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Original article

MOLECULAR DETECTION AND PHYLOGENETIC ANALYSIS OF *MYCOPLASMA SPP.* ISOLATED FROM AWASSI SHEEP IN AL-MUTHANA PROVINCE, IRAQ

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Summary

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This study was carried out for molecular detection and phylogenetic analysis of *Mycoplasma* spp. in Awassi sheep in Al-Muthana province, Iraq. A total of 270 milk samples and swabs were collected from infected sheep. A polymerase chain reaction (PCR) technique was performed to detect a specific 16S rRNA gene of *Mycoplasma spp.* in these samples. Forty-four positive samples (16.2%) were identified, from which eight samples were selected for partial-gene sequencing. Then, alignment, comparison with referencing isolates in GenBank, and phylogenetic tree were performed using mean (UPGMA tree) in a MEGA software. The analyses revealed high homology between the current Iraqi isolates and American and Sweden *Mycoplasma* strains. The present molecular study showed that the studied Iraqi Awassi sheep were infected with *Mycoplasma* spp. with higher detection percentage from ocular swabs than from other types of samples. The phylogenic analysis registered eight Iraqi isolates with accession numbers in the GenBank with high similarity to five referencing *Mycoplasma* species.

Key words: Iraq, *Mycoplasma*, PCR, phylogeny, sequence, sheep

INTRODUCTION

Mycoplasma is a genus of bacteria which lack a cell wall around their cell membrane. *Mycoplasma* infection is one of the most important diseases in small ruminants that has great economic, veterinary, and medical importance worldwide (Ruffin, 2001). Mycoplasmae cause a wide range of diseases in both humans and animals like mastitis, conjunctivitis, pneumonia, arthritis, infertility, and abortions (Nicolas *et al*., 2008).

About five *Mycoplasma* species are associated with these diseases in sheep namely *Mycoplasma agalactiae, Mycoplasma mycoides* subsp. *mycoides* LC (large colony), *Mycoplasma capricolum* subsp. *capricolum*, *Mycoplasma mycoides*

subsp. *Capri*, and *Mycoplasma putrefaciens* (Winn *et al.,* 2005).

The *Mycoplasma* infections are difficult to diagnose due to limitations in the current diagnostic tests together with the similarities in the diseases that they cause. Mycoplasmae take weeks to be cultivated, and many serological tests are non-specific and insensitive. More recently, PCR has been used to detect a number of *Mycoplasma* species (Waites *et al*., 2000).

However, the detection of mycoplasmae by molecular techniques is more reliable than those depending on the clinical and classical microbiological methods. PCR is particularly useful for detection of pathogens which are difficult to grow *in vitro* or require a long cultivation period like mycoplasmae (Timenetsky *et al*., 2006). Genomic data coupled with phylogenetic methods have enhanced the ability to detect infectious diseases, including mycoplasmosis (Achtman, 2008)

Because the lack in the definitive studies about mycoplasmae in sheep in Mid-Euphrates of Iraq, the present study was carried out for the detection of *Mycoplasma* spp. in sheep and giving interpretation about some diseases that do not respond to treatment.

MATERIALS AND METHODS

Sampling

During the period from January to March 2016, two hundred and seventy samples were collected from 37 Awassi sheep flocks from different areas in Al-Muthana province, middle of Iraq.

The samples included 120 milk samples from sheep that had clinical signs of mastitis, 50 ocular swabs from infected sheep with keratoconjunctivitis, 50 nasal swabs from sheep that developed respiratory signs, and 50 ear swabs from sheep that suffered from mild otitis and marked ear irritations. These swabs were suspended in a *Mycoplasma* transport media.

All samples were incubated at 37 °C/ 1 h. Milk samples were centrifuged, and about 0.3 mL from milk sediment and from other swabs (nasal, ocular, and ear) was transported into 3 mL PPLO broth in a tightly closed screw-capped tube. Then, all PPLO-injected samples were incubated at 37 °C up to 10 days and growing indicators such as change of pH indicated by a colour change, turbidity in the media, or purple ring formation, were observed daily. Slight and gradual shifts in the pH indicator via marked colour change for a 3- to 5-day period without gross turbidity suggested true-positive culture. Those with no signs of growth were discarded after 10 days of incubation (Nicolas, 2002). The positive PPLOs were submitted to *Mycoplasma* spp. detection using a PCR assay.

