ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS Biotechnology

ISSN 1682-296X
DOI: 10.3923/biotech.2018.142.150

Research Article
Molecular Characterization of Two New *Mycoplasma* Species Isolated from Chickens in Saudi Arabia

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Abstract

Background and Objective: Avian *Mycoplasma* infections pose a problem in poultry breeding. The main objective in this study was to isolate and identify new endemic *Mycoplasma* species that cause poultry diseases in Taif, Saudi Arabia by using both traditional microbiological methods and modern molecular methods. **Materials and Methods:** One hundred forty lung tissue samples from native chickens were collected from a Taif poultry abattoir and subjected to culture isolation, pathological and molecular characterization. **Results:** Sixteen isolates (11.4%) were identified as *Mycoplasma* species based on their cellular, morphological and biochemical characteristics. Furthermore, *Mycoplasma* isolates were identified by 16S rRNA gene sequencing; seven *Mycoplasma* isolates were identified as *Mycoplasma gallinaceum*, which infects turkeys. Only one of these isolates was identified as *Mycoplasma fermentans*, which infects human and eight isolates were unknown *Mycoplasma* sp. They may be trans located between farmers and chickens or between turkeys and chickens in the same farm. This is the first report of isolation *M. gallinaceum* from native chickens in the Taif region, Saudi Arabia. **Conclusion:** These results confirmed that polymerase chain reaction could be used to rapidly diagnose and identify *Mycoplasma* and is sensitive to molecular detection. These obtained isolates could be applied in control strategies and vaccine development in Saudi Arabia.

Key words: Avian, *Mycoplasma*, new endemic *Mycoplasma* species, *Mycoplasma gallinaceum*, 16S rRNA sequence

Citation: Magdy Hassan Yassin, Alaa Ahmed Mohamed, Mohamed Mahmoud Hassan, Ahmed Abd El-Aziz Baiomy and Amena Maher Ibrahim, 2018. Molecular characterization of two new *Mycoplasma* species isolated from chickens in Saudi Arabia. Biotechnology, 17: 142-150.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Mycoplasma is the common name for prokaryotes belonging to the class Mollicutes. These organisms are bacteria that lack cell walls, making them resistant to antimicrobials that act on this cell structure, such as penicillin[1]. *Mycoplasma* infection is responsible for economic losses in the chicken and turkey rearing industries; in many countries, the major control strategy is based on eradicating the pathogen from the breeding stock[2]. Avian mycoplasmosis is caused by several pathogenic *Mycoplasma* species. Moreover, *Mycoplasma* are also associated with many other diseases[3-5], representing a major problem in avian breeding and are related to bacterial infections[6,7].

It is difficult to diagnose mycoplasma infection in poultry based on clinical signs. *Mycoplasma* diagnosed from serological assays have shown false-positive results in previous studies[2,8]. Accordingly, these techniques were recently replaced by 16S rDNA sequencing for rapid, sensitive identification of *Mycoplasma*[9-11]. The 16S rRNA gene method has been successfully used to identify bacterial strains under both experimental and field conditions and to track epidemiologically related isolates in the field[2]. Alternatively, the repetitive element sequence-based polymerase chain reaction (rep-PCR) method can be applied to classify a wide variety of bacteria[12,13]. However, this method has not yet been used to isolate and identify *Mycoplasma* species in chickens in Saudi Arabia.

Therefore, the main aim of this study was to use microbial and molecular techniques to isolate and identify new strains of *Mycoplasma* from native chickens in Taif Governorate, Saudi Arabia.

MATERIALS AND METHODS

Samples: One hundred forty lung tissue samples were collected from Taif poultry abattoir, Taif, Saudi Arabia between September, 2016 to November, 2017, then specimens submitted for laboratory examination were divided into three portions for isolation, pathological studies and PCR analysis.

Isolation and identification of *Mycoplasma* species: *Mycoplasma* spp. was isolated as described by Shklair *et al.*[14] using PPLO media with incubation at 37°C in an atmosphere containing 5% CO_2. *Mycoplasma* colonies were subjected to biochemical analyses, such as catabolism of glucose, hydrolysis of arginine and digitonin sensitivity tests, as previously described by Ruhnke and Rosendal[15], for species identification.

