Ehtisham-ul-Haque et al., 2011; Ehtisham-ul-Haque et al., 2015). Consequently, a qPCR technique was developed with a FAM-labeled probe where a TaqMan VIC probe served as an internal positive control (IPC). In one study, a black hole quencher was used as a non-fluorescent quencher (NFQ), attached to the middle of the probe for the detection of the mgc2 gene specific for MG (Sprygin et al., 2010). In contrast, the technique used in the present study incorporated an NFQ that

combined with the MGB (minor groove binder) moiety at the 3' end. It has been indicated that the use of the MGB probe in combination with NFQ leads to improved specificity in the detection of dual infections by diminishing the background fluorescence obtained through the use of fluorescent quenchers (Farkas et al., 2009). Previous studies have already confirmed that the qPCR assay is a successful tool for sensitive and specific detection of MG in commercial chicken flocks (Raviv and Kleven, 2009; Sprygin et al., 2010; Fraga et al., 2013). The present study describes a new TaqMan RT-PCR assay for the concurrent detection of MG occurring in commercial poultry flocks in Saudi Arabia. Application of this method in the poultry industry laboratories would definitely aid in the development of Mycoplasma control programs.

These isolates were divided into three groups, according to the specimen sites of birds suffering from respiratory distress: group A, group B and group C. According to the present results, tiamulin was shown the highest sensitivity percentage (93.33%) against various isolates of MG (Hannan, 2000; Islam et al., 2009; Gharaibeh and Al-Rashdan, 2011). The MIC_{90} of tiamulin for group A, B and C of the MG isolates was 1 µg/ml (Table 5), which is possibly a result of the fact that it has not been formerly used to treat chicken in Saudi Arabia. The exceptional sensitivity of MG isolates to tiamulin makes it an attractive selection as an effective antimicrobial agent in the field for poultry farms which do not incorporate ionophore antibiotics in their feed. In addition, similar findings showed it to be effective against MG isolates in chicken in Iran (Ghaleh Golab Behbahan et al., 2008) and Jordan (Gharaibeh and Al-Rashdan, 2011). In contrast, gentamycin demonstrated a high resistant level (91.67%), against all groups of MG isolates at low concentrations (MIC_{90} = 16 µg/ml). Similar results have been obtained by other researchers, (Gharaibeh and Al-Rashdan, 2011) who determined the MIC of 13 antimicrobial agents against two groups of MG (MIC_{90} = ≥ 64 µg/ml). Therefore, gentamycin is not frequently used to treat MG in chicken due to its poor intestinal absorbability, which decreases its interaction with MG in the chicken respiratory system. The macrolides group is recognized as having a high in vitro and in vivo activity against Mycoplasma species. In the present study, after tiamulin, tylosin exhibited a good activity against 85 % of the total 60 isolates with MIC_{50} ranged from 0.032 to 1 µg/ml. Although tylosin was employed for several years before the introduction of tilmicosin, it still exhibits better action against MG isolates. Similar results have been obtained in previous research (Ghaleh Golab Behbahan et al., 2008). In contrast, there was a momentous increase in resistance to tilmicosin and erythromycin among all groups, particularly for erythromycin, where its MIC_{90} for group A, B and C were ≥ 64 µg/ml, ≥ 32 µg/ml and ≥ 64 µg/ml, respectively (Table 5). The quinolones like enrofloxacin and ciprofloxacin showed an important decrease in activity against all groups of MG isolates (Table 5). The MIC_{90} of ciprofloxacin and enrofloxacin for all groups was 8 µg/ml and 4 µg/ml, respectively (Table 5). Similar earlier studies in Israel (Gerchman et al., 2009) and Iran (Ghaleh Golab Behbahan et al., 2008) had established the increased resistance of MG isolates to enrofloxacin. The MIC_{90} of oxytetracycline for group A, B and C was ≥ 16 µg/ml, 8µg/ml and ≥ 16 µg/ml, respectively. In this study, it was also noted that MG isolates, probably developed a slight resistance to oxytetracycline, possibly as a result of its frequent usage in Saudi Arabian chicken farms over a long period of time. Furthermore, all groups of MG isolates were resistant to penicillin G. This confirms that the lack of a cell wall makes the Mycoplasma resistant to the beta-lactam group of antibiotics and emphasizes the fact that penicillin G can be used in antimicrobial susceptibility testing only as a negative control.

