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Virulence Traits and Antimicrobial Sensitivity Testing of Untyped *Mycoplasma* species Recovered From Sheep and Goats in Egypt

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ABSTRACT

Many *Mycoplasma* species are involved in caprine and ovine pneumonia all over the world causing serious economic losses. These species have been reported to cause pneumonia, mastitis, arthritis, conjunctivitis and genital infection. The objective of the present study was to detect *Mycoplasma* species in the respiratory system of sheep and goats. So, a total of 400 samples (250 from sheep and 150 from goat) were examined bacteriologically, biochemically, by PCR, sequencing, in addition to the antibiotic susceptibility profiles screening. Only 13 isolates could be identified as *Mycoplasma* species. One of these sequences was submitted to the Genbank taking the accession number MK910041; *Mycoplasma* species 'ovine/caprine serogroup 11'. Only 7 isolates were weak biofilm producers and the other 6 isolates were non biofilm producers; 8 isolates were positive for the catalase test and 5 were negative. H_2S production was recorded in 10 isolates; haemolysis was detected in only 6 isolates. In addition, the minimum inhibitory concentrations (MICs) for seven antimicrobial agents, including danofloxacin, tulathromycin, tylosin, streptomycin, lincomycin, florfenicol and oxytetracycline, were determined. All the isolates (100%) were sensitive to tulathromycin and tylosin, streptomycin and oxytetracycline; 38.5% were sensitive to danofloxacin, 69.2% were sensitive to florfenicol, while 69.2% of isolates were resistant to lincomycin. As all the isolates were recovered from apparently healthy and clinically diseased animals and identified as untyped *Mycoplasma* species, more investigations will be done to identify these isolates and discover their roles in infection.

Keywords: Biofilm-formation, Goat, Sheep, Untyped-*Mycoplasma*-species, Virulence-genes.

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INTRODUCTION

Mycoplasmas are the smallest, self-replicating bacteria that lack cell walls; therefore, they have intrinsic resistance to penicillin and B- lactam antibiotics. They affect all animal species such as chicken flocks **(El-Ashram *et al.*, 2021),** sheep, goats **(Adehana *et al.*, 2006),** and human beings, causing serious pathological conditions including respiratory problems and mastitis, arthritis and genital disorders. *Mycoplasma* species have been reported as a cause of pneumonia, mastitis, arthritis, conjunctivitis and genital infection. Sheep are important household animals and play a major role in the economic status of farmers in Egypt like many other developing countries. They constitute an important component of Egypt's food security plan**.** The most pathogenic *Mycoplasma* species affecting sheep and goats is *Mycoplasma capricolum* subsp. *capripneumoniae (Mccp)*; the main cause of contagious caprine pleuropneumonia (CCPP) **OIE (2017)** List disease; and *M. agalactiae;* the cause of classical contagious agalactia (CA).

Other *Mycoplasma* species have been isolated from sheep and goats suffering from respiratory problems and apparently healthy animals (*M. arginini, M. ovipneumoniae,* and *M. mycoides* subsp. *capri* and *M. capricolum* subsp. *capricolum* **(Nicholas *et al.*, 2008).** Respiratory affection in sheep is a major multifactorial problem causing significant economic losses; many bacterial and viral species can cause it in addition to *Mycoplasma* species. The most commonly recorded *Mycoplasma* species with respiratory infection worldwide is *M. arginini* and *M. ovipneumoniae* **(Azizi *et al.*, 2011; Chinedu *et al.*, 2014; Abdel Halium *et al.*, 2019).** The infection may

be caused by a combination of these species, especially for young lambs **(Niang *et al*., 1999).**