Histological and histochemical studies using light microscopy: The lung tissues were dissected, fixed in 10% formal saline and embedded in paraffin wax and 6-µm-thick sections were then cut for histological analysis and stained with hematoxylin and eosin as described by Kieranan[16]. Masson's trichrome staining was performed according to the method of Drury and Wallington[17] and collagen fiber evaluation and periodic acid-Schiff (PAS) staining for mucopolysaccharides were performed according to the methods of Bancroft and Gamble[18].

Antibiotic susceptibility test: Drug sensitivity tests were conducted using the standard disk diffusion technique with 9 antibiotic disks (Bioanalyse, Ankara, Turkey): ciprofloxacin, oxacillin, clindamycin, erythromycin, ceftazidime, amoxicillin/clavulanic acid, tetracycline, ampicillin and amoxicillin as described by Sharma *et al.*[19].

DNA extraction: All *Mycoplasma* isolates were cultured in PPLO broth overnight at 37°C. DNA was extracted from 5 mL liquid culture using a bacterial DNA extraction kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Amplification of the 16SrRNA gene of *Mycoplasma*: The PCR amplification of the 16S rRNA gene was achieved as described by Lauerman *et al.*[20], using the forward primer GPO-3and reverse primer MGSO. PCR was performed using Go *Taq* Green Master Mix (Promega) according to the methods of Hassan and Belal[13].

Sequencing of the 16S rRNA gene: Approximately 280-bp 16S rDNA fragments were purified using a QI Aquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and then sequenced using the same primers and sequencer (Gene Analyzer 3121; Macrogen Co., Seoul, South Korea). The *Mycoplasma* 16S rDNA sequences obtained were compared with known 16S rDNA sequences in GenBank using BLAST and a phylogenic tree was constructed using MEGA program version 7.10.

Rep-PCR analysis: Ten different rep-PCR primers, including BOX A1 and GTG_5 were used for the molecular characterization of *Mycoplasma* isolates. PCR amplification of

the designed rep-PCR primers was performed according to the method of De Vuyst *et al.*[21]. The total reaction volume was 20 µL and all components for PCR were included as described by Gaber *et al.*[22].

Data analysis: The similarity matrix was subjected to cluster analysis by an unweighted pair group method and a dendrogram was constructed using the program NTSYS-PC version[23] 2.01.

RESULTS

Several *Mycoplasma* species were suggested as possible causes of respiratory diseases alone, in synergy with other microorganisms, or influenced by noninfectious factors, such as climatic conditions and management related problems.

The disease spreads horizontally by direct and indirect contact and by vertical transmission. In the present study, 16 of the 140 lung tissue samples (11.4%) were positive for *Mycoplasma* and were subjected to biochemical assays. Biochemical characterization revealed that all isolates were sensitive to digitonin and glucose fermentation but were negative for arginine deamination (Table 1).

The lung tissues of negative birds showed normal alveoli with moderate thickening of the interalveolar septa without congested pulmonary blood vessels (Fig. 1a and b). The lung tissues of infected birds showed significant thickening of the interalveolar septa because of the presence of perivascular edema and extensive round cell infiltration (Fig. 1c). Most bronchioles showed partial shedding of the mucosal lining and the appearance of cellular debris inside with leukocytic infiltration in the lamina propria (Fig. 1d). Numerous areas of cellular infiltration of mononuclear cells, including lymphocytic cells and macrophages were detected in the connective tissue surrounding the lung bronchioles, along with congested pulmonary blood vessels and severe fibrinous inflammation of the alveolar tissue (Fig. 1e and f).

Lung tissue sections from negative chickens showed a weak PAS reaction (magenta red stain) in the basal lamina of the alveolar epithelium and magenta red granules in the cytoplasm of the epithelial lining of bronchioles and lung alveoli (Fig. 2a). Lung tissue sections from *Mycoplasma* infected specimens exhibited a strong PAS reaction in the thickened interstitium, bronchiolar mucosa and mucopolysaccharides in the bronchioles (Fig. 2b).