5. CONCLUSION

The success of MG control programs depends on rapid and accurate diagnostic techniques. Therefore, the ABI-7500 fast real-time PCR assay was developed for the detection of MG infection in chicken flocks. The assay was highly specific, sensitive, and reproducible, and allowed the quantification of MG template copies directly from clinical samples. In the future, the TaqMan RT-PCR assay can be used as a diagnostic and research tool for the detection and quantification of MG DNA isolated from clinical samples. In addition, among the tested antimicrobial agents, tiamulin, tylosin, doxycycline and spectinomycin exhibited the best in vitro antimicrobial activity against the identified field MG isolates from naturally infected chicken, whereas all of the field isolates showed a higher percentage of resistance against gentamycin, timlicosin, erythromycin, ciprofloxacin, oxytetracycline and doxycycline.

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7. REFERENCES

Barua, S.R., Prodhan, A.M., Chowdhury, S. 2006. Study on *Mycoplasma gallisepticum* in Chicken in selected areas of Bangladesh. Banglad. J. Vet. Med. 4: 141–142.

Bradbury, J.M., Yavari, C.A., Giles, C.J. 1994. In vitro evaluation of various antimicrobials against *Mycoplasma gallisepticum* and *Mycoplasma synoviae* by the micro-broth method, and comparison with a commercially prepared test system. Avian Pathol. 23: 105–115.

Brittain-Long, R., Nord, S., Olofsson, S., Westin, J., Anderson, L., Lindh, M. 2008. Multiplex real-time PCR for detection of respiratory tract infections. J. Clin. Virol. 41: 53–56.

Card, R., Zhang, J., Das, P., Cook, C., Woodford, N., Anjuma, M.F. 2012. Evaluation of an Expanded Microarray for Detecting Antibiotic Resistance Genes in a Broad Range of Gram-Negative Bacterial Pathogens. Antimicrob. Agents Chemother. 57: 458-465.

Carli, T., Eyigor, A. 2003. Real-time polymerase chain reaction for *Mycoplasma gallisepticum* in chicken trachea. Avian Dis. 47: 712–717.

Charlton, B.R., Bermudez, A.J., Boulianne, M., Eckroade, R.J., Jeffrey, J.S., et al. Avian Disease Manual. In: Charlton, B.R. (Ed.), American Association of Avian Pathologists, Kennett Square, Pennsylvania: USA; 1996, p. 115–125.

Chiu, C., Ou, J.T. 1996. Rapid identification of Salmonella serovars in feces by specific detection of virulence genes, invA and spvC, by an enrichment broth culture-multiplex PCR combination assay. J. Clin. Microbiol. 34: 2619–2622.

Ehtisham-ul-Haque, S., Rahman, S.U., Siddique, M., Qureshi, A.S. 2011. Involvement of *Mycoplasma synoviae* in respiratory distress cases of broilers. Pak. Vet. J. 31: 117–119.

Ehtisham-ul-Haque, S., Rahman, S.U., Khan, M.I., Younus, M., Awais, M.M., Nasir, A. 2015. A simplified duplex real-time PCR incorporating TaqMan minor groove binder (MGB) probes and an exogenous internal positive control for the simultaneous detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* cultures. J. Vet. Med. 60: 268–273.

Farkas, T., Szekel, E., Belak, S., Kiss, I. 2009. Real-time PCR-based pathotyping of Newcastle disease virus by use of TaqMan minor groove binder probes. J. Clin. Microbiol. 47: 2114–2123.

Fraga, A.P., de Vargas, T., Ikuta, N., Fonseca, A.S., Celmer, A.J., Marques, E.K., Lunge, V.R. 2013. A Multiplex realtime PCR for detection of *Mycoplasma gallisepticum* and *Mycoplasma synovie* in clinical samples from Brazilian commercial poultry flocks. Braz. J. Microbiol. 44: 505–510.

Freire-Moran, L., Aronsson, B., Manz, C., Gyssens, I.C., So, A.D., Monnet, D.L., Cars, O. 2011. Critical shortage of new antibiotics in development against multidrug-resistant bacteria-Time to react is now. Drug Resist. 14: 118–24.

