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Research Article

Haemolysin, Catalase and Hydrogen Sulphide Production as Unique Phenotypic Virulence Determinants, Biofilm Formation and Defense against Antibiotics among *Mycoplasma arginini* and *Mycoplasma ovipneumoniae* Isolated from Pneumonic Lungs of Domestic Sheep (*Ovis aries*)

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Abstract | This research is an endeavor to extend the understanding of factors implicated in the pathogenicity of *Mycoplasma arginini* (*M. arginini*) and *Mycoplasma ovipneumoniae* (*M. ovipneumoniae*). It shed light on the current knowledge gap in the role of two potential virulence determinants *viz* H_2S production and catalase activity, for their possible roles in biofilm formation, antibiotic resistance and hemolytic activity of *M. ovipneumoniae*. The recovered sheep isolates of *M. arginini* and *M. ovipneumoniae* were examined bacteriologically and by PCR using *Mycoplasma* species-specific primers. MIC testing of the axenic isolates showed that 19 of them were 100% susceptible to tulathromycin, *streptomycin,* oxytetracycline and tylosin and highly resistant to danofloxacin, *l*incomycin and florfenicol. Some interactions demonstrated significance ($p < 0.05$), such as hemolysis/ tulathromycin (-0.607), tylosin/streptomycin (0.519), lincomycin/ florfenicol (0.808), lincomycin/ oxytetracycline (0.47) and oxytetracycline/H_2S (0.76). Our study distinctly elucidates that although H_2S production and hemolysis are not relevant (0.26) yet, H_2S and antibiotic resistance for oxytetracycline was relevant (0.76). On the other hand, the irrelevance between catalase and biofilm formation (-0,27) as summarized in our study brings to light quite clearly that the presence of catalase has a beneficial impact on biofilm formation. The *xer* virulence gene, the quinolone resistance-determining region (QRDR) genes *par*C, *par*E and *gyr*A and the macrolide and lincomycin resistance genes, *rpl*D and *rpl*V genes were undetected. The role of H_2S production in the pathogenesis of *M. arginini* and *M. ovipneumoniae* needs further in *vivo* studies.

Keywords | Antibiotic-resistance, Biofilm, H_2S, *Mycoplasma*, Sheep

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INTRODUCTION

Mycoplasma is one of the most pathogenic bacterial species recorded in different animal species such as sheep (Halium et al., 2019), camels (Abdelazeem et al., 2020), chickens (El-Ashram et al., 2021). Sheep can be infected with a variety of infectious microbes. Respiratory diseases are of the most important affections of sheep (Marcondes et al., 2011; Radostits et al., 2017) due to its impact on economic losses (Goodwin-Ray et al., 2008). This has been reported in many investigations in different geographic regions such as Jordan (Al-Momani et al., 2006), Brazil (Viana et al., 2007), Argentina (Suárez and Busetti, 2009), Ethiopia (Garedew et al., 2010), India (Mellau et al., 2010), and in Egypt (Osman et al., 2021)

M. ovipneumoniae was isolated from both sheep and goats andit has been recovered from the respiratory passages of both healthy and diseased sheep worldwide. Lamb pneumonia was also reported in Universidad Federal do Mato Grosso do Sul (Almeida et al., 2013) and in 1974 in New Zealand (APHIS, 2015). Recently, McRae et al. (2016) reported pneumonic lesions in lambs at slaughter in New Zealand, and Swedish flocks (Tauni, 2017).

M. ovipneumoniae is not normally highly pathogenic, but it makes sheep highly susceptible to other respiratory infections (Besser et al., 2012) by lymphocyte quelling or interference with normal ciliary activity in the respiratory tract (APHIS, 2015). The control of these microbes is encountering some difficulties of drug resistance. There is a great need for potent mycoplasma vaccines both in the veterinary and medical fields (Gautier-Bouchardon, 2018; Kirby, 2018).