The number of collagen fibers with decreased thickness and the staining of elastic fibers in the peribronchiolar area surrounding the damaged bronchioles in tissues from positive birds were greater than those in tissues from negative birds (Fig. 2c and d).

All the isolates were resistant to erythromycin (E), oxacillin (OX), ceftazidime (CAZ), amoxicillin (AX), amoxicillin/clavulanic acid (AMX, Augmentin) and ampicillin (AM) but they were susceptible to ciprofloxacin (CIP), clindamycin (DA) and tetracycline (TE; Table 2).

Mycoplasma strains were isolated from lung tissue samples using broth medium after 24 h of incubation and identified by PCR. PCR of all broth cultures (purified) tested in this study using universal *Mycoplasma* primers resulted in a band at 280 bp. Eight of 16 culture samples showed one specific band at approximately 280 bp and were identified as *Mycoplasma* spp. by 16S rDNA sequencing, whereas the other eight cultures were identified as unknown *Mycoplasma* isolates. These eight samples were selected for further studies. The partial sequences of the 16S rRNA gene of the eight selected *Mycoplasma* isolates were deposited in the GenBank database and the 16S rDNA nucleotide sequences showed 99% similarity (Fig. 3). When the sequences were aligned with the database sequences, seven *Mycoplasma* isolates were identified as *M. gallinaceum*, which infects turkeys and one isolate was identified as *M. fermentans*, which infects humans; this isolate showed 97% 16S rRNA sequence similarity with *M. fermentans* NR-044666.2. The phylogenetic relationships among the studied *Mycoplasma* strains based on comparisons of their partial sequences were inferred using the neighbor-joining method. Phylogenetic analysis revealed 97% similarity between *M. gallinaceum* and *M. fermentans*.

The genetic diversity of the eight mycoplasma isolates was investigated using rep-PCR for the first time. The eight strains produced different banding patterns showed in Fig. 4 and 5, whereas one of the M8 isolates produced a different rep-PCR profile using the same primers (lanes 9). In total, 167 fragments were generated, the total number of bands as shown in Table 3 varied from 12 bands with primer rep-R1 to 26 bands with primer Rep-I5. The total of monomorphic amplicons was 84 and the total of polymorphic amplicons was 83, with average percentage 50.3 and 49.7%, respectively.

Table 1: Number of *Mycoplasma* isolated and their biochemical characterization

Samples	No. of *Mycoplasma* isolates	Digitonin sensitivity	Glucose fermentation	Arginine deamination
140	16 (11.4%)	16 (11.4%)	16 (11.4%)	0%

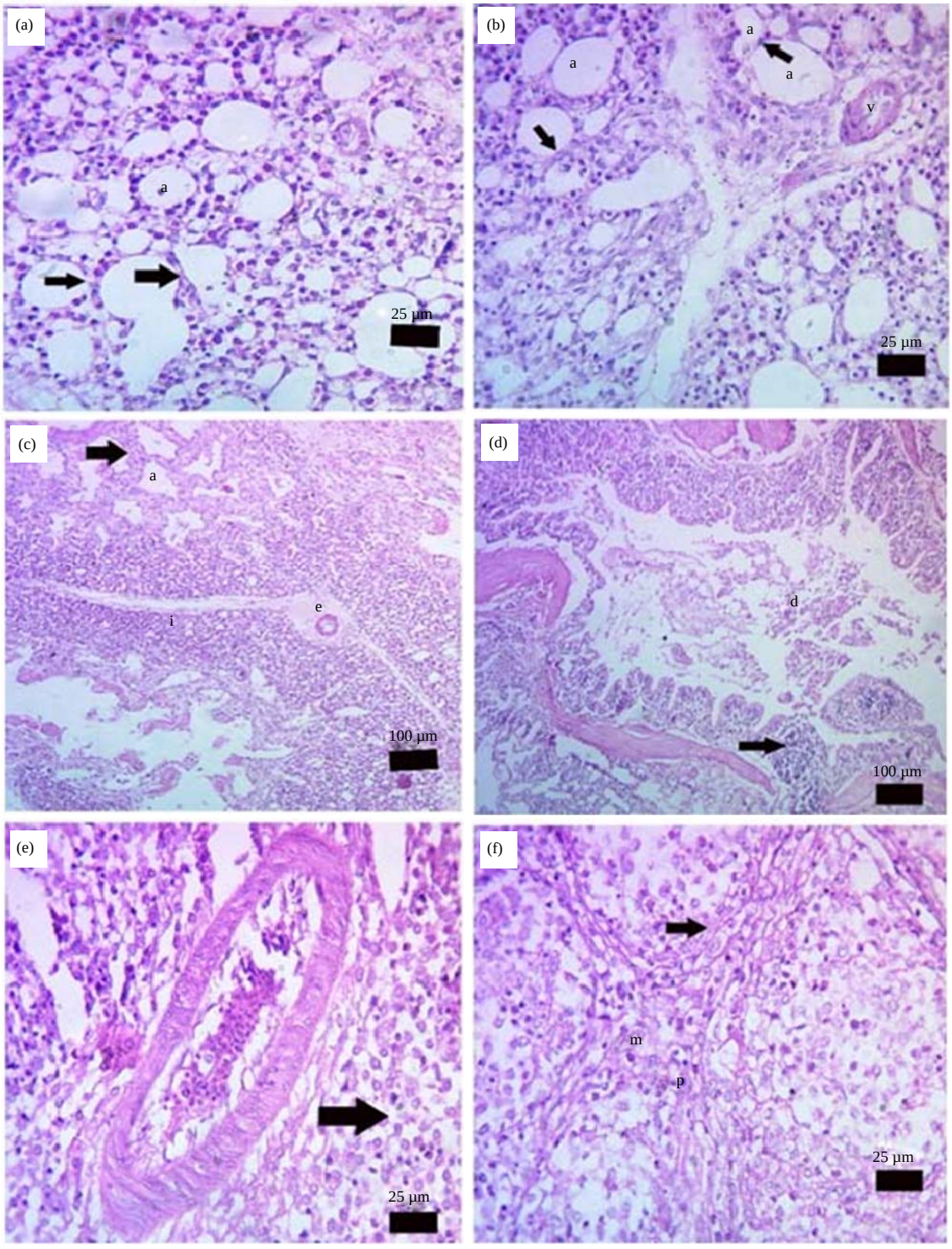

Fig. 1 (a-f): Lungs of negative birds showed normal alveoli whereas, (a) Lungs with moderate thickening of the interalveolar septa, (b) Lungs of the control group showed normal alveoli, (c) Lungs of infected birds showed significant thickening of the interalveolar septa, (d) Bronchiole showing partial shedding of the mucosal lining and the appearance of cellular debris inside, (e) Numerous areas of cellular infiltration and (f) Severe fibrinous inflammation of the alveolar tissue

