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Research Article

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Isolation and molecular identification of *Mycoplasma gallisepticum* from chicken flocks

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ABSTRACT

Since Mycoplasma Gallisepticum (MG) is one of the most pathogenic Mycoplasma, and caused tremendous economic loss in the poultry industry. Therefore, the present study aimed to isolate MG from chicken tracheas samples and to confirm molecular identification the MG strain using PCR. Mycoplasma gallisepticum which isolated was identified by the polymerase chain reaction (PCR). Banding patterns that were clearly identical from the common MG Ts-11 strain and in MG isolated from the field at 888-938 bp. Our data demonstrated an excellent technique to diagnose and investigate MG infections in chickens. The isolated MG can be used as autogenous vaccines in the future.

Keywords: Mycoplasma gallisepticum (MG), Polymerase Chain Reaction (PCR).

INTRODUCTION

Mycoplasma gallisepticum is the most economically significant mycoplasma pathogen of poultry such as pheasants [1], partridges [2], songbirds [3] quail [4], ducks [5], and geese [6, 7], and has a world-wide distribution. Mycoplasmas are the smallest free-living organisms and have complex nutritional requirements. They lack a cell wall, which results in Pleomorphism, Penicillin resistance, and susceptibility to environmental factors [2]. The first successful cultivation of *Mycoplasma* was the agent of bovine pleuropneumonia (BPP), in 1898 [8]. In chickens, the first isolation was in 1935 [9, 10]. The condition caused by mycoplasma designated "chronic respiratory disease" (CRD) was described in 1943 [11]. Different strains of pleuropneumonia like organisms of avian origin, which are now, designated as different species of mycoplasma were described in 1957 [12].

The pathology associated with this disease is characterized by severe air sac infection where MG is the primary pathogen followed by secondary infections with *Escherichia coli* (*E. coli*) and/or viruses such as infectious bronchitis (IBV) virus or Newcastle disease virus (NDV) [2].

Serological monitoring of a representative sample of the flock is performed periodically, and isolation or DNA-based detection methods are used to confirm suspected MG infection. Currently, the most widely used method to differentiate MG strains is arbitrarily primed PCR analysis [2]. These methods have been used to identify vaccine strains in MG vaccinated flocks [3, 13] and for epidemiological studies. Also, MG strains can be differentiated by polyacrylamide gel electrophoresis of cellular proteins, southern blot analysis, restriction fragment length polymorphism (RFLP) of whole cell-DNA and amplified fragment length polymorphisms (AFLP) [2, 13].

Severity of clinical manifestations of MG infection varies widely. Although severity is strongly influenced by undercurrent infections and environmental factors, strains of MG show significant variability in several characteristics. For example, strains of MG vary widely in virulence and serological response [14, 15, 16]. Variability in tissue tropism is indicated by reports of infection in turkeys showing neurological signs [17]. There

are significant variations in antigenic makeup, especially when measured by hem agglutination inhibition test [18, 19]. Strains can be differentiated by polyacrylamide gel electrophoresis of cellular proteins [20], southern blot analysis [21], or Restriction Fragment Length Polymorphism (RFLP) of whole cell-DNA [22, 23]. More recently, Random Amplified Polymorphic DNA (RAPD) techniques have been used for rapid identification MG strains [24].
In Jordan, there is a serious respiratory disease in chickens causing catastrophic economical losses to farmers and chicken companies. Some cases of this respiratory disease in chickens have been diagnosed as MG infection on the basis of clinical signs and gross lesions only. The aim of this study was to isolate and molecular identification of the MG strain using molecular techniques.

EXPERIMENTAL SECTION

2.1. Sample collection
During a period for 3 months between December 2014 and March 2015, commercial chicken flocks with respiratory symptoms breeder flocks located in Zarqa region were examined, tracheas were collected aseptically. All of these flocks were not vaccinated against MG.

2.2. Isolation of MG
2.2.1. MG culture media
For the growth of MG the following broth and agar media were used:

• Part A: Pleuropneumonia-like organism (PPLO) broth base without crystal violet (Difco) (14.7 g); distilled or deionized water (700 ml).
• Part B: Pig serum (heated at 56°C for 30 minutes) (150 ml): 25% (w/v) fresh yeast extract (100 ml); 10%(w/v) glucose solution (10 ml); 5% (w/v) thallous acetate (10 ml); 200,000 International Units (IU)/ml penicillin G (5 ml); and 0.1% (w/v) phenol red solution (20 ml). The pH is adjusted to 7.8, the pig serum may be replaced by horse serum, but it is important to ascertain that it supports the growth of MG.

Part A was autoclaved at 121°C, 1 atmosphere for 15 minutes and, after cooling, was added to Part B, which has been previously sterilized by filtration.

For the corresponding solid medium, 10 g of purified agar, known to support the growth of MG, is added to part A above. The mixture was autoclaved as previously mentioned and kept in a water bath at 56°C. The constituents of part B, omitting the phenol red, are mixed separately and then incubated at 56°C. Parts A and B were mixed carefully to avoid the production of air bubbles, and are dispensed into 50 mm dishes using 7-9 ml/dish. Excess surface moisture was removed by a short incubation at 37°C. Plates were stored in an airtight container at approximately 4°C for up to 2 weeks.

Fresh yeast extract is commercially available, although it is preferable to prepare it 'in-house' by taking active dry baker's yeast (250 g) and suspending it in distilled water (1 litter). It was heated to boiling point, cooled and then centrifuged for 20 minutes at 3000 g. the supernatant fluid is decanted and adjusted to pH 8.0 with 0.1 M NaOH.

This is clarified by centrifugation or by filtration, and then sterilized by filtration. The extract is stored at -20°C.

Reagent grade glucose (10 g) is dissolved in distilled or deionised water (100 ml) and adjusted to pH 7.8-8.0 with 0.1 M NaOH. It is sterilized by filtration and stored at 4°C.

Reagent grade thallous acetate (5 g) was dissolved in 100 ml distilled or deionized water, filter-sterilized and stored at -20°C. Penicillin solution (106 IU benzyl penicillin in 5 ml distilled water) was stored at 4°C, and has a shelf life of 1 week. Phenol red (0.1g) was ground in 0.1 M NaOH (2.8 ml), made up to 100 ml in sterile distilled water, autoclaved at 115°C, 1 atmosphere for 30 minutes and stored at 4°C [25].

2.2.2. Isolation of MG
MG was isolated according to a previously described procedure [26]. In brief: tracheal swabs were pooled and inoculated into 3-5 ml of broth cultures, then incubated at 37°C with 5% CO2. 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 were inoculated with PCR positive broth, using microbiological loop, and incubated at 37°C with 5 % CO2 for 5-7 days.

2.3. Purification of MG isolates
One colony from the agar plate was inoculated into broth medium, incubated and then tested by PCR. Agar plates were inoculated with PCR positive broth. This purification of cultures was repeated two more times.

2.4. Identification of MG isolates by PCR

2.4.1. DNA extraction

DNA extraction was -performed according to a previously described procedure [27], in brief: 1 ml of mycoplasma broth culture was centrifuged at 13,000 rpm for 10 min. The 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 TS-11 strain vaccine was used as a positive control.

2.4.2. PCR

Primers used specifically to detect MG were previously described by Lauerman [27], and their sequences are listed in table 1. The PCR mix was prepared in a volume of 25 µl containing 0.2 µl *Taq* polymerase (50 units/ml), 0.5 µl dNTP (10 mM), 2.5 µl $MgCl_2$ (25 mM) (Promega Corp, Madison, USA), 15.3 µl nuclease free water (Promega Corp, Madison, USA), 1 µl of forward 10 mM and 1 µl reverse primers 10 mM (Alpha DNA- Canada), 2.5 µl PCR buffer (500 mM KCL, 100 mM Tric CL , 15 mM $MgCL_2$, pH: 8.3 at room temperature) and 2 µl of DNA extract. Amplification was carried out for 45 cycles: 94°C for 30 seconds, 48°C for 2 min, and 72°C for 2 min, and final extension 72°C 1 for 5 min. PCR products were determined by agarose gel electrophoresis (1% agarose gel contain 0.5 µg/ml ethidium bromide). The DNA ladder and the PCR amplicons for MG were visualized using UV trans-illuminator (Bio Rad, UK).