García, M., Ikut, N., Levisohn, S., Kleven, S.H. 2005. Evaluation and comparison of various PCR methods for detection of *Mycoplasma gallisepticum* infection in chicken. Avian Dis. 49: 125–132.

Gautier-Bouchardon, A.V., Reinhardt, A.K., Kobisch, M., Kempf, I. 2002. In vitro development of resistance to enrofloxacin, erythromycin, tylosin, tiamulin and oxytetracycline in *Mycoplasma gallisepticum*, *Mycoplasma iowae* and *Mycoplasma synoviae*. Vet. Microbiol. 88: 47–58.

Gerchman I., Levisohn, S., Mikula, I., Lysnyansky, I. 2009. In vitro antimicrobial susceptibility of *Mycoplasma bovis* isolated in Israel from local and imported cattle. J. Vet. Microbiol. 137: 268–275.

Gerchman I., Lysnyansky, I., Perk, S., Levisohn, S. 2008. *In vitro* susceptibilities to fluoroquinolones in current and archived *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates from meat-type turkeys. J. Vet. Microbiol. 131: 266–276.

Ghaleh Golab Behbahan, N., Asasi, K., Afsharifar, A.R., Pourbakhsh, S.A. 2008. Susceptibilities of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates to antimicrobial agents in vitro. Int. J. Poult. Sci. 7: 1058–1064.

Gharaibeh, S., Al-Rashdan, M. 2011. Change in antimicrobial susceptibility of *Mycoplasma gallisepticum* field isolates. Vet. Microbiol. 150: 379–383.

Glew, M.D., Browning, G.F., Markham, P.F., Walker, I.D. 2000. pMGA phenotypic variation in *Mycoplasma gallisepticum* occurs in vivo and is mediated by trinucleotide repeat length variation. Infect. Immun. 68: 6027–6033.

Grodio, J.L., Dhondt, K.V., O'Connell, P.H., Schat, K.A. 2008. Detection and quantification of *Mycoplasma gallisepticum* genome load in conjunctival samples of experimentally infected house finches (*Carpodacus mexicanus*) using real-time polymerase chain reaction. Avian Pathol. 37: 385–391.

Guo, Y., Cheng, A., Wang, M., Shen, C., Jia, R., Chen, S., Zhang, N. 2009. Development of TaqMan® MGB fluorescent real-time PCR assay for the detection of anatid herpesvirus-1. J. Virol. 6: 71–78.

Hannan, P.C.T. 2000. Guidelines and recommendation for antimicrobial minimum inhibitory concentration

(MIC) testing against veterinary mycoplasma species. Vet. Research. 31: 373–395.

Hannan, P.C.T., Windsor, G.D., de Jong, A., Schmeer, N., Stegemann, M. 1997. Comparative susceptibilities of various animal-pathogenic mycoplasmas to fluoroquinolones. Antimicrob. Agents Chemother. 41: 2037–2040.

Hu, Y., Li, F. 2014. Development of a novel real-time polymerase chain reaction (PCR) assay by amplification of double target genes for quantitative detection of *Mycoplasma gallisepticum*. Afr. J. Biotechnol. 13: 2766–2773.

Islam, K.M.S., Klein, U., Burch, D.G. 2009. The activity and compatibility of the antibiotic tiamulin with other drugs in poultry medicine—A review. Poult. Sci. 88: 2353–2359.

Kaboli, K., Bijanzad, P., Moggadam, A.R.J., Shahbazi, M., Hosseini, H. 2013. Evaluation of *Mycoplasma gallisepticum* Infection diagnosis in rural poultry by 16S rRNA PCR methods. Eur. J. Zool. Res. 2: 63–66.

Kawahara, F., Taira, K., Nagai, S., Onaga, H., Onuma, M., Nunoya, T. 2008. Detection of five avian Eimeria species by species-specific real-time polymerase chain reaction assay. Avian Dis. 52: 652–656.

Kempf, I. 1998. DNA amplification methods for diagnosis and epidemiological investigations of avian mycoplasmosis. Avian Pathol. 27: 7–14.