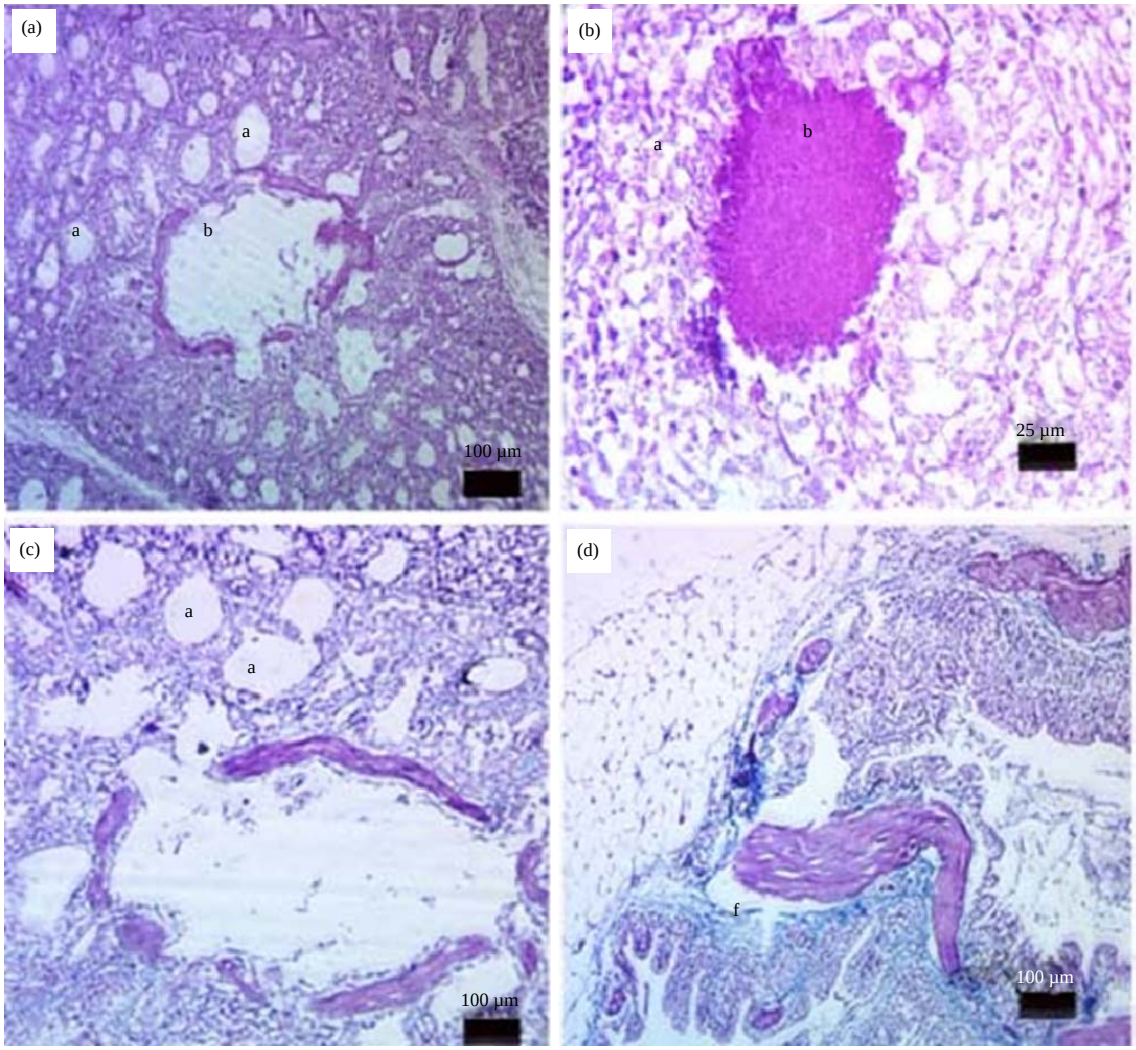

Fig. 2(a-d): Lung tissues section of infected birds and control staining with periodic acid-Schiff reaction and Masson's trichrome staining whereas, (a) Lung sections from negative lungs revealed weak PAS reaction, (b) Lung sections from the infected group revealed strong PAS reaction, (c) Lung sections showed few collagen fibers around the bronchioles and alveoli (d) Lung sections showed excessive collagen fibers with decreased thickness and staining of elastic fibers

Table 2: Inhibitory zones (mm) of tested antimicrobials against *Mycoplasma* field isolates (n = 8)

Antibiotic (µg)	Inhibitory zone
Ciprofloxacin (CIP) (5)	33.5
Clindamycin (DA) (2)	30.3
Tetracycline (TE) (30)	25.4
Erythromycin (E) (15)	8.3
Ceftazidime (CAZ) (30)	7.5
Amoxicillin/clavulanic acid (AMC) (30)	7.4
Oxacillin (OX) (1)	7.3
Ampicillin (AM) (25)	6.6
Amoxicillin (AX) (25)	6.4

The dendrogram based on the rep-PCR banding patterns divided the *Mycoplasma* isolates into two clusters with 59% genetic similarity (Fig. 6). These results were similar to those of 16S rDNA sequencing, indicating that rep-PCR fingerprints could be used for typing of bacterial strains, including *Mycoplasma* spp.

DISCUSSION

Mycoplasmosis in birds is caused by many pathogenic *Mycoplasma* species. Only *M. galisectem* and *M. synoviae* have been reported previously[24,25]. In the present study, 16 of 140 lung tissue samples (11.4%) were positive for *Mycoplasma* and were tested for digitonin, glucose fermentation and

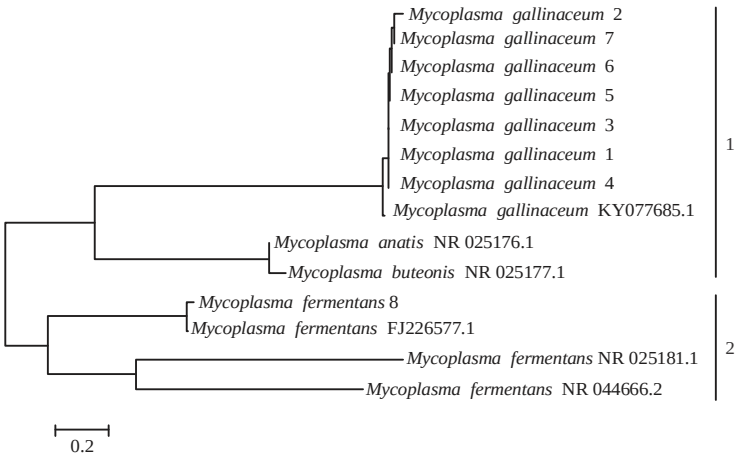

Fig. 3: Neighbor-joining phylogeny based on 16S rRNA gene sequences of eight *Mycoplasma* isolates and related *Mycoplasma* species obtained from a BLAST search of the NCBI database

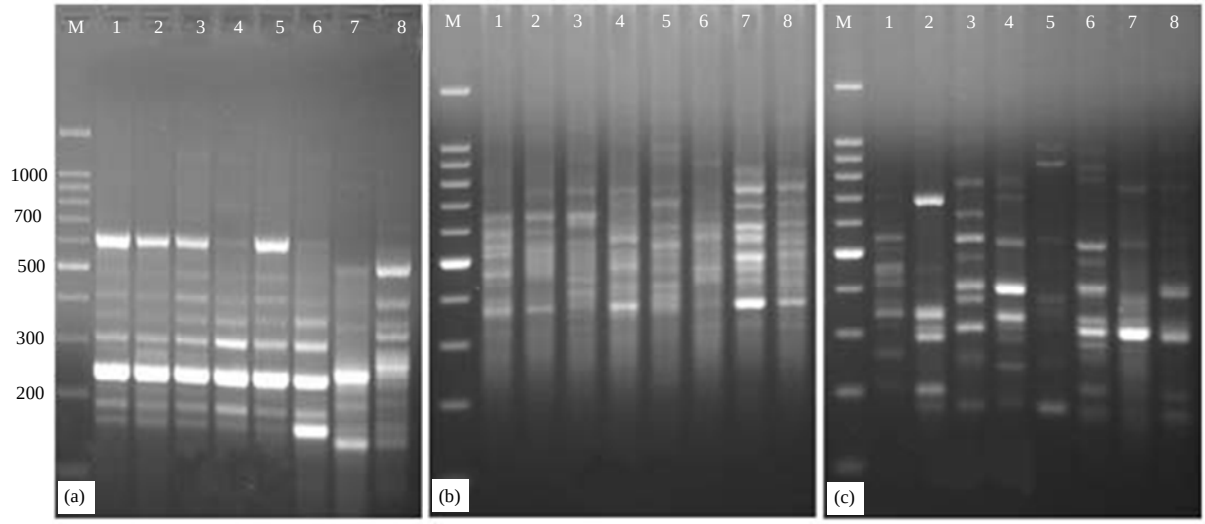

Fig. 4: Rep-PCR profiles of eight *Mycoplasma* isolates (a) BOX AI, (b) Rep-I10 and (c) Rep-I12
Lane1 = M: 100-bp DNA ladder and Lane 2-9: *Mycoplasma* strains

arginine deamination. These samples were negative for arginine and positive in the other tests. Similar results were obtained in previous studies[2,24-27]. The different *Mycoplasma* isolates from chickens and turkeys were then subjected to biochemical assays.

Lung tissues of infected birds showed significant thickening of the interalveolar septa due to the presence of perivascular edema and extensive round cell infiltration. *Mycoplasma* are chiefly found in the respiratory tract and organs of the reproductive tract[28,29]. The pattern of lesion development was similar to that reported in previous studies[10]. Airs acculates was observed in *M. gallinaceum*-infected birds; this condition which was initially mild but subsequently changed to a thick cloudy form, as described by Kleven *et al.*[30] and Nascimento *et al.*[31] Lesions, such as congestion and focal necrosis were present on the surface of the lungs, as reported by Rodriguez *et al.*[32] Under chronic conditions, the characteristic extensive fibrosis of the lung surface was the same as that documented by Bajwa *et al.*[33]. The lung tissues in the present study showed congestion, leukocyte infiltration and fibrinous inflammation of the alveolar tissue, consistent with the findings of Branton *et al.*[34].

All isolates were resistant to all tested antibiotics. These results were inconclusive because the development of antibiotic resistance by *Mycoplasma* was associated with essential changes in the genome and proteome. This process involves many genes and proteins related to fundamental cellular processes and virulence[35]. Appropriate

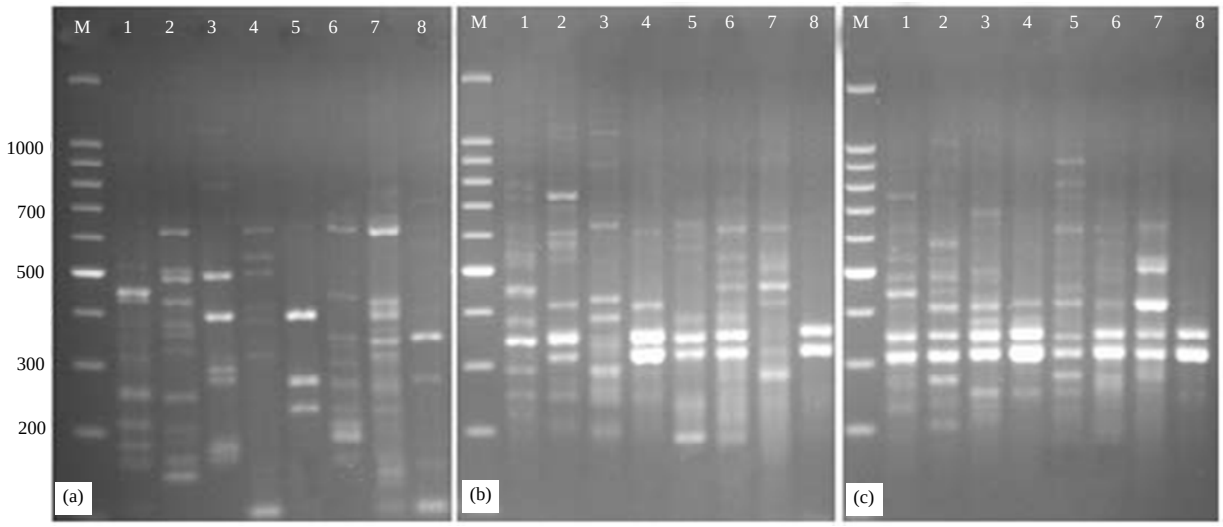

Fig. 5: Rep-PCR profiles of eight mycoplasma isolates, (a) Rep-I5, (b) Rep-I7 and (c) Rep-I8
Lane1 = M: 100 bp DNA ladder and Lane 2-lane 9: *Mycoplasma* strains

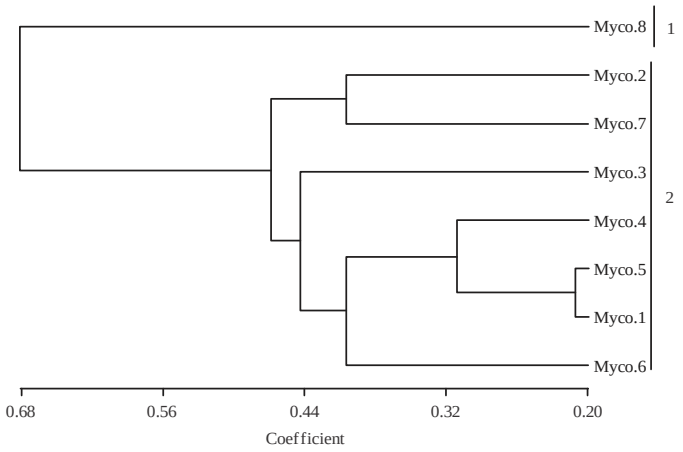

Fig. 6: Dendrogram based on cluster analysis of rep-PCR data among the eight *Mycoplasma* isolates