Table 1. PCR primer used for MG

Type of primers	Primers Name	Sequences	Expected size
Universal	Forward	5'ACACCATGGGAGCTGGTAAT3'	888-938 bp
	Reverse	5'CTTCATCGACTTTCAGACCCAAGGCAT3'	

2.5. Confirmation of MG isolates purity by PCR

Primers used to confirm the purity of MG isolates were previously described by Lauerman *el al*. [27] and their sequences are listed in table1. These universal primers were used for detection of nine avian mycoplasmas by amplification of the 16s/23s ribosomal RNA (rRNA) intergenic space in Mollicutes, and will give different PCR product size for different avian mycoplasmas. MG gives a PCR product size ranging from 838 to 938 bp. Other avian mycoplsmas give different product size [27], DNA extraction was performed as described above. The PCR mix was prepared in a volume of 50 µl containing 25 µl master mix (*Taq* polymerase 50 units/ml, 400 mM of dNTP, and 3 mM $MgCl_2$) (Promega Corp, Madison, USA), 19 ul nuclease free water (Promega Corp, Madison, USA), 0.5µl 100 picomoles) of forward and reverse primers (Alpha DNA- Canada), and 5 µl of DNA extract. 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.

RESULTS AND DISCUSSION

3.1. Isolation and PCR analysis

The media were used in this study for MG isolation have been reported to be the most commonly used for the isolation of mycoplasma. Some strains of MG, like Ts-11 (temperature sensitive strain-11), and some investigators have reported that medium is superior for isolation of fastidious mycoplasma [25], based on these reports, it was expected that medium, with the enrichments, will provide superior efficacy for primary isolation of MG from infected chickens. However, the results of this study clearly demonstrated that media agreed on isolation results.

Traditionally, fluorescent antibody (FA) test is used to identify MG colonies on agar medium [28, 29, 30]. We used PCR with species-specific primers [26, 31] for identification of MG in cultures. This technique was very convenient for us because FA reagents were not available. This is in agreement with findings of other researchers who showed that PCR provides a rapid diagnosis and identification of MG, and also showed that PCR assay has several features that simplify the diagnosis of MG infections [32]. Also PCR can be used to detect MG in an air-dried mucosal swab without special transport requirements other than a container to prevent cross contamination between flock [32].

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. Purity of MG cultures was also continued by PCR using universal primers for avian mycoplasmas.

MG were isolated from tracheas samples using broth media and tested to identify MG using PCR as in the figure 1. We find six samples positive out of nine samples with MG.

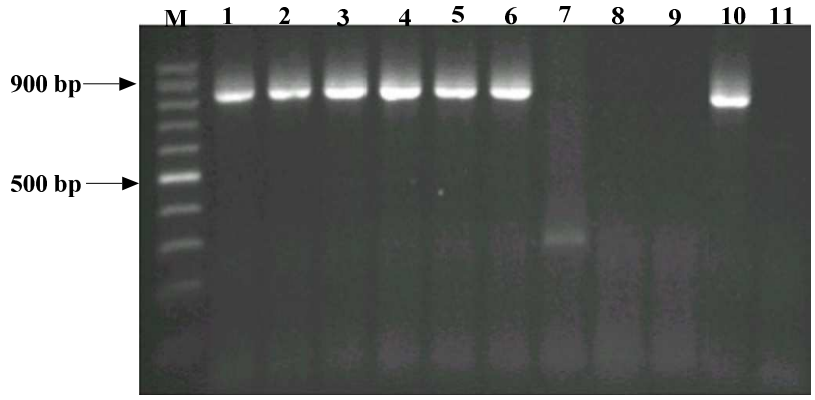

Figure 1. PCR amplicons of MG isolates using universal primers. Lane M: 100 bp DNA marker; Lanes 1-9: tracheas samples culture; Lane 10: MG TS 11 strain vaccine; Lane 11: negative control

3.2. Confirmation of MG isolates purity by PCR

The effective method for culture identification is direct immunoflourescence employing colonies on the surface of agar plate or colony imprints [29, 30, 33]. Agar gel precipitin [34, 35], iminunoperoxidase [36], and growth inhibition [34, 37] are also used to identify cultures of MG. To differentiate MG strains from one another, direct comparison of protein binding patterns result from Sodium DodecyI-Sulfate-polyacrylaminde gel electrophoresis (SDS-PAGE) [20], AFLP [2], And RFLP [22, 23]. These methods are especially useful for identification of vaccine strains of MG and for epidemiological investigations of MG outbreaks [2]. We have adapted an excellent technique to diagnose and investigate MG infections in chickens in Jordan, by detecting the MG at the molecular level of DNA using the PCR method.

All broth cultures (purified) and MG TS 11 vaccine strain were tested in this study using universal mycoplasmas primers gave a band with a size ranging from 888-938 bp confirming the purity of MG cultures as in the figure 2.

To increase sensitivity of MG detection, PCR based on specific sequences of nucleotides has been used [27, 38, 39, 40, 41, 42]. Multiplex PCR, PCR-RFLP also developed for MG detection and identification [33]. PCR with arbitrary HI test [43, 44, 45, 46]. ELISA test has been used to detected MG antibodies in respiratory tract washing, and in egg yolk sample [47]

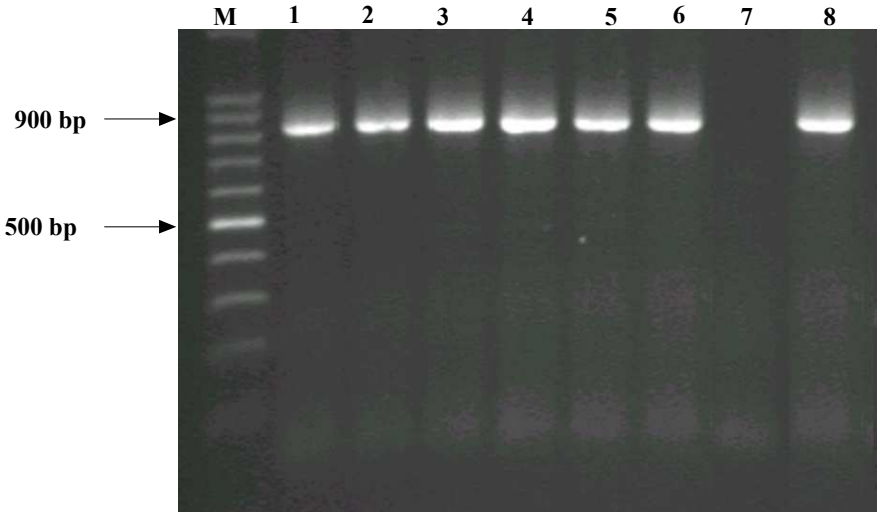

Figure 2. Electrophoresis analysis (1% agarose gel) of PCR products of MG isolates Amplified by universal primer. Lane M = 100 bp DNA marker; Lanes 2-7 = MG pure samples; Lane 7: negative control; Lane 8 = MG TS 11 strain vaccine

Recently, live MG vaccines which use strains 6/85 and ts-11 (temperature sensitive 11 strains) has been used and has been shown to possess little or no virulence for chicken or turkey [48, 49]. The strains of MG which isolated in this study will be the first isolates in Jordan and will gave the way for further studies on this bacteria and its control. Future work may include the possibility of making autogenous vaccines in Jordan or the Middle East from these isolates

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