Genomic DNA extraction

DNA was extracted from all positive PPLO cultures by using Presto™ Mini gDNA Bacteria Kit (Geneaid, USA) according to the company instructions.

PCR amplification

PCR assay was carried out using a specific primer (163-bp fragment of a highly conserved region of the *16S rRNA* gene in all *Mycoplasma*) which was designed by Pourbakhsh *et al.* (2010). The 16S-rRNA forward primer was GCTGCGGTGAATA CGTTCT and the 16S-rRNA reverse primer: TCCCCACGTTCTCGTAGGG, both provided by Bioneer Company, Korea. Then, the PCR master mix was prepared using AccuPower® PCR PreMix kit (Bioneer, Korea). The PCR premix tube contained a freeze-dried pellet of Taq DNA polymerase 1U, dNTPs 250 μM,

Tris-HCl (pH 9.0) 10 mM, KCl 30 mM, $MgCl_2$ 1.5 mM, stabiliser, and a tracking dye. The PCR master mix reaction was prepared according to kit instructions in 20 µL of total volume by adding 5 µL of purified genomic DNA, 1.5 µL of 10 pmol of forwarding primer, and 1.5 µL of 10 pmol of reverse primer, and then the volume was completed with deionised PCR water. After that, it was briefly mixed by Exispin vortex centrifuge (Bioneer, Korea). The reaction was performed in a thermocycler (Techne TC-3000, USA).

The thermal cycle included three steps as followed: as the first step, initial denaturation was performed at 94 ^{o}C for 5 min. In the second step, 30 cycles in which each cycle included denaturation at 94 ^{o}C for 30 s, annealing at 56 ^{o}C for 30 s, and extension at 72 ^{o}C for 45 s were performed. In the third step, the final extension was conducted at 72 ^{o}C for 7 min.

The PCR products were loaded in a 1.5% agarose gel stained with ethidium bromide that was electrophoresed for 1 h at 100 V and 80 A. The PCR products were visualised under a UV illuminator.

DNA sequencing and phylogenetic analysis

The 163-bp PCR products were purified from agarose gel using EZ-10 Spin Column DNA Gel Extraction Kit (Biobasic, Canada). Eight-purified PCR-products (2 of each sample type) were sent out to Bioneer Company, Korea for performing the DNA sequencing using both forward and reverse 16S rRNA primers by AB DNA sequencing system.

These sequences were aligned and compared with other available closely referencing sequence at the NCBI BLAST (Basic Local Alignment Search Tool) from GenBank by using ClustalW2 online programme. The phylogenetic tree was created using Neighbour Joining Method by MEGA software (Tamura *et al*., 2007).

Statistical analysis

The results of the present study were analysed by SPSS software (version 19).

RESULTS

Of the 270 different samples examined, forty-four samples were scored to be positive for the *Mycoplasma* spp. 16S rRNA, and the *Mycoplasma* infection percentage was 16.2% (Fig. 1). In nasal swabs, *Mycoplasma* spp. detection was significantly higher: 24% (50/12) than that in mastitis milk samples 15% (120/18), in ocular swabs 16% (50/8), and in ear swabs: 12% (50/6) (Table 1).

Sequence confirmation and examination were checked using 6 referencing sequences of the 16S rRNA gene of *My-*

Table 1. PCR positive *Mycoplasma spp.* of different sample types

Sample type	Number	PCR-positive for *Mycoplasma* spp.	%
Mastitic milk	120	18	15 ^{A}
Nasal swabs	50	12	24 ^{B}
Ocular swabs	50	8	16 ^{A}
Ear swabs	50	6	12 ^{A}
Total	270	44	16.2

The different lowercase letters refer to statistically significant differences ($P \leq 0.05$).

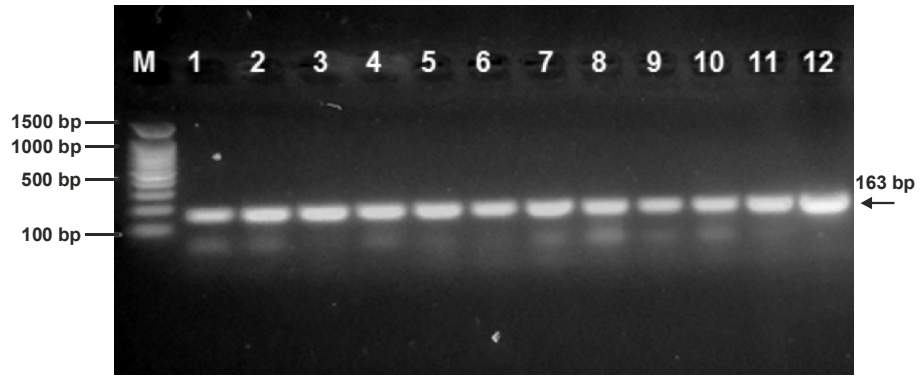

Fig. 1. Agarose gel electrophoresis image showing the PCR product of *16S rRNA* gene in *Mycoplasma* gene of the *Mycoplasma* genus positive isolates; M– marker (1500–100 bp); lanes 1 to 12 are positive for *Mycoplasma genus* showing a 163bp sized PCR product.

coplasma agalactiae, *M. arginini*, *M. mycoides* subsp. *mycoides*, *M. carpicolum*, *M. putrefacines*, *M. mycoides* subsp. *Capri*. Gene sequencing data were recorded in The GeneBank and compared to the referencing isolates to find the similarity scores of our isolates with referencing strains.

The results of the local *Mycoplasma spp.* (A7 and A3) showed a close matching to the NCBI-Blast-based *Mycoplasma agalactiae* at an identity score of 100%. On the other hand, the results of other local *Mycoplasma* spp. (A1, A6, and A8) revealed a close similarity to the NCBI-Blast-based *Mycoplasma mycoides* subsp. *mycoides* at an identity score of 100%. Moreover, the results of the local *Mycoplasma* spp. (A2) demonstrated a close identity to the NCBI-Blast-related *Mycoplasma arginini* at an identity score of 99%. In addition, the results of our local *Mycoplasma* spp. (A4) declared a close matching to the NCBI-Blast-based *Mycoplasma capricolum* at an identity score of 100%. Finally, the results of our local *Mycoplasma* spp. (A5) showed a close similarity to the NCBI-Blast-related *Mycoplasma mycoides* subsp. *capri* at an identity score of 100% (Table 2).

The results of the multiple sequence arrangement of the partial DNA sequencing regarding the current 8 Iraqi *Mycoplasma* isolates showed high similarity and identity scores ranging from 99–100% with the reference strains.

The phylogenetic tree analysis was generated based on the 163-bp-sized region of the *16S rRNA* gene partial sequencing data using Unweighted Pair Group Method with Arithmetic Mean (UPGMA tree) in MEGA v. 6.0. The local *Mycoplasma* isolates (A1, A6, and A8) showed a close similarity to the NCBI-Blast *Mycoplasma mycoides* subsp. *mycoides* (EU016367.1). The local *Mycoplasma* isolates A2 revealed a close matching to the NCBI-Blast *Mycoplasma arginini* (NR_041743.1). The local *Mycoplasma* isolates A3 and A7 demonstrated a close identity to the NCBI-Blast *Mycoplasma agalactiae* (AF332751.1). The local *Mycoplasma* isolate A4 declared a close matching to the NCBI-Blast *Mycoplasma capricolum* (NR_118796.1). Finally, the local *Mycoplasma* isolate A5 showed a close similarity to the NCBI-

Blast *Mycoplasma mycoides subsp. capri* (NR_118794.1) (Fig. 3).

In addition, Table 3 shows the NCBI-based accession numbers of the current local *Mycoplasma* isolates and the identity scores between our isolates and the world reference isolates. The results of our phylogenic tree and the sequencing analyses showed *Mycoplasma mycoides* (A1, A6, and A8) isolated from milk, eyes, and ears of sheep in Al-Muthana province, Iraq. The tree showed *Mycoplasma agalactiae* (A3 and A7) isolated from nasal swabs and milk of sheep. It also revealed *Mycoplasma arginine* (A2) isolated from nasal swabs of sheep in the province. Moreover, the analysis demonstrated *Mycoplasma carpicolum* (A4) isolated from ear swabs of sheep in Al-Kudher district (Al-Muthana province, Iraq) and finally, *Mycoplasma mycoides* subsp.*capri* (A5) in nasal swabs of tested sheep.