Table 3: Polymorphic band patterns and percentages of polymorphism for each repetitive element sequence-based polymerase chain reaction (rep-PCR) primers for eight *Mycoplasma* strains

Primers	Total bands	No. monomorphic bands	No. polymorphic bands	Monomorphic bands (%)	Polymorphic bands (%)
(GTG) 5	16	12	4	75.0	25.0
BOX A1	16	9	7	56.3	43.7
Rep-R1	12	3	9	25.0	75.0
Rep-I5	26	5	21	19.2	80.8
Rep-I7	23	6	17	26.1	73.9
Rep-I8	19	7	12	36.8	63.2
Rep-I9	18	15	3	83.3	16.7
Rep-I10	17	14	3	82.4	17.6
Rep-I12	20	13	7	65.0	35.0
Total	167	84	83	50.3	49.7

antibiotic therapy may significantly reduce the population of *Mycoplasma* in the respiratory tract of chickens[35-36].

Eight of 16 *Mycoplasma* isolates were identified as *Mycoplasma* spp. based on 16S rDNA analysis, whereas the other eight cultures were identified as unknown *Mycoplasma* isolates using rep-PCR. In previous studies, some authors have shown that PCR can be used to rapidly diagnose and identify *Mycoplasma* and that PCR has several features that simplify

this process[1,9]. In this study, analysis of the 16S rDNA nucleotide sequences showed that seven *Mycoplasma* isolates were *M. gallinaceum*, which infects turkeys, whereas only one isolate was *M. fermentans*, which infects human. Phylogenetic analysis revealed 97% similarity in both *M. gallinaceum* and *M. fermentans*. Manso-Silvon *et al*.[37] compared 16S rRNA-derived phylogenies among different *Mycoplasma* species. The sequence of the 16S rRNA gene is important for studying the evolution of microorganisms and is widely used to reconstruct the phylogenetic relationships of bacteria[12,13].

The dendrogram based on the rep-PCR banding patterns divided the *Mycoplasma* isolates into two clusters with 59% genetic similarity. These results were similar to those of 16S rDNA sequencing, indicating that rep-PCR fingerprints could be used for typing of bacterial strains, including *Mycoplasma* spp. Comparison of rep-PCR with other methods showed that rep-PCR produced more reliable results than other molecular methods[38-39]. These findings confirmed that PCR could be used to rapidly diagnose and identify *Mycoplasma* and was more sensitive than classical methods. These *Mycoplasma* strains were the first to be isolated from local chickens in Saudi Arabia and could have applications in developing control strategies and vaccines in Saudi Arabia.

CONCLUSION

The detection of *Mycoplasma* DNA using PCR has great potential for improving the diagnosis and control of avian mycoplasmosis. Moreover, the obtained *Mycoplasma* strains were the first to be isolated from native chickens in Saudi Arabia using polymerase chain reaction technique and could be used in control strategies and vaccine development in Saudi Arabia. These results confirm that PCR could be used to rapidly diagnose and identify *Mycoplasma*. This recently developed technology will provide the poultry industry with invaluable tools for improving the control of avian mycoplasmosis.

SIGNIFICANCE STATEMENT

This study discovers the new seven *Mycoplasma* isolates were identified as *Mycoplasma gallinaceum*, which infects turkeys not chickens, that can be beneficial for applying in control strategies and vaccine development in Saudi Arabia. This study will help the researcher to uncover the critical areas of How transfer of *Mycoplasma* species among different hosts that many researchers were not able to explore. Thus, a new theory on transfer *Mycoplasma* among different hosts that They live together may be arrived at.

ACKNOWLEDGMENTS

The authors would like to express their thanks and appreciation to Taif University, KSA, for funding this work. This work was supported by Taif University, KSA under project no. 1-437- 5177.

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