Pathogenic bacteria have multiple mechanisms which guarantee their survival and spread to consequently cause diseases in their hosts. Since infectious diseases represent a major cause of death worldwide, identifying and understanding their pathogenicity mechanisms is a key step for successful combating. Despite the existence of plenty of many pathogenic microbes' infection strategies have turned out to be remarkably similar. However, diseases caused by mycoplasmas continue to be a serious problem for human health as well as livestock production with significant socio-economic divergence worldwide (Deeney et al., 2021).

Effective control measures of mycoplasmas are wanting, mainly due to shortage of knowledge about their pathogenicity mechanisms. They lack typical pathogenicity features found in other bacteria, and despite the genomes of many important mycoplasma pathogens having been sequenced, questions related to their virulence and survival remain (Citti and Blanchard, 2013).

Hemolysis is an important aspect of bacterial pathogenicity which can have severe effects in both the human and animal hosts (Orf and Cunnington, 2015). Since H_2S has been proven to play a role in hemolytic activity of other pathogenic bacteria (Großhennig et al., 2016), *M. arginini* and *M. ovipneumoniae* were also to be tested for production of additional hemolytic compounds, like hydrogen sulfide. Therefore, previous findings and reports of several researchers concerning the incorporation of certain *Mycoplasma* species with pneumonia in sheep and their possible hazardous influence on public health are to be considered (Watanabe et al., 2012) and wildlife conservation (Sumithra et al., 2013; Highland et al., 2018; Kamath et al., 2019; Manlove et al., 2019; Wolff et al., 2019). Hence, this research is an endeavor to evaluate two potential virulence determinants *viz* H_2S production and catalase activity, for their possible roles in virulence, biofilm formation, resistance to antibiotics and hemolytic activity of *M. arginine* and *M. ovipneumoniae*, an unexplored issue until now.

MATERIALS AND METHODS

Ethical issue: The Department of Microbiology, Faculty of Science, Cairo University approved all animal experiments (CU-04-20), which adopted all national standards for the use of animals in scientific testing, and the basic procedure defined by the OIE (2020 Edition).

Samples collection and processing

The samples were collected from domestic sheep (*Ovis aries*) at Cairo slaughterhouse during 2018 and transported while cold to the Animal Health Research Institute (AHRI), Dokki, Egypt as quick as possible. Nasal swabs were collected from live, apparently healthy animals (n=50); in addition to samples from pneumonic (n=200). Mycoplasma isolation, detection, characterization, DNA extraction and molecular typing were carried out in AHRI, Department of Mycoplasmology, following the standard mycoplasma isolation and identification procedures (Nicholas and Baker, 1998) including PCR assays and 16S rRNA gene sequencing. Culture-enriched nasal swab eluates for mycoplasma culturing were inoculated into Friis' medium (McAuliffe et al., 2003) and incubated at 37°C with a daily follow up for color change. Several PCR techniques were used to define the conformity of the culture isolates. As a primary step, conventional PCR for *Mycoplasma* species isolates were processed (Parker et al., 2017), then followed by an *Acholeplasma laidlawii* conventional PCR technique (Supplementary Table S1). To confirm the *M. ovipneumoniae* and *M. arginini* culture purity, 16S ribosomal (rRNA) genes were amplified using

specific primers, as per previous reports (Supplementary Table S1) using previously described PCR assays (Supplementary Table S1) applied to the extracted DNA from broth culture media. After assuring that the isolates were mycoplasmas, the isolates were identified to the species level. The presence of *M. ovipneumoniae* and *M. arginini* were detected by previously described PCR methods (Supplementary Table S1) applied to the DNA extracted from broth culture media. PCR-positive *M. ovipneumoniae* and *M. arginini* extracts were genotyped by targeting genetic loci of the partial DNA sequences from the small ribosomal subunit (16S). Protocols and primers for PCR amplification of these loci was described previously (Supplementary Table S1). DNA sequencing of amplified PCR products was conducted by bidirectional Sanger sequencing. After that, each aligned sequence of the independent locus in MUSCLE72 were done, following the default parameter settings of Geneious R10.1.3 (http://geneious.com, Biomatters, Ltd.).

Phenotypic virulence traits

After species identification, each isolate was subjected to phenotypic virulence traits assays and antimicrobial susceptibility testing.

Hydrogen sulfide assays with lead acetate test strips

M. ovipneumoniae and *M. arginini* isolates were incubated in 1 ml 1x PBS, pH 7.4 or in MP-medium supplemented with different cysteine concentrations in 2 ml Eppendorf reaction tubes. Lead acetate test strips (Aldrich) were fixed under the lid of the tube (without contact to the liquid) to catch the produced hydrogen sulfide. The black PbS formed by the reaction between the lead covering the strips and the sulfide, results in dark staining of the test paper. After an overnight incubation at 37°C, the coloration of the test strips was examined and photographed (Großhennig et al., 2016).

Catalase enzyme activity

The procedure of Pritchard et al. (2014) was implemented. Mid-log phase cultures of each *M. ovipneumoniae* and *M. arginini* isolate were collected, centrifuged at 20,000×g for 20 minutes then washed 3 times with cold phosphate-buffered saline (PBS). Cells were spread onto a clean microscope slide then one drop of 3% H_2O_2 was added. Catalase activity was indicated by the production of bubbles.

Assays for detection of hemolytic activity

To analyze hemolytic activity of *M. ovipneumoniae* and *M. arginini*, the colonies were overlayed onto 5% sheep blood agar, after that the plates were incubated for 1-2 days at 37°C (Großhennig et al., 2016).

For analyzing the hemoxidative or hemolytic activities of the isolates of *M. arginini* and *M. ovipneumoniae*, the cultures were incubated with 5% defibrinated sheep blood or 2% washed sheep RBCs in PBS (pH 7.4) and various supplements (1 mM glycerol, 1 mM glycerol-3-phosphate and 1 or 10 mM L-cysteine) in a total volume of 1 ml followed by sample incubation at 37°C for several hours (100-110 rpm). After incubation, the tubes were centrifuged (1,400 x g at 4°C), the supernatant was transferred carefully into a new tube. The pellets were re-suspended and lysed in 1 ml dH_2O. The spectra of both supernatant and pellets were recorded photometrically from 370 to 700 nm (Großhennig et al., 2016).

Analysis of biofilm production by crystal violet staining

Biofilm production was quantified by scoring the absorbance (560 nm) of 100 ml of the solubilized crystal violet in a microtiter plate (McAuliffe et al., 2006).

Antimicrobial susceptibility testing

The broth microdilution method was used for determination of MIC of *M. ovipneumoniae* and *M. arginini* isolates recovered from the nasal and lung samples. The Broth microdilution method following the procedure of Hannan (2000) was implemented taking into consideration that the suggested breakpoint interpretations provided herein were derived from three sources: Clinical and Laboratory Standards Institute (CLSI) criteria for veterinary pathogenic bacteria in cattle (CLSI, 2002), the MIC breakpoints of aminoglycosides according to (Jelinski et al., 2020), preceding publications and the MIC data generated in the current study. The *M. ovipneumoniae* (n=5) and *M. arginini* (n=14) isolates were examined for their sensitivity to seven antimicrobial agents by the disc diffusion method. The antimicrobial agents utilized in this study included: fluoroquinolone: danofloxacin (5 ug); macrolides: tulathromycin (30 µg) and tylosin (30 ug); phenicol: florfenicol (30 ug); aminoglycoside: streptomycin (10 ug); lincosamide: lincomycin (2 ug); tetracycline: oxytetracycline (30 ug). The type strain *M. bovis* ATCC 25523/ PG45 was used as quality control.

Detection of the virulence, QRDR, Macrolide and Lincomycin resistance genes

DNA was extracted by using the simple boiling procedure (100°C for 5 min) to liberate the DNA. The supernatant was used as PCR templates to detect the presence of *gyr*B, *Par*C, *Par*E, *rpl*D, *rpl*V and *xer* genes. Primers and PCR conditions for these genes were previously declared and summarized in Supplementary Table S1.

Nucleotide sequencing and sequence analysis

PCR products were purified using the Gene Jet PCR

purification kit; Fermentas (Thermo Fisher Scientific). The purified amplicon of each isolate was sequenced in both forward and reverse directions using the amplification primers (Supplementary Table S1). Amplicons were sequenced in an automated sequencer (Macrogen Company 24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea). Sequence data similarity searches were analyzed by using NCBI-BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). The comparisons of the obtained nucleotide sequences and associated multiple alignments were performed using the BioEdit sequence alignment editor (CLUSTALX software version 7.0.9.0) (6/27/07) for multiple sequence alignment.

Molecular phylogenetic analysis by Maximum Likelihood method

Based on Hasegawa et al. (1985), the evolutionary history was deduced by using the Maximum Likelihood method for revealing the tree. Next to the branches, the trees' percentage with clustered associated taxa was shown. The Neighbour-Joining method was used to obtain the guiding Initial trees. The tree was drawn and the analysis involved 22 nucleotide sequences. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. In the final dataset, there were a total of 2765 positions. The analyses of evolution was done using MEGA6 (Tamura et al., 2013). Sequences (Supplementary Figure S1) were then submitted to NCBI GenBank using BankIt (http://www.ncbi.nlm.nih.gov/WebSub/?tool=genbank) under the accession numbers of *Mycoplasma arginini*: (MK640677.1*, MK640679.1*, MK643127.1*, MK774823*).

The online pipeline NGpyologeny.fr was used for constructing the 16S phylogenetic tree (Figures 3 and 4) (Lemoine et al., 2019). Within this pipeline, sequences were aligned using MUSCLE, ambiguous aligned regions were removed with GBLOCK, and a phylogenetic tree was constructed with PhyML+SMS using GTR model and bootstrap 100 times (Edgar, 2004; Talavera and Castresana, 2007; Lefort et al., 2017). The heatmap Phylogenetic tree was visualized and annotated with iTOL (Letunic and Bork, 2016).

Statistical analysis

As a first step, the data were treated using descriptive statistics. After that, the heatmap graphs confirm the analyses performed by principal component analysis (PCA) as well as by the analysis of variance (ANOVA), using the outcome variables as factors (e.g., Virulence traits and antibiotics) for each tissue of interest (e.g., lung and nasal swab). In addition, Pearson's correlation was verified for all variables. The results were analyzed using Microsoft Excel and GraphPad Prism 8.

RESULTS AND DISCUSSION

Prevalence of *Mycoplasma arginini* and *Mycoplasma ovipneumoniae* in samples recovered from nasal swabs and lungs of sheep

Out of 50 nasal swabs of apparently healthy sheep and 200 samples of pneumonic lungs, 19 isolates were confirmed to be positive for putative mycoplasmas identified to the species level as five *Mycoplasma ovipneumoniae* (5/19) isolated from the pneumonic lungs and 14 *M. arginini* isolated from the nasal swabs (5/14) and the pneumonic lungs of the sheep (9/14) (Supplementary Table S2).

Phenotypic virulence traits of *Mycoplasma arginini* and *Mycoplasma ovipneumoniae*

The four phenotypic virulence traits are recorded in Supplementary Table S2. Regarding the virulence traits, Figure 1 demonstrates the effect of *M. arginini* (A) and *M. ovipneumoniae* (C) on the lungs and *M. arginini* for the nasal swab (B). Be that as it may, no significant differences were recognized ($p > 0.05$).

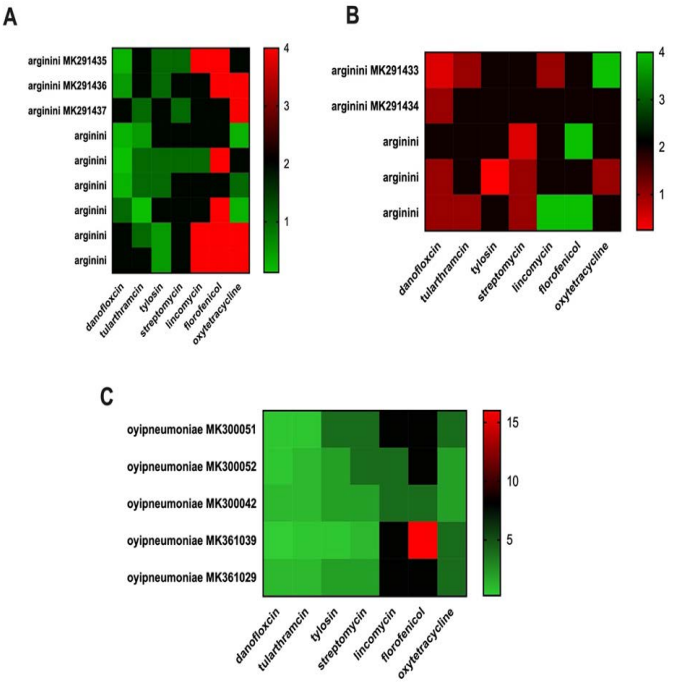

Figure 1: Clustering heatmap of virulence traits phenotypes by lungs and nasal swab.

Hydrogen sulphide production

The recorded hemolytic activities of the *M. arginini* and *M. ovipneumoniae* isolates in the presence of cysteine suggested that the bacteria produce H_2S from cysteine which in turn causes hemolysis. Yet, 8/14 of the *M. arginini* isolates (4/5, from the nasal swabs and 4/9, from the pneumonic lungs) were unable to produce H_2S. In addition, 3/5 *Mycoplasma ovipneumoniae* isolated from pneumonic sheep lungs were also unable to produce H_2S.

Detection of hemolytic activities

After two days of incubation on blood agar it was revealed that 14 isolates (10 *Mycoplasma arginini* and 4 *Mycoplasma ovipneumoniae*) exhibited clear zones of α-hemolysis around their colonies. The effect of *M. arginini* and *M. ovipneumoniae* on RBCs was also examined in a liquid environment.

Catalase activity

Five out of nine *M. arginini* isolated from pneumonic sheep lungs were impuissant to indicate any catalase activity. Two out of the five *M. arginini* isolated from nasal swabs of apparently healthy sheep were unable to indicate any catalase activity. The catalase activity was not obvious in 3/5 of the *M. ovipneumoniae* that were isolated from the pneumonic lungs of sheep.

Biofilm formation capability

The ability of *M. arginini* strains adherence to polystyrene multiwall plates were found to be weak. A very low spectrophotometric values (A560 (0.187-0.292)) were recorded for the isolates from the nasal swabs of apparently healthy sheep indicating poor adhesion and biofilm formation abilities. On the other hand, two isolates had a relatively moderate ability for biofilm formation ((A560 (1.099-1.153)) while the remaining seven isolates isolated from pneumonic lungs, were in their normal range of weak biofilm formation (A560, 0.216-0.564). The five *M. ovipneumoniae* isolated from the pneumonic lungs also exhibited a weak tendency for biofilm formation (A560, (0.347-0.400)).

Antimicrobial resistance among the *Mycoplasma arginini* and *Mycoplasma ovipneumoniae* isolates

The susceptibility of 19 field isolates of *M. ovipneumoniae* and *M. arginini* isolated from sheep, to seven different antimicrobial agents representing six antimicrobial groups is displayed in Supplementary Table S2. MIC testing of the seven antimicrobials showed that the 19 mycoplasmas were 100% susceptible to tulathromycin, streptomycin, oxytetracycline and tylosin and highly resistant to danofloxacin, lincomycin and florfenicol. Moreover, the antimicrobial resistance combinations of *M. arginini* and *M. ovipneumoniae* to various antibiotics displayed six different combinations of antibiotics representing three classes, with the lincosamide (lincomycin) being the most obviously seen in four out of the six combinations (Table 1). Danofloxacin, lincomycin, florfenicol combination was the most recorded in ten out of fourteen isolates; furthermore, the multidrug-resistant (MDR), and extensively drug-resistant (XDR) isolates were apparent in three (21.4%) and five (35.7%) isolates, respectively.

In this sense, Figure 2A demonstrates the role of different antibiotics on *M. arginini* in the lungs, which showed other actions among the tested antibiotics [F (6, 48) = 8,409, p <0.0001]. About the effects on the nasal swabs, no significant differences were identified (Figure 2B, p = 0.06). When *M. ovipneumoniae* was verified in the lungs (Figure 2C), differences between the studied antibiotics were demonstrated [F (6, 48) = 11.48, p <0.0001].

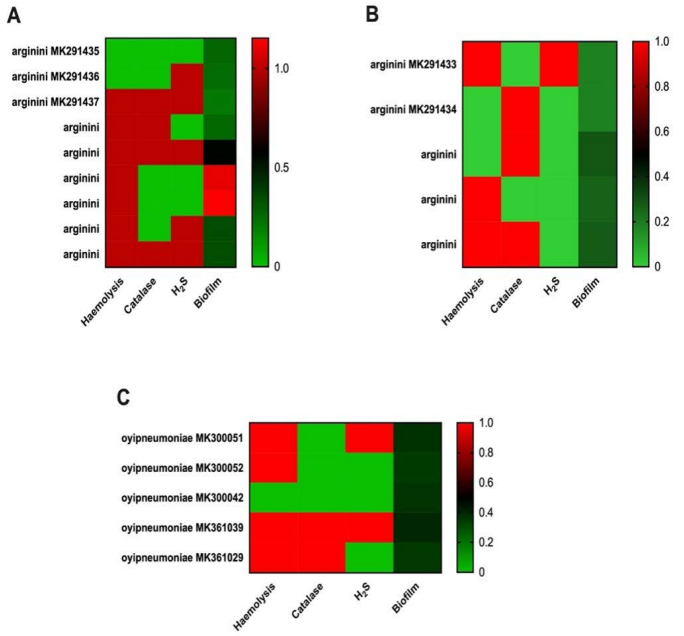

Figure 2: Clustering heatmap of antimicrobial resistance by lungs and nasal swab.

Table 1: The antimicrobial resistance combinations of *Mycoplasma arginini* and *Mycoplasma ovipneumoniae* to various antibiotics.

Antibiotics	n= resistant antibiotics	n= antibiotic classes	n= of isolates	Extensive drug resistance (XDR)	Multiple drug resistance (MDR)	Pan drug resistance (PDR)
Danofloxacin	1	1	1	0	0	0
Lincomycin	1	1	2	0	0	0
Florfenicol	1	1	1	0	0	0
Danofloxacin, lincomycin	2	2	3	3	0	0
Lincomycin, florfenicol	2	3	2	2	0	0
Danofloxacin, lincomycin, florfenicol	3	3	10	0	3	0

Table 2: Pearson's correlations matrix from PCA analysis from nasal swabs and lung isolates.

	Tulathromycin	Tylosin	Streptomycin	Lincomycin	Florfenicol	Oxytetracycline	Hemolysis	Catalase	H_2S	Biofilm
Danofloxacin	0.288	-0.077	-0.223	-0.069	-0.243	0.281	-0.045	0.248	0.110	-0,204
	Tulathromycin	-0.398	-0.323	-0.292	-0.352	0.092	-0.607*	0.050	-0.094	-0.454
		Tylosin	0.519*	0.225	0.013	0.014	-0.024	-0.011	-0.126	-0.058
			Streptomycin	0.320	0.179	0.127	0.208	-0.447	0.056	0.088
				Lincomycin	0.808*	0.470*	0.182	0.083	0.107	-0.109
					Florfenicol	0.389	0.186	0.137	0.224	-0.005
						Oxytetracycline	0.068	0.100	0.760*	-0.455
							Hemolysis	0.088	0.268	0.281
								Catalase	0.045	-0.271
									H_2S	-0.203

Distribution of the virulence, QRDR and macrolide and lincomycin resistance genes

Again, to our surprise, we were unable to detect any of the investigated genes, the *xer* virulence gene, the QRDR genes *par*C, *par*E and *gyr*A and the Macrolide and Lincomycin resistance genes, *rplD* and *rplV* genes by PCR in any of the 19 *Mycoplasma arginini* and *Mycoplasma ovipneumoniae* isolates.

When performing the PCA analyses, Pearson's correlation matrix was generated (Table 2). Thus, it is observed that some interactions demonstrated significance ($p < 0.05$), such as Hemolysis/ Tulathromycin (-0.607), tylosin/ streptomycin (0.519), lincomycin/ florfenicol (0.808), lincomycin/ oxytetracycline (0.47) and oxytetracycline/ H_2S (0.76).

Phylogenetic analysis of 16S rRNA of *M. arginini* isolates from sheep, goat, cattle, camel and human in Egypt with *M. arginini* isolates recovered from different countries

M. arginini cattle isolate (MF101758.1*): show no 100% identity to any other sequence but show high similarity (98%) to: MK789480.1 (sheep *M. bovigenitalium* from Turkey), LC158833.1 (Bovine *M. bovigenitalium* from Japan) and HQ661819.1 (sheep *M. bovigenitalium* from South Africa).

M. arginini sheep isolates (MK291433.1*, MK291434.1*, MK291435.1*, MK291436.1*, MK291437.1*) show 100% identity to each other' and to goat *M. arginini*, Egypt sequences (MK774823.1*, MK643127.1*, MK640679.1*), sheep *M. arginini* from Turkey), (MK789487.1,) and HQ661822.1 (sheep *M. arginini* from South African) and show high similarity 99.66% to Camel *M. arginini* from Egypt (MK271638.1*, MK271640.1*, MK271641.1*), human *M. arginini* from Hungary (HM179556.1), and Feline, *M. arginini*, USA (U15794.1).

All goat *M. arginini* isolates with accession numbers (MK640677.1*, MK640679.1*, MK643127.1*, MK774823*) show 100% identity to each other's and to sheep *M. arginini* isolates from Turkey (MK789491.1 and MK789487.1) but show 99.66% similarity to: Camel *M. arginini*, Egypt (MK271638.1*, MK271640.1*, MK271641.1*), human *M. arginini* from Hungary (HM179556.1), and Feline, *M. arginini*, USA (U15794.1).

The human *M. arginini* from Hungary (HM179556.1) show 100% identity to *M. arginini* isolated from tick infesting *Camelus dromedarius*, India (MG564230.1, MG564231.1, MG564232.1, MG564233.1) but show similarity with sheep *M. arginini* from Turkey (MK789485.1); 99.71%, (MK789486.1); 99.70%, and 99.66% to HQ661819.1 (sheep *M. arginini*, South Africa), 99.58% to U15794.1 (Feline, *M. arginini*, USA), 99.52% to HQ661828.1 (sheep *M. arginini*, South Africa), 99.33% to MK271641.1 (camel *M. arginini*, Egypt), 99.30% to (KP742976.1) *M. arginini* of Dromedary Camel from Sudan and 98.31% to camel, *M. arginini*, Egypt (JQ859817.1 1). The human *M. arginini* from Hungary (HM179555.1) show no 100% identity to any other published sequence but show similarity to goat *M. arginini* sequences: 97.85% (KP685373.1), and 97.71% to (KP685376.1, KP685375.1 and KP685374.1). Camel *M. arginini* isolates (MK271638.1*, MK271640.1*, MK271641.1*) show 100% identity to each other and show high similarity 99.66% to sheep *M. arginini*, turkey (MK789491.1, MK789487.1, sheep, *M. arginini*, Egypt (MK291434.1*, MK291435.1*, MK291436.1*, MK271639.1*), camel *M. arginini*, Egypt (MK640677.1*), goat *M. arginini*, Egypt, and sheep *M. arginini*, South Africa (HQ661825.1 and HQ661826.1). Camel *M. arginini* (M0K271639.1*) show no 100% identity with any other published sequences but show 99.66% similarity to: MK271640.1*, MK271641.1*, 99.32% to: sheep *M. arginini*, turkey (MK789491.1, MK789487.1), sheep *M. arginini*, Egypt (MK291433.1*, MK291434.1*, MK291435.1*, MK291436.1*), and goat *M. arginini*,

Egypt (MK640677.1*, MK640679.1*) (Figures 3 and 4). N.B. *(these sequences are from our preceding studies).

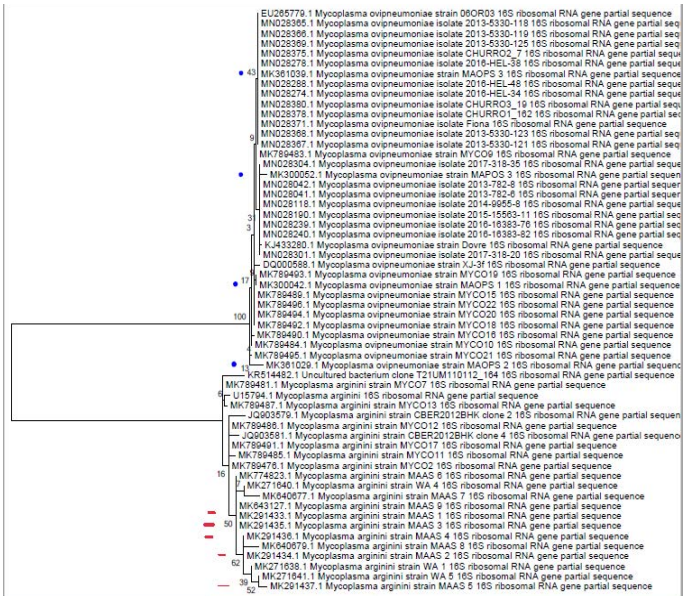

Figure 3: Maximum Likelihood phylogenetic tree of 16S ribosomal RNA gene, partial sequence of sheep *Mycoplasma arginini* (five isolates) and *M. ovipneumoniae* (four isolates) in comparison with other representative strains of *M. arginini* and *M. ovipneumoniae* available on NCBI.

Figure 4: Maximum Likelihood phylogenetic tree of 16S ribosomal RNA gene, partial sequence of goat *M. arginini* (four strains) and sheep *M. arginini* (five strains), Cattle *M. arginini* (one strain), Camel *M. arginini* (four strains) in comparison with human *M. arginini* (two strains) on Genbank, and other representative strains of *M. arginini* available on NCBI. Camel —, Sheep ●, Goat □, Human — and cattle □ isolates.

This research aimed to look into and evaluate three potential virulence determinants: *viz* hemolysis, H2S production and catalase activity for their possible roles in virulence, biofilm formation, antibiotic resistance, and hemolytic activity of *M. arginini* and *M. ovipneumoniae*, an issue unexplored until now. *M. ovipneumoniae* is the most commonly isolated mycoplasma from the sheep respiratory tract and is associated with atypical pneumonia affecting sheep in their first year of life. Its first recorded isolation was in New Zealand in 1974, and from this time, it has been recovered from the respiratory tracts of healthy (Alley et al., 1999) and symptomatic sheep worldwide (APHIS, 2015). Its association with *M. arginini* has been often reported in sheep (Güler, 1993; Niang et al., 1998a; Ayling and Nicholas, 2007; Sheehan et al., 2007; APHIS, 2015) leading to paroxysmal coughing, rectal prolapse and reduced weight gain in lambs. The temperature, velocity of wind, clouds, precipitation, and explosion of volcanoes usually incorporate in the aerographic conditions._The prevalent climatic conditions have a considerable influence on the pathogens survival (Rahal et al., 2014). An alteration in weather conditions of a geographical area has always witnessed an outburst of infectious diseases and has been labeled as predisposed to disease epidemics. Small ruminants are well adapted to extreme temperatures, with their body hair coats providing insulation against cold and heat (Rahal et al., 2014). Any modulation in the environmental temperature influence the incubation period, the life cycle (the time between infection and sporulation), and the contagious period (the time during which the pathogen continues to propagate the disease). At higher temperatures, the pathogen's life cycle usually gets hastened consequently, epidemics develop at a faster rate. In colder conditions, the pathogens produce dormancy, and the progress of the pandemic is slower, leading to a decline in the disease incidence and seriousness. During times of stress or hot weather, the subclinical infection may predispose sheep to acute fibrinous pneumonia, pulmonary abscessation, or pleurisy leading some investigators to suggest that *M. ovipneumoniae* is actually a commensal of sheep (Alley et al., 1999).

Transmission occurs by means of aerosol spread and close contact with infected animals. The nasal cavity of adult animal is considered the predilection seat of *M. ovipneumoniae*, from where it spreads to lambs, causing transