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The Use of Molecular Techniques in Isolation and Characterization of *Mycoplasma gallisepticum* from Commercial Chickens in Jordan

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Abstract: Commercial chickens in Jordan suffer from respiratory disease of undetermined etiology. This study was designed to document the involvement of *Mycoplasma gallisepticum* (MG) in this respiratory disease. Seventy six different chicken flocks with respiratory symptoms (48 broiler flocks, 21 layer flocks and 7 broiler-breeder flocks) were examined serologically by commercial MG ELISA kit and bacterial isolation for Mycoplasma. Twenty four MG isolates from 76 flocks were cloned and the presence of MG in the cloned cultures was confirmed by polymerase chain reaction. The isolates were further examined using Random Amplified Polymorphic DNA (RAPD) technique. The prevalence of MG in the flocks examined was 73.5% and 31.6% by ELISA and isolation, respectively. This high prevalence in flocks with respiratory disease, confirms the endemic nature of the disease in Jordan. RAPD testing of the 24 isolates revealed the presence of 5 banding patterns that were different than the common MG F strain vaccine used in the field. This is the first study to isolate and characterize MG from chickens in Jordan.

Key words: *Mycoplasma gallisepticum*, Jordan, RAPD, PCR and chickens

Introduction

Avian mycoplasmosis causes considerable economical losses to the poultry industry, especially in chickens and turkeys all over the world. *Mycoplasma gallisepticum* (MG) is responsible for what is called chronic respiratory disease in chickens and infectious sinusitis in turkeys. In broilers, it causes reduction in weight gain, decrease in feed conversion efficiency, increase in mortality rate and increased condemnations in slaughter houses. In breeders and layers, the disease causes a drop in egg production and an increase in embryo mortality (Ley, 2003). Vertical transmission of MG has been documented and it leads to infected progeny flocks (Bradbury, 2001). MG also causes respiratory disease including sinusitis and conjunctivitis in turkeys, pheasants, partridges, quail, ducks, geese and other avian species (Ley, 2003).

Vaccination is practiced as a control measure in some countries (Ley, 2003). The commercially available live MG vaccines include the F strain (Carpenter *et al*., 1981), 6/85 strain (Evans and Hafez, 1992) and temperature sensitive strain - 11 (ts - 11) (Whithear, 1996; Whithear *et al*., 1990). In Jordan, F strain is the most commonly used vaccine because it gives a better displacement of the field strains (Kleven *et al*., 1998) and a better protection after challenge (Abd-El-Motelib and Kleven, 1993) compared to the other live vaccines.

Historically, chicken flocks are usually monitored for MG infection by serology using rapid plate agglutination then confirmed by ELISA, hemagglutination inhibition, or isolation (Kleven, 1998). Isolation of MG was usually restricted to specialized laboratories that have specific conjugated antibodies against different types of mycoplasma species and these antibodies are used to identify the unknown colonies by fluorescent antibody test to help picking up positive MG colonies and later making pure cultures of these colonies (Talkington and Kleven, 1983). Polymerase chain reaction (PCR) test can be performed on clinical samples without the need for isolation. Its high sensitivity and fast turnaround time is making it the most frequently used test to monitor MG infection (Garcia *et al*., 2005). Differentiation of isolates and tracing MG infections for epidemiological studies is usually done by molecular techniques such as random amplified polymorphic DNA (RAPD) (Fan *et al*., 1995a; Geary *et al*., 1994), restriction fragment length polymorphism analysis (RFLP) (Fan *et al*., 1995b), gene targeted sequencing (Ferguson *et al*., 2005), or amplified fragment length polymorphism (AFLP) (Hong *et al*., 2005). Hong *et al*. (2005) demonstrated that AFLP results correlated well with DNA sequence analysis and RAPD with AFLP analysis having a much higher discriminatory power and reproducibility. However, RAPD analysis is still widely accepted for strain differentiation of MG isolates.

Because immunofluorescence conjugated antisera was not available for us, this study was designed to isolate and characterize MG from chicken flocks with respiratory disease using molecular techniques instead of the routine mycoplasma bacteriology procedures.

Materials and Methods

Sample collection: During the period of 7 months, between July 2004 - January 2005, 76 commercial

Table 1: PCR primers used for MG detection

Type of primers	Primer's Name	Sequences	Expected PCR product size
Specific (Nascimento *et al.*, 1991)	Upstream	5'GGATCCCATCTCGACCACGAGAAAA3'	
	Downstream	5'CCTTCAATCAGTGAGTAACTGATGA3'	732 bp
Universal (Lauerman *et al.*, 1995)	Forward	5'ACACCATGGGAGCTGGTAAT3'	
	Reverse	5'CTTCATCGACTTTCAGACCCAAGGCAT3'	888-938 bp (for MG)
RAPD (Geary *et al.*, 1994)	Geary 1254	5'CCGCAGCCAA3'	Variable

chicken flocks with respiratory symptoms (48 broiler flocks, 21 layer flocks and 7 broiler-breeder flocks) located in three Northern Jordanian governorates (Irbid, Jarash and Mafraq) were examined. Broiler flocks had respiratory signs, decreased feed intake and retarded growth with mortality rate ranging from 1-15%. Gross lesions in these flocks were of tracheal congestion, airsaculitis and polyserositis. Layer and breeder flocks had decreased in feed intake and egg production (10-11%) with gross lesions of keratoconjunctivitis, catarrhal tracheitis and salpingitis. Six tracheas were collected randomly and aseptically from each flock and kept at-80°C until they were used for MG isolation. Nine to fourteen days after trachea collection, 12-15 random serum samples from 49 of the above 76 flocks (27 from broiler flocks, 15 from layer flocks and 7 from broiler breeder flocks) were collected for serology testing. All of these flocks were above five weeks of age and not vaccinated against MG.

Serology: Presence of antibodies against MG in serum samples was evaluated using the ProFLOK® MG ELISA kit (SynBiotics Corp, San Diego, CA and USA). ELISA testing was performed according to the manufacturer's instructions. A flock was considered positive when antibody titer was greater than 744 (titer group 2) in two out of the 15 samples tested as previously described (Giovanni *et al.*, 2004), regardless of the geometric mean titer (GMT) of the flock. The absorbance was read at 405 nm wave length on ELx800 ELISA reader (BIO-TEK Instruments Inc, Winoski VT, USA).

MG isolation: Culture media used in this study were Frey's (Frey *et al.*, 1968) and SP_4 (Fritz *et al.*, 1991). MG was isolated according to a previously described procedure (Kleven, 1998). Briefly, tracheal swabs from each flock were pooled and inoculated into 3-5 ml of broth cultures (SP_4 and Frey's media), then incubated at 37°C with 5% CO_2. When color of the cultures changed from red to orange or yellow, or became turbid, the cultures were tested for MG presence by PCR. Agar plates (SP_4 and Frey's media), were inoculated with PCR positive broth, using microbiological loop and incubated at 37°C with 5 % CO_2 for 5-7 days.

Cloning of MG isolates: Because RAPD analysis depends on the whole genome of the organism investigated and not a certain specific sequence, it was crucial to make pure cultures of MG by cloning to eliminate any possible contamination. One colony from the agar plate was inoculated into a broth medium (SP_4 and Frey's media), incubated and then tested by PCR. Agar plates (SP_4 and Frey's media), were inoculated with PCR positive broth. This cloning procedure was repeated two more times and final broth culture was named cloned tertiary culture.

Identification of MG isolates by PCR: DNA extraction was performed according to a previously described procedure (Liu *et al.*, 2001). Briefly, one milliliter of mycoplasma broth culture was centrifuged at 13,000 rpm for 10 min. The cell pellet was then washed twice with 1 ml of phosphate-buffered saline (PBS) (pH 7.2) and resuspended in a final volume of 20 µl of PBS. The cell suspension was heated in a dry block at 110°C for 10 min and placed on ice for at least 10 min. After cooling, the lysate was centrifuged at 13,000 rpm for 2 min. The supernatant containing DNA was collected and stored at-20°C until used. MG F strain vaccine (Scherring Plough, NJ and USA) was used as a positive control.
PCR was used to specifically detect MG using primers previously described (Nascimento *et al.*, 1991) and their sequences and product size are listed in Table 1. The PCR mix was prepared in a volume of 50 µ containing 25 µl master mix (*Taq* polymerase 50 units/ml, 400 mM of dNTP and 3 mM $MgCl_2$) (Promega Corp, Madison, WI, USA), 18 µl nuclease free water (Promega Corp, Madison, WI, USA), 1 µl (50 µM) of each upstream and downstream primers (Alpha DNA, Canada) and 5 µl of DNA extract. Amplification was carried out in a Mycycler thermal cycler (BioRad, Hercules, CA , USA), for one cycle of 5 min at 94°C for one cycle, followed by 94°C for 1 min, 55°C for 1 min, 72°C for 2 min for 35 cycles. PCR products were separated on a 1.5% agarose gel (Promega Corp, Madison, WI, USA) and stained by ethidium bromide (Promega Corp, Madison, WI, USA). Agarose gel electrophoresis unit (BioRad, Hercules, CA, USA), was run for 45 min at 100 V, 400 mA and the gel was visualized under UV light (Alphamager) (Alphainnotech, San Leandro. CA, USA).

Confirming the presence of MG in the cloned cultures by PCR: PCR was used to confirm the presence of MG in cloned broth culture for each isolate using primers previously described by Lauerman *et al.* (1995) and their sequences are listed in Table 1. These universal

primers were used for detection of nine avian mycoplasmas by amplification of the 16s/23s ribosomal RNA (rRAN) intergenic space in *Mollicutes* and will give different PCR product sizes for different avian mycoplasmas. MG gives a PCR product size ranging from 838-938 bp. Other avian mycoplsmas give different product size. A local *M. synoviae* (MS) isolate was used to verify the technique and its expected band size ranges from 473-497 bp. The PCR mix was carried out as above. Amplification was carried out for 45 cycles of 30 seconds at 94°C, 2 min at 48°C and 2 min at 72°C and for 1 cycle of 5 min at 72°C. PCR products were separated and visualized as described above.

RAPD analysis: RAPD primer used to differentiate between isolates was previously described by Geary *et al.* (1994) and its sequence is listed in Table 1. The PCR mix was prepared in a volume of 50 µl, 5 µl 10X PCR buffer (100 mM KCl, 100 mM $(NH4)_2SO_4$, 200 mM Tris, 20 mM $MgSO_4$, 0.1% Triton X-100 buffer) (GenScript Corp, USA), 0.5 µl (2.5 units) Taq DNA polymerase (GenScript Corp, USA) and 1 µl dNTP mix (10 mM) (GenScript Corp, USA), 2 µl $MgCl_2$ (25 mM) (Promega Corp, Madison, WI, USA), 400 ng of primer, 40 ng of DNA extract from MG cloned cultures and the volume completed to 50 µl by nuclease free water (Promega Corp, Madison, WI, USA). Amplification was carried out as follows; 1 cycle at 97°C for 5 min, followed by 4 cycles at 94°C for 5 min, 40°C for 5 min, 72°C for 1 min, followed by 30 cycles of 94°C for 1 min, 39°C for 1 min, 72°C for 1 min, followed by 1 cycle of 72°C for 10 min. RAPD patterns were determined to be unique if one or more fragment differences were detected on agarose gel. Gel pictures were analyzed for presence of distinct patterns using the Jaccard coefficient and unweighted pair group method with average linkage by AlphaEase software (Alphainnotech, San Leandro. USA).

Statistical analysis: Presence of any relation between ELISA results (GMT), isolation results, type of birds, locations of isolates and RAPD banding patterns were investigated by analysis of variance statistical test (ANOVA) using SAS program software (SAS, 1996).

Results

Serology: Thirty six out of the 49 chicken flocks (73.5%) tested by ELISA had positive antibody titer for MG. MG ELISA was positive in 19 broiler flocks (70.4%), 12 layer flocks (80%) and 5 broiler breeder flocks (71.4%).

Isolation: MG was isolated in broth and gar media (Frey's and SP_4) and confirmed by PCR (Fig. 1) from 24 out of 76 flocks (31.6%). There was no difference in isolation sensitivity between Frey's and SP_4 media. MG was isolated from 15 broiler flocks (31.3%), 8 layers flocks (38.1%) and 1 broiler-breeder flock (14.3%).

Confirming the presence of MG in the cloned cultures by PCR: All cloned cultures tested using universal *Mycoplasma* primers gave a band with a size ranging from 838-938 bp which is only consistent with MG. A local MS isolate gave a different product size of slightly less than 500 bp as expected (Fig. 2).

RAPD analysis of MG isolates: Analysis of RAPD banding patterns of 24 isolates revealed a total of 5 distinct patterns (A to E). Banding patterns A, B, C, D and E, comprise 1, 5, 3, 6 and 9 isolates respectively. These results were reproducible by repeating the procedure on these isolates on different dates. These 5 patterns have a few similar fragments and several different fragments that were qualitatively sufficient to differentiate between the banding groups and the MG F strain vaccine (Fig. 3). Fig. 4 shows the similarity of RAPD banding patterns of MG isolates groups and MG F strain vaccine.

Statistical analysis: There was a statistically significant difference between ELISA GMT of isolation positive (1827.44±342.63) and negative (261.52±238.57) groups with a *P*-value of 0.0005. However, there was no significant relation between ELISA results (GMT), type of birds, location of isolation and RAPD banding patterns (Data not shown).

Discussion

Traditionally, fluorescent antibody (FA) test is used to identify MG colonies on agar medium (Bass and Jasper, 1972; Talkington and Kleven, 1983). We used PCR with species-specific primers (Nascimento *et al.*, 1991) for identification of MG in cultures. This technique was very convenient for us because FA reagents were not available. In the present study, PCR provided a rapid diagnosis and identification of MG when it was performed on broth cultures inoculated in the traditional manner (Fig. 1). MG presence was confirmed in the tertiary cloned cultures by PCR using universal primers for avian mycoplasmas (Lauerman *et al.*, 1995) (Fig. 2). In this study, rate of MG infection in the examined flocks was 73.5% by ELISA and 31.6% by isolation. This high infection rate confirms the endemic nature of the disease in Northern Jordan in the absence of any control strategy. *Mycoplasma gallisepticum* seroprevalence in chickens has been reported to be high in many countries with no control strategy or in countries before the implementation of a control strategy. For example, MG seroprevalence was 73% in layers in Southern California in 1984 (Mohammed *et al.*, 1986). In the Middle East region, significant seroprevalence of the disease was evident in Egyptian chickens (Saif-Edin, 1997).

Although not statistically significant, layer flocks had higher infection with MG compared to the other types of chickens. This is probably due to the longer life span of

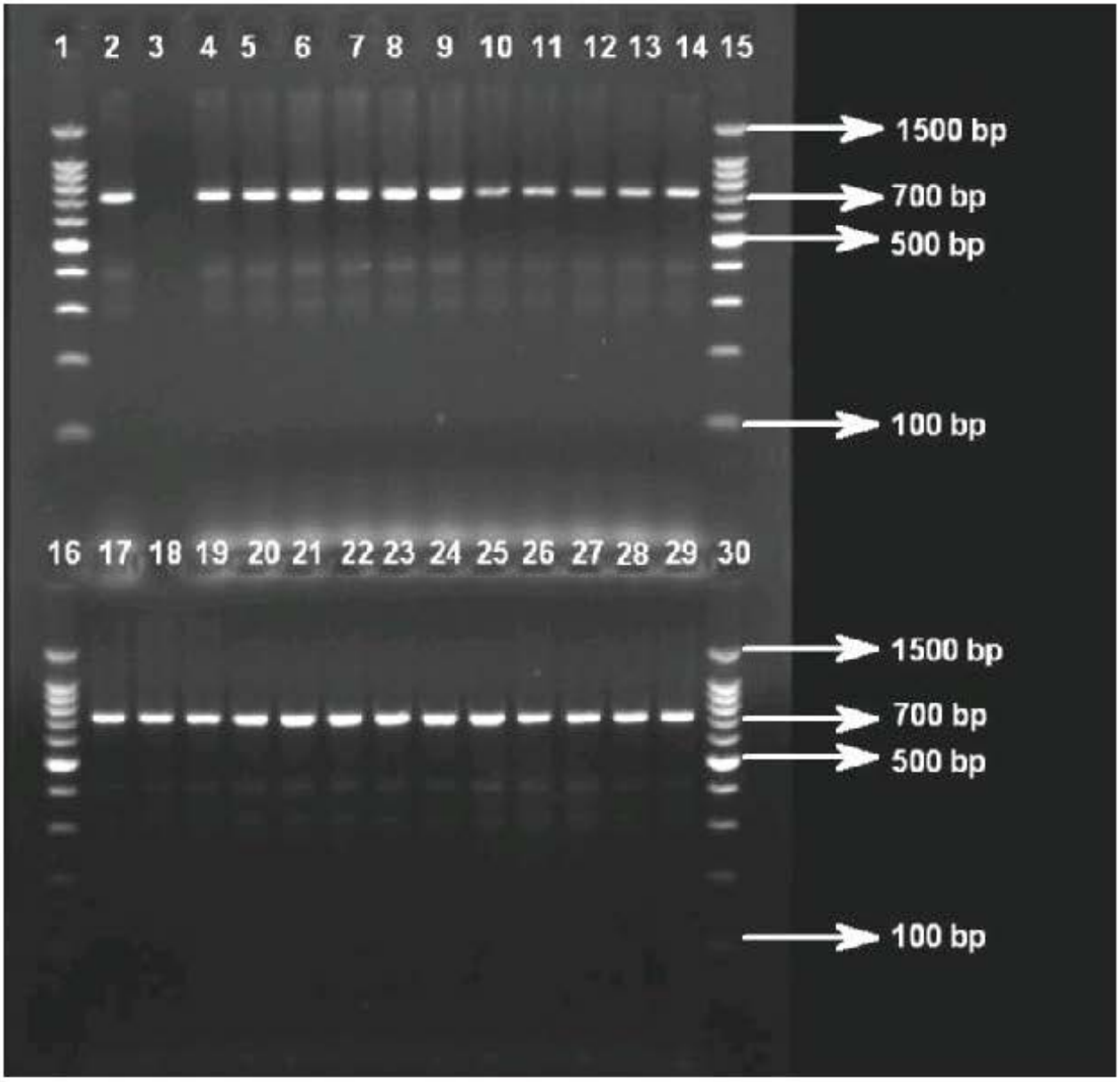

Fig. 1: Electrophoresis analysis (1.5% agarose gel) of PCR products of 24 MG isolates amplified by MG species-specific primers (Nascimento *et al.*, 1991). Lane 1, 15, 16, and 30 = 100 bp DNA marker (Promega Corp, Madison, WI, USA); Lane 2 = MG F strain vaccine; Lane 3 = negative control; Lanes 4-14 and 17-29 = positive MG cultures.

layer than broiler flocks increasing the chance for MG field exposure. While breeder flocks are kept as long as layers, they are usually given periodical prophylactic treatments with tylosine. None of the flocks tested were less than 5 weeks of age and this excludes that the titers are due to maternal antibodies from parent flocks. All the flocks that were positive by isolation were also ELISA positive and their ELISA GMT titers were significantly higher than flocks that were negative on isolation. This is expected because serum samples were collected 9-14 days after collection of tracheas for isolation and these flocks had time to seroconvert. Some of the flocks were isolation negative and ELISA positive. These titers are may be due to an earlier MG infection in these flocks. This also may indicate failure of isolation due to antibiotic treatment as farmers tend to start medication as soon respiratory signs develop and some of these flocks were already on antibiotics when tracheas for isolation were collected.

Frey's and SP4 media were used in this study for MG

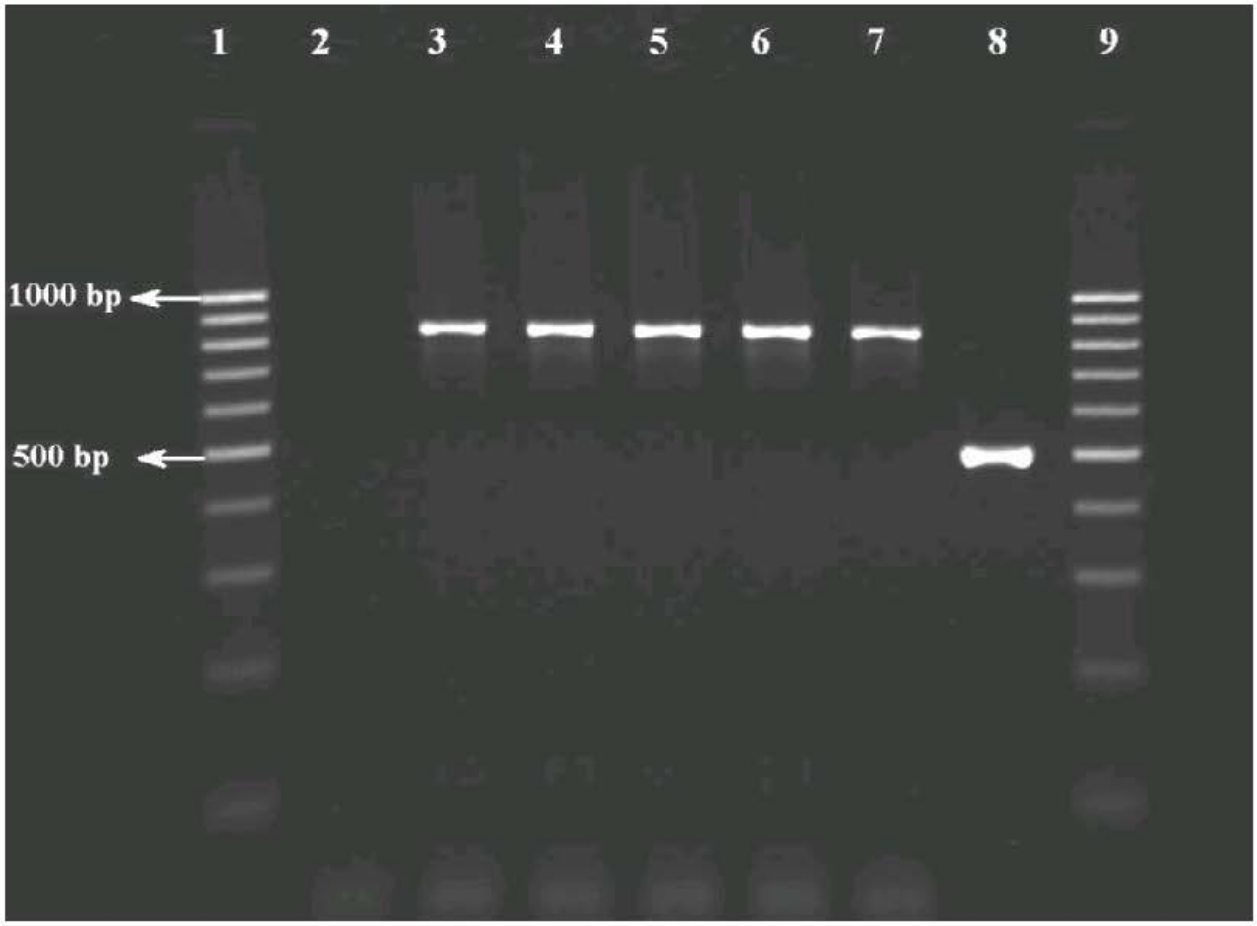

Fig. 2: Electrophoresis analysis (1.5% agarose gel) of PCR product of amplified by universal primers (Lauerman *et al.*, 1995). Lanes 1, and 9 = PCR 100 bp low DNA ladder (Promega Corp, Madison, WI, USA); Lane 2 = negative control; Lane 3 = MG F strain vaccine; Lanes 4-7 = samples of cloned MG cultures; Lane 8 = MS.

isolation, because these two media have been reported to be the most commonly used for the isolation of mycoplasma (Frey *et al.*, 1968; Fritz *et al.*, 1991; Kleven, 1998; Ley, 2003). Although some strains of MG like ts-11 strain will grow in SP_4 medium but not in Frey's medium (Cluss and Somerson, 1986) and some investigators have reported that the SP_4 media is superior for isolation of fastidious mycoplasma (Fritz *et al.*, 1991), based on these reports, it was expected that the SP_4 media, with the enrichments (yeastautolysate, CMRL 1066), will provide superior efficacy for primary isolation of MG from infected chickens. However, the results of this study clearly demonstrated that both media were equally sensitive for primary isolation of MG and none of the isolates were isolated in one medium and not in the other. This may be due to the absence of fastidious low pathogenic MG strains in the field in Northern Jordan or simply because the samples were collected from clinically diseased flocks which is probably caused by pathogenic MG isolates which will easily grow in the commonly used Frey's medium as well as the sensitive SP_4 medium.

It has been usually noted that when culturing from commercial layers, it is difficult to isolate MG because the cultures become overgrown with nonpathogenic mycoplasmas that grow very fast and vigorously. However, this problem was not an issue using the procedure described in this study. May be because we only cultured MG PCR positive broth into agars and many separate colonies were selected for subculture into different broth cultures and only PCR positive ones were further selected for second and third round of purification.

Various techniques have been applied for MG differentiation and these include protein profiles analysis using SDS-PAGE (Khan *et al.*, 1987), RFLP (Kleven, 1998), amplified fragment length polymorphism (AFLP) (Hong *et al.*, 2005), gene-targeted sequencing (Ferguson *et al.*, 2005) and RAPD (Fan *et al.*, 1995a; Geary *et al.*, 1994). AFLP and gene-targeted sequencing

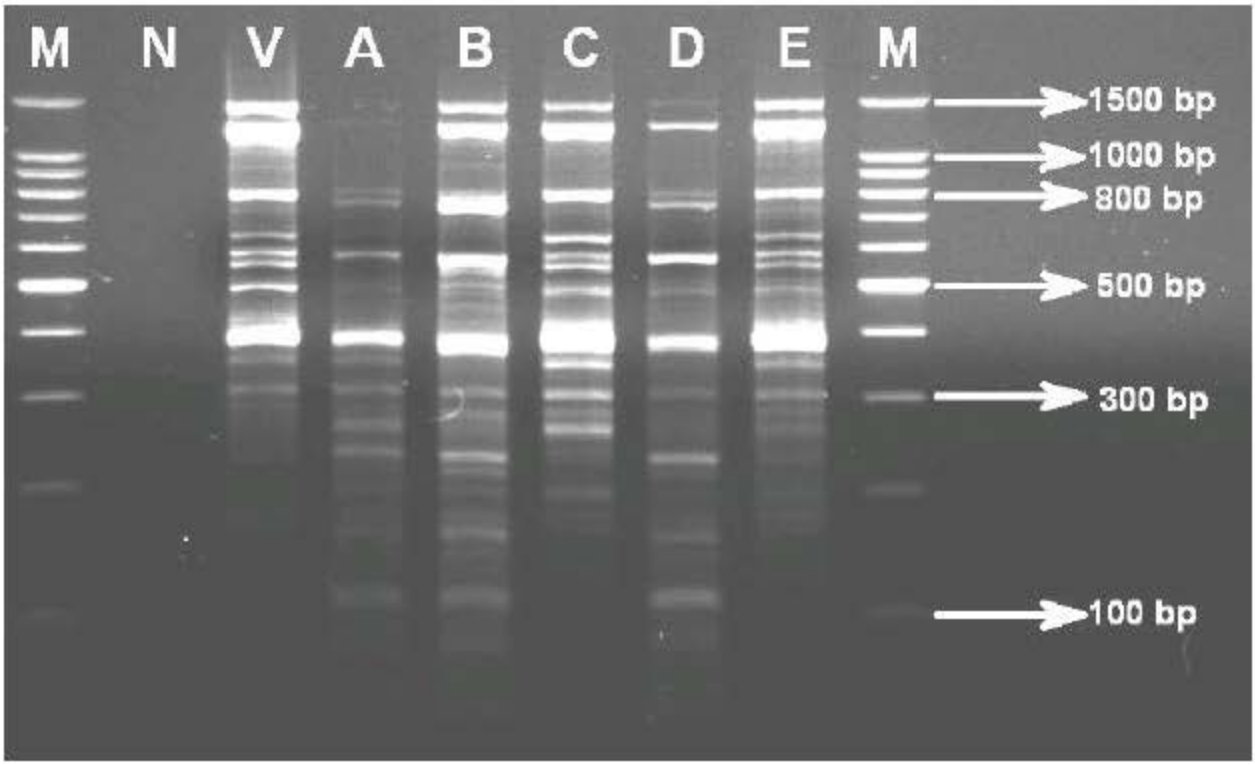

Fig. 3: Electrophoresis analysis (1.5% agarose gel) of RAPD patterns of MG isolate groups using Geary 1254 primer (Geary *et al*., 1994). Lane M = 100 bp DNA marker (Promega Corp, Madison, WI, USA); Lane N = negative control; Lane V = MG F strain vaccine; Lane A = represents 1 MG isolate; Lane B = represents 5 MG isolates; Lane C = represents 3 MG isolates; Lane D = represents 6 MG isolates; Lane E = represents 9 MG isolates.

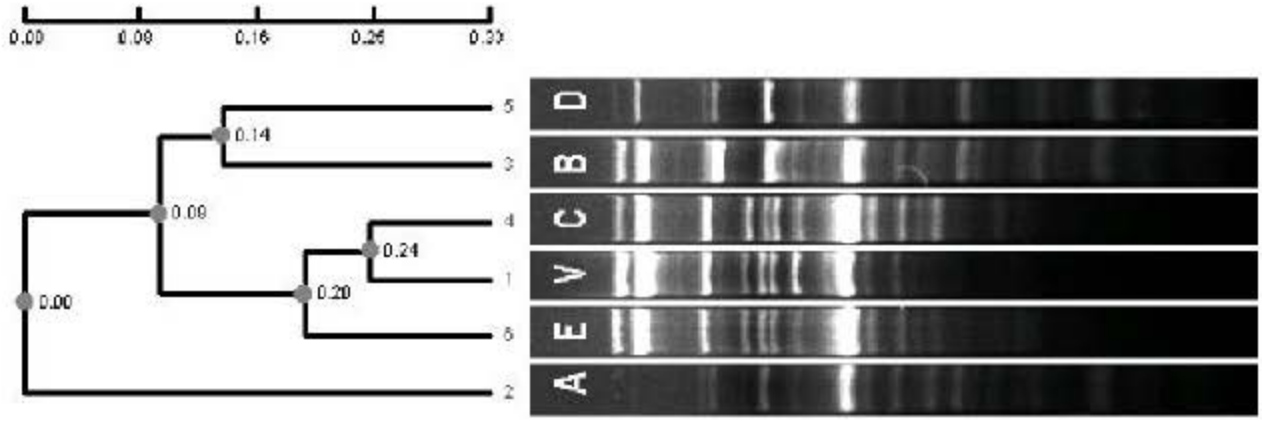

Fig. 4: Dendrogram showing relatedness of MG RAPD pattern groups with Jaccard coefficient and unweighted pair group method with average linkage using Alphaease software (Alphainnotech, San Leandro. CA, USA).

methods have a much higher discriminatory power and reproducibility than RAPD, these two techniques make it possible to compare various strains without the need for including them in the same run (Ferguson *et al*., 2005; Hong *et al*., 2005). In this study, we used the more simpler technique (RAPD) (Geary *et al*., 1994) and it was an excellent tool to distinguish between isolates of MG and demonstrated the presence of 5 RAPD banding patterns for our MG isolates that were different from the MG F strain vaccine (the common vaccine used in

Jordan). Therefore, the ongoing MG outbreak in commercial chickens in Jordan appears to be caused by different strains of MG. We were unable to confirm the presence of a trend for these banding patterns according to statistical analysis in relation to type of chicken, isolation location and ELISA GMT titers. The presence of the same banding patterns in different locations in Jordan could be due to the closeness of the areas covered in this study and most probably due to the vertical transmission of MG and that breeder progeny are being sold in different parts of the country. RAPD banding pattern C which represents 3 of the isolates in this study is very similar to the vaccine pattern (Fig. 4). This might indicate that these isolates might actually be F vaccine strain or mutated vaccine isolates and this requires further investigation to determine their pathogenicity or sequencing of the intergenic region to compare it with the vaccine sequence (Raviv *et al*., 2007).

This study provides a practical method by the use of PCR technology to aid in production of cloned MG cultures for laboratories that do not have the facility or reagents to do FA testing on routine MG cultures. The strains of MG isolated in this study will be the first isolates in Jordan and will pave the way for further studies on this bacteria and its control. Future work may include the sensitivity of Jordanian MG isolates to different antibiotics and the possibility of making autogenous vaccines from these isolates.

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