Kempf I., Blanchrd, A., Gesbert, F., Guittet, M., Bennejean, G. 1994. Comparison of antigenic and pathogenic properties of *Mycoplasma iowae* strains and development of a PCR-based detection assay. Res. Vet. Sci. 56: 179–185.

Khalifa, K.A., Abdelrahim, E.S., Badwi, M., Mohamed, A.M. 2013. Isolation and Molecular Characterization of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in Chicken in Sudan. J. Vet. Med. 2013: 1–4.

Kleven, S.H. 1997. Changing expectations in the control of *Mycoplasma gallisepticum*. Acta Vet. Hung. 45: 299-305.

Kutyavin, I.V., Afonina, I.A. Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., Hedgpeth, J. 2000. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28: 655–661.

Lauerman, L.H., Chilina, A.R. Closser, J.A., Johanssen, D. 1995. Avian mycoplasma identification using PCR amplification and restriction fragment polymorphism analysis. Avian Dis. 39: 804–811.

Levisohn, S., Kleven, S.H. 2000. Avian mycoplasmosis (Mycoplasma gallisepticum). Rev. Sci. Tech.19: 425–442.

Nouzha, H., Ammar, A., Bakir, M., Ahmed, K.L. 2013. Comparison of Three Diagnostics Methods of *Mycoplasma gallisepticum* in Batna Governorate (Algeria). J. Vet. Advances. 3: 125–129.

Pakpinyo, S., Sasipreeyajan, J. 2007. Molecular characterization and determination of antimicrobial resistance of *Mycoplasma gallisepticum* isolated from chicken. J. Vet. Microbiol. 15: 59–65.

Papazisi, L., Gorton, T.S., Kutish, G., Markham, P.F., Browning, G.F., Nguyen, D.K., Swartzell, S., Madan, A., Mahairas, G., Geary, S.J. 2003. The complete genome sequence of the avian pathogen *Mycoplasma gallisepticum* strain R(low). Microbiology, 149: 2307–2316.

Purswell, J.L., Evans, J.D., Leigh, S.A., Collier, S.D., Olanrewaju, H.A., Kim, E.J., Pharr, G.T., Peebles, E.D., Branton, S.L. 2012. *Mycoplasma gallisepticum* transmission: Comparison of commercial F-strain vaccine versus layer complex-derived field strains in a tunnel ventilated house. Poult. Sci. 91: 3072–3079.

Raviv, Z., Callison, S., Noel, F., Kleven, S.H. 2008. Strain differentiating real-time PCR for *Mycoplasma gallisepticum* live vaccine evaluation studies. J. Vet. Microbiol. 129: 179–187.

Raviv, Z., Kleven, S.H. 2009. The development of diagnostic real-time TaqMan PCRs for the four pathogenic avian mycoplasmas. Avian Dis. 53: 103–107.

Reda, L.M., Abd El-Samie, L.K. 2012. Some Studies on the Diagnosis of *Mycoplasma gallisepticum* in Chicken. Nature and Science. 10: 247–251.

Sabry, M.Z. 1968. Characterization and classification of avian Mycoplasmas. Ph.D. Thesis, Cornell University USA; P. 244.

Sabry, M.Z., Ahmed, A.A. 1975. Evaluation of media and cultural procedure for the primary isolation of Mycoplasma from female genitalia of farm animals. J. Egypt. Vet. Med. Assoc. 35: 18–36.

Sambrook, J., Russel, D.W. Molecular cloning: A laboratory manual (3rd Ed.). Cold Spring Harbor N.Y., USA: Cold Spring Harbor Press; 2001.

Sprygin, A.V., Andreychuk, D.B. Kolotilov, A.N., Volkov, M.S., Runina, I.A., Mudrak, N.S., Borisov, A.V., Irza, V.N., Drygin, V.V., Perevozchikova, N.A. 2010. Development of a duplex real-time TaqMan PCR assay with an internal control for the detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* in clinical samples from commercial and backyard poultry. Avian Pathol. 39: 99–109.

Winner, F., Rosengarten, R., Citti, C. 2000. In vitro cell invasion of *Mycoplasma gallisepticum*. Infect. Immun. 68: 4238–4244.