Table 2. NCBI-BLAST homology sequence identity for 16S ribosomal RNA gene in local *Mycoplasma* spp. isolates and NCBI-BLAST local *Mycoplasma* species

Local *Mycoplasma* sp.	NCBI-BLAST homology sequence identity											
	Mycoplasma agalactiae (AF332751.1)		*Mycoplasma arginini* (NR_041743.1)		*Mycoplasma putrefaciens* (U26055.1)		*Mycoplasma capricolum* (NR_118796.1)		*Mycoplasma mycoides* subsp. *capri* (NR_118794.1)		*Mycoplasma mycoides* subsp. *mycoides* (EU016367.1)	
	Identity	Total score	Identity	Total score	Identity	Total score	Identity	Total score	Identity	Total score	Identity	Total score
A1											100%	855
A2			99%	716								
A3	100%	992										
A4							100%	779				
A5									100%	855		
A6											100%	855
A7	100%	992										
A8											100%	814

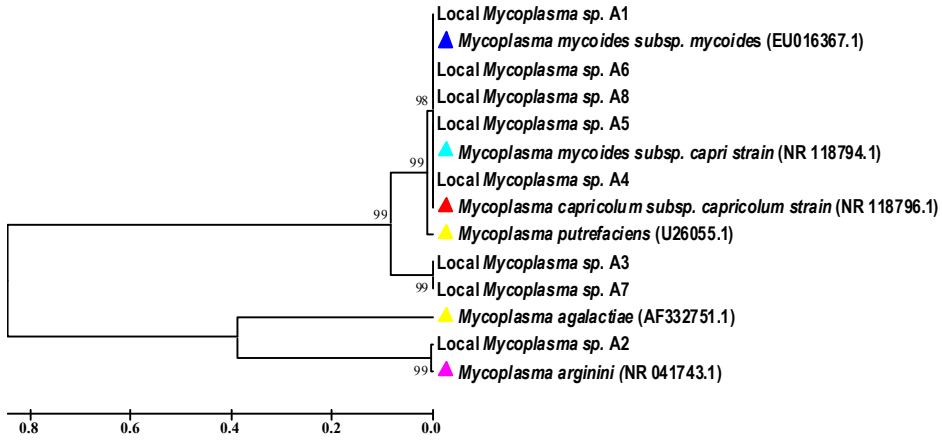

Fig. 2. Phylogenetic tree based on the 16S rRNA gene partial sequencing of *Mycoplasma species* typing.

Table 3. Summary of the sequencing of current local *Mycoplasma isolates* by NCBI-Blast-based analysis

Samples		Local genotype	Accession number BankIt1922298
Number	Type		
A1	Milk	*Mycoplasma mycoides*	KX272662
A2	Nasal	*Mycoplasma arginini*	KX272663
A3	Nasal	*Mycoplasma agalactiae*	KX272664
A4	Ear	*Mycoplasma capricolum*	KX272665
A5	Ocular	*Mycoplasma mycoides* subsp. *capri*	KX272666
A6	Ocular	*Mycoplasma mycoides*	KX272667
A7	Milk	*Mycoplasma agalactiae*	KX272668
A8	Ear	*Mycoplasma mycoides*	KX272669

DISCUSSION

The detection of *Mycoplasma* spp. by molecular techniques is more reliable than that depending on the clinical and classical microbiological methods.

Forty-four *Mycoplasma spp.*. were distinguished via the use of the *16S rRNA* gene as a genetic target for the identification of *Mycoplasma* genus focusing on a 163-bp sized region in this gene. A conventional PCR technique was used for this detection targeting milk, nasal, ear, and ocular samples that resulted in various percentages. This is in agreement with a report by Radostits *et al*. (2007) of mycoplasmae that infected all ruminants and could be isolated from acute and chronic infections of different body organs.

The infection rate of 16.2% was higher in nasal infection more than that in otitis. These present results are lower than what was previously recorded, 76.2%, by Khezri *et al*. (2015) in Iran. However, it was higher than the rate (9.6%) reported by Oravcova *et al*. (2009).

First, not all positive cultivated samples that appeared in characteristic fried-egg-like colonies on PPLO agar gave positive PCR results for *Mycoplasma genus*. This agrees with the results by Khezri *et al*. (2015) who recorded only 76.2% positive PCR results from positive PPLO cultures for *Mycoplasma* genus in Iran.

The significantly higher *Mycoplasma* PCR detection in nose than in ears and milk was similar to the findings of Khezri *et al*. (2015). However in another study in Kurdistan, Iran, the PCR detection was higher in milk samples, 73.7%, than that in other samples (Khezri *et al.,* 2012). Pooladgar *et al*. (2015) showed comparable findings to our present results with higher detection in eye swabs than in milk samples.

In many laboratories, PCR is used as a reliable, rapid, and sensitive test. However, in conventional PCR, the negative results are not considered definite negative. The negative results were attributed to the presence of inhibitory substances in biological samples which excite significant reduction or even blockage of the amplification activity of DNA polymerases that eventually affect the sensitivity degree of PCR as well as the skilled personnel carrying out the test (Vaneechoutte & Van Eldere, 1997; Lo & Kam, 2006).

The DNA sequencing study is very important approach to understand the epidemiology of different infectious diseases

and to comparing diagnostic microorganisms with global isolates. In our current study, the sequencing and the phylogenetic analyses of *Mycoplasma* species comparing them to other *Mycoplasma* species in the Gene Bank gave us information about a new strain of *Mycoplasma* species, its emergence, and the molecular epidemiology of this new strain. This is very important in our country to help in the control of the disease.

The eight sequenced samples from sheep of different regions in Al-Muthana province showed good results of the forward nucleotide sets such as sequencing of the *16s rRNA* gene (163–375) or wave-like sequencing via the use of the due-terminator sequencing method.

Our data of the DNA sequencing were processed for confirm the nucleotide sequencing and evaluate relationships of the current isolates with other world strains using the online NCBI-BLAST website – considered as the best data processing tool to measure the identity and similarity of our strains with the global isolates.

The phylogenic analysis that was carried out using the alignment of our study strains of sheep with the reference world strains in the GeneBank give accurate degrees of similarity and identity with the world strains, done for the first time on mycoplasmae in sheep in Iraq.

The detailed tree analysis that was based on the 163-bp sized region of the *16S rRNA* gene partial sequencing, so the phylogenic tree constructed using Unweighted Pair Group method with Arithmetic mean (UPGMA tree) in MEGA 6.0 software, exhibited that our isolates from Iraqi sheep numbered A1, A6, and A8 under accession numbers KX272662, KX272667, and KX272669, respectively showed 100% identity with *Mycoplasma mycoides* subsp. *mycoides* (EU016367.1) isolated in Sweden in 1995 and USA in 2014. The local isolate A2 under accession number KX272663 showed 99% identity with *Mycoplasma arginini* (NR_041743.1) that was isolated in Sweden in 1999 and USA in 2011. The A3 and A7 isolates with accession numbers KX272664 and KX272668, respectively showed 100% identity with *Mycoplasma agalactiae* (AF332757.1) isolated in Sweden in 2000. Our strain A4 under accession number KX272665 showed 100% identity with *Mycoplasma capricolum* (NR_118796.1) that was isolated in Sweden in 1995 and USA in 2014. Finally, the local strain A5; with accession number KX272666) showed 100% identity with *Mycoplasma mycoides* subsp. *capri* (NR_118794.1) isolated in Sweden in 1995 and USA in 2014.

The results of the present study suggested that the *Mycoplasma* species recorded for the first time in sheep of Iraq and deposited in the GeneBank might have come to Iraq from neighbour countries or via transport of contaminated goods and feedstuff from endemic areas. We expect its transmission to small ruminants in Iraqi Mid-Euphrates because sheep of Al-Muthana are mixed with other animals in rearing on natural pasture and lands in Al-Samawa and Al-Najaf deserts during the previous winter season.

In conclusion, *Mycoplasmas* infection in Iraqi Awassi sheep are common and there are five genotypes of *Mycoplasma* species registered in the GeneBank that isolated from different samples.

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