M. ovipneumoniae is a serious and highly infectious pathogen causing lethal pneumonia in sheep and goats which appear in all ages, especially in lambs **(Besser *et al*., 2013).** Clinical observations showed difficult breathing with dyspnea, followed by death in newborn kids and those up to 2 months of age. In young lambs, it can be associated with a severe paroxysmal cough that predisposes to rectal prolapses, termed coughing syndrome. Between flocks, this disease is chronic and lasts for several weeks, with variable morbidity and mortality rates **(Nicholas *et al*., 2008).** In many reports, the number of isolated *Mycoplasma* species was not identified by the known species-specific primers **(Kılıc *et al.*, 2013).**

The present study was designed for isolation and molecular characterization of *Mycoplasma* species affecting sheep and goats, detection of biofilm formation and evaluation of the antimicrobial susceptibility of the obtained isolates by MIC test.

MATERIIALS AND METHODS

Sample collection and preparation:

A total of 400 sheep and goats samples were randomly collected between December 2017 till May 2018 from El-Basatin slaughterhouse in Cairo, Egypt. Pneumonic lung samples (n=240), referring to those showing various degrees of congestion, fibrin deposition, and/ or pleural adhesion to the lung, were collected then transported to the Microbiology laboratory- Faculty of Veterinary Medicine, Cairo University in a special ice-filled container. A hot spatula seared the outer surface of the lung sample before cutting the lung's inner surface to avoid any contamination. Very small parts of the lung were cultured on PPLO broth and incubated at 37°C for 24-48 hours and sub-cultured twice to minimize the contamination, then were cultured on PPLO agar medium by the running drop technique, incubated at 37°C, with 5-10% CO_2 for 7-14 days. Mild turbidity in a liquid medium and the appearance of fried egg colonies on the solid media indicate suspected Mollicutes growth. Nasal swabs (n=160) collected from apparently healthy animals were taken by sterile cotton swabs deeply embedded in the nostrils, then kept in an icebox and submitted to the laboratory. Samples were then processed as lung samples but incubated for 7-21 days and examined regularly.

Digitonin sensitivity test for differentiation between *Mycoplasma* and Acholeplasma: using a filter paper disc impregnated in 0.2 ml of 1.5% (w/v) ethanol solution of digitonin. *Mycoplasma* species are digitonin sensitive, while Acholeplasma is resistant to digitonin **(Freundt, 1973).** Further identification for the isolates using biochemical tests as glucose fermentation and arginine deamination tests were done **(Erno, 1987).**

Phenotypic virulence traits of *Mycoplasma* species isolates:

Four assays were used for demonstration of the phenotypic virulence traits of *Mycoplasma* isolates using: semi-quantitative analysis of biofilm formation using crystal violet staining by measuring the absorbance (620nm) of 100 ml of the solubilized crystal violet in a microtiter plate according to **McAuliffe *et al.*, (2006),** hemolytic and haemoxidative activity of the isolates according to **Großhennig *et al.*, (2016),** catalase activity assay according to **Pritchard and Mitchell, (2015),** hydrogen sulfide production by the isolates using lead acetate detection strips according to **Shatalin *et al.*, (2011).**

Molecular identification:

Preparation of samples for DNA extraction:

Five ml of 24 hours broth culture of each isolate was centrifuged at 8000 rpm (JOUAN) for 15 min in a 1.5ml microtube. The obtained pellets were washed three times with Phosphate-Buffered Saline pH 7.4, heated at 100ºC for 10 min in a heat block (TECHNE DB-3A) followed by freezing for 10 min, finally, centrifuged for 5 min. Then, the supernatant containing chromosomal DNA was collected and stored at -20°C until used.

Mycoplasma species identification:

The isolates were confirmed as mycoplasmas by PCR amplification using *Mycoplasma* genus-specific primer according to **Van Kuppeveld *et al.*, (1992)** using the following primer sequences [Gpo 3 (5'-ACTCCTACGGGAGGCAGCAGTA-3') and Mgso (5'-TGCACCATCTGTCACTCTGTTAACCTC-3') giving a final amplification product at 264 bp. PCR reaction was performed in a total of 50 µl reaction volume consisting of 5 µl of 50 ng of genomic DNA,1 µl of 50 pM of each primer, and 25ul PCR master mix (Thermo fisher scientific),18 µl of DNase- RNase- free, deionized water. Thermal conditions in (GTC96S cleaver scientific) one cycle of denaturation step at 94°C for 5 min, 40 cycles of denaturation at 94°C for 45s, annealing at 55°C for the 60s and extension at 72°C for 60 s, and a final extension step at 72°C for 10 min. The amplified products were examined in UV transilluminator after electrophoresis in 1.5% agarose gel containing 0.005% ethidium bromide in 1× Tris/borate/EDTA buffer at 7 V/cm. The amplified PCR products were sequenced (AHRI, EGYPT). One of these sequences was submitted to NCBI-Genbank taking the accession number: MK910041.1. The assembled DNA sequence was subjected to BLAST analysis. After aligning the sequences using the CLUSTAL W algorithm method, phylogenetic analysis

was done using the neighbor-joining method using MEGA 6.0 software (Fig. 1).

Antimicrobial sensitivity testing:

It was performed and the number of colour-changing units (CCU) was determined by the MIC method **(Felde *et al*., 2018).** The selected antimicrobial agents are those frequently used in Egypt: Quinolones: danofloxacin; Phenicol: florfenicol; Macrolides: tylosin and tulathromycin; Aminoglycosides: streptomycin; Lincosamide: lincomycin; Tetracycline: oxytetracycline. All the strains were tested in duplicates and all the plates contained a duplicate of the type strain (NCTC 10110) as a quality control. The results were interpreted according to **TerLaak *et al*., (1993); Hirose *et al.* (2003); and CLSI (2011).**

RESULTS

Out of 400 samples from sheep and goats, only 13 (3.25%) were identified as *Mycoplasma* species represented as 2 isolates (1%) from sheep lung samples, 3 isolates (6%) from sheep nasal swabs, 3 isolates (7.5%) from goat lung samples and 5 isolates (4.5%) from goat nasal swabs. Therefore, the incidence of *Mycoplasma* species isolation from goats 5.3% was higher than that from sheep 2% (Table, 1).

Table1: Results of *Mycoplasma* species isolation from the examined samples

Type of sample	No of examined samples	*Mycoplasma* species	
		No	%
Sheep (lung tissue)	200	2	1
Sheep (nasal swab)	50	3	6
Total Sheep samples	250	5	2
Goat (lung tissue)	40	3	7.5
Goat (nasal swab)	110	5	4.5
Total goat samples	150	8	5.3
Total samples	400	13	3.25

Biochemical testing of the isolates showed that they were negative for arginine hydrolysis but were positive for both digitonin and glucose fermentation tests. Virulence characteristics revealed that 7 isolates (53.8%) were biofilm producers while 6 (46.2%) were non biofilm producers. Catalase activity was detected in 8 isolates (61.5%) and was absent in 5 isolates (38.5%). We observed that 6 isolates (46.2%) were hemolytic and the other isolates (53.8) were non-haemolytic. H_2S was detected in 10 isolates (76.9%) and was not revealed in 3 isolates (23.1%) (Table 2).

All the isolates could produce amplified DNA products with the suspected amplicon size **(photo. 1)**. One of the sequence results of *Mycoplasma* isolates was sent to the Genbank taking the accession number "MK910041.1".

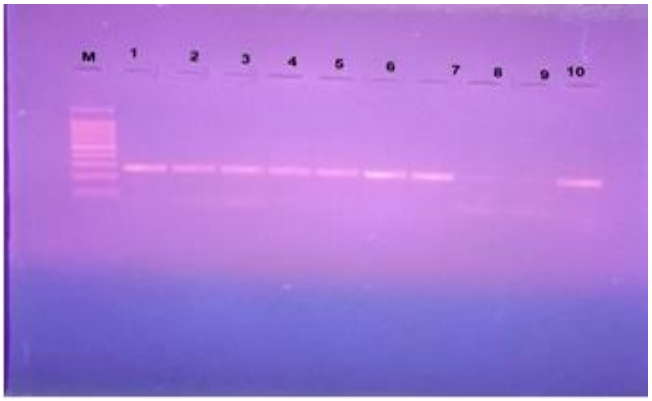

Photo 1: Electrophoresis of PCR products of ten tested *Mycoplasma* isolates showing amplified products at 264 bp represented as Lane M: molecular marker (100-1500bp), Lanes (1-10) representative positive samples.

Table 2: Virulence characteristics and biochemical activity of the isolates

Species	N=number	Biochemical activity			Virulence traits							
		Digitonin test	Glucose fermentation	Arginine hydrolysis	Biofilm		Catalase		Hemolysis		H_2S	
					N	N*	N	N*	N	N*	N	N*
Untyped *Mycoplasma*	13	+	+	-	7	6	8	5	6	7	10	3

N: number of positive samples N*: number of negative samples

Table 3: Antibiotic susceptibility testing results of sheep *Mycoplasma* isolates

Antimicrobial agent	*Mycoplasma* isolates (n=13)					
	S		I		R	
	No	%	No	%	No	%
Danofloxacin	5	38.5	5	38.5	3	23
Tulathromycin	13	100	0	0	0	0
Tylosin	13	100	0	0	0	0
Streptomycin	13	100	0	0	0	0
Lincomycin	4	30.8	0	0	9	69.2
Florfenicol	9	69.2	4	30.8	0	0
Oxytetracycline	13	100	0	0	0	0

R: resistant, I: intermediate, S: sensitive % was calculated to the total examined *Mycoplasma* species.

Table 4: Antimicrobial sensitivity testing**,** virulence characteristics of all Mycoplasma species isolated from sheep and goat:

Mycoplasma Species	Source	Animal	Virulence traits				Antimicrobial sensitivity						
			Biofilm	Catalase	Hemolysis	H_2S	Danofloxacin	Tulathromycin	Tylosin	Streptomycin	Lincomycin	Florfenicol	Oxytetracycline
1	lung	sheep	0.593	+	+	-	0.125	1	1	1	2	2	1
2	lung	Goat	0.328	-	-	+	1	2	2	2	2	2	2
3	lung	sheep	0.268	-	+	+	0.5	2	1	1	1	4	4
4	Nasal swab	sheep	0.370	+	-	+	0.5	1	0.5	2	2	2	2
5	Nasal swab	sheep	0.255	+	+	+	1	1	1	2	1	2	4
6	Nasal swab	Sheep	0.350	+	+	+	2	1	2	2	2	2	4
7	Lung	Goat	0.312	+	-	+	2	0.5	2	2	4	4	4
8	Lung	Goat	0.296	-	+	+	1	0.5	2	2	4	2	2
9	Nasal swab	Goat	0.414	+	-	+	2	2	0.25	1	4	0.5	2
10	Nasal swab	Goat	0.221	-	-	-	1	0.125	1	1	1	4	4
11	Nasal swab	Goat	0.300	+	-	+	0.5	1	2	1	1	4	2
12	Nasal swab	Goat	0.236	-	-	+	1	1	2	0.25	2	2	2
13	Nasal swab	Goat	0.211	+	+	-	0.5	0.25	2	2	4	2	4

Mycoplasma species 2 carries the accession number "MK910041**".**

By testing the sequence result (MK910041.1) against the other sequences on Genbank, it showed 100% identity to the strains: MK615054.1, LC158833.1, NR_113690.1, HQ661809.1, AY121109.1, AY121108.1, AY121107.1, AY121106.1, AY121105.1, AY121104.1, AY121103.1, and AY12109.1, but showed 99.62 similarity with the strains: AY121100.1, MK789488.1, MK789480.1, MK789479.1, MK789478.1 and MK789477.1.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 100 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 19 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 263 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 **(Tamura *et al.*, 2013)** (Fig. 1).

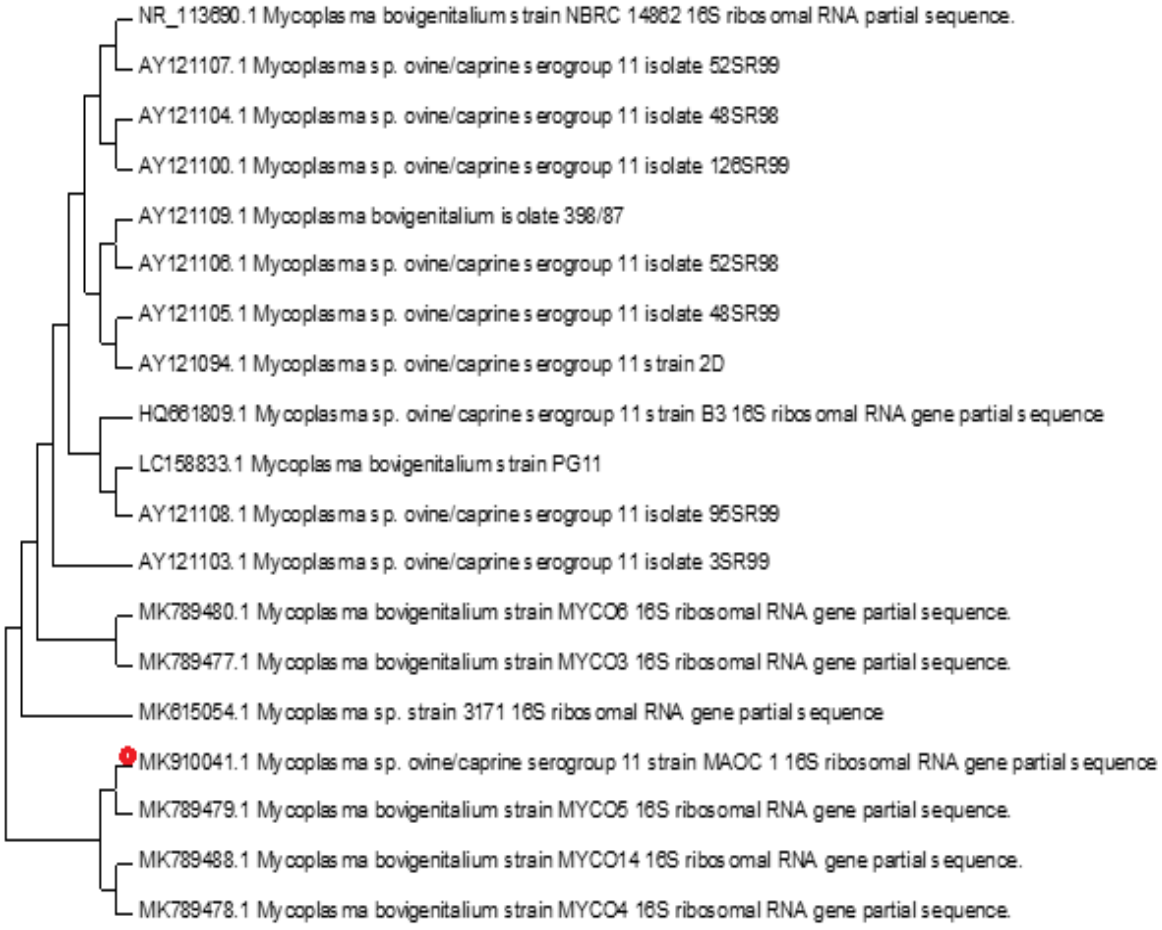

Fig.1: Evolutionary relationships of taxa

DISCUSSION

Mycoplasmas are highly fastidious, difficult to be cultured and slow-growing bacteria. Many species are veterinary pathogens causing respiratory infection, mastitis, conjunctivitis, arthritis, and occasionally abortion. The World Association for Animal Health **(OIE, 2017)** listed four important diseases of most livestock because of their socio-economic impacts. They comprise Contagious Bovine Pleuro-Pneumonia (CBPP), Contagious Agalactia, Contagious Caprine Pleuro-Pneumonia (CCPP) and Avian Mycoplasmosis. Others include enzootic pneumonia, costly worldwide disease of pigs, bovine mycoplasmosis, which is characterized by respiratory distress, mastitis and arthritis, and community-acquired atypical pneumonia in humans. New *Mycoplasma* disease outbreaks in desert tortoises and bighorn sheep have attracted the attention for the future of these endangered species **(Nicholas, 2017).**

For sheep and goats, mycoplasmal pneumonia has been associated with *M. ovipneumoniae, M. capricolum* subsp. *capricolum, M.*

mycoides subsp. *mycoides* LC and *M. arginini* **(Ikheloa *et al*., 2004).** *M. ovipneumoniae* is the most commonly isolated *Mycoplasma* from the upper respiratory tract of normal sheep and can be significant in respiratory disease in both sheep and goats **(Grand *et al*., 2004).** During stressful times, a subclinical infection may predispose sheep to atypical pneumonia with paroxysmal coughing **(Niang *et al*., 1999).** The infection is sometimes associated with *M. haemolytica, Parainfluenza-3* virus or *M. arginini*. In the present study, the isolation rate of *Mycoplasma* species was 3.25%, which agrees with that mentioned by **Maksimović *et al*. (2013).**

***Mycoplasma* isolation rate:**

was 3.4% of the examined samples. Also, this result was near to that recorded by **Kılıc *et al.* (2013)** who studied *Mycoplasma* in the lungs of sheep and lambs with pneumonia with an incidence of 8.33%**. Abdel Halium, *et al.*, (2019)** identified undifferentiated *Mycoplasma* species with an incidence of 41.6 % from total positive *Mycoplasma* isolates while it was 2.98% from the total collected samples.

Concerning the haemolytic activity of *Mycoplasma* species recovered from sheep and goat, 6 isolates (46.2%) were hemolytic and 7 isolates (53.8%) were non-haemolytic and that agrees with **Cole *et al*., (1968)** who tested the haemolytic activity of various animal and human *Mycoplasma* on erythrocytes from various sources (sheep- rabbit- chicken- guinea pig- duck). Also, these results agree with **Großhennig *et al.*, (2016)** who studied this activity in more detail; he incubated *M. pneumoniae* M129 on MP agar plates and overlaid the sheep blood agar with the growing bacteria.

The submitted sequence to the Genbank was identified as *M. ovine/caprine* serogroup 11. This is the first time for isolation of this bacterial species from the goat respiratory tract. But, it matches with that previously isolated from the lung of goats by **DaMassa *et al*. (1992),** known as *Mycoplasma* 2D that usually causes genital disorders in ruminants. Little information is known about the pathogenicity and epidemiology of this strain.

In recent literature, *M. ovine/caprine serogroup* 11 was classified as *M. bovigenitalium* **(Nicholas *et al*., 2008)** documented that the comparison of 16s and 23s rRNA sequences of 10 *M. ovine/caprine serogroup* 11 and 6 strains of *M. bovigenitalium* showed that they shared 98-100% similarity between all the tested strains but only 86-95% to other *Mycoplasma* species.

CONCLUSION

In conclusion, not only the identified *Mycoplasma* species but also the untyped ones can be isolated from apparently healthy as well as clinically diseased sheep and goats. Due to its hazardous role in infection, more studies about their pathogenicity and genetic characters are needed.

Declaration of Conflicting Interests

The authors revealed that there was no potential conflicts of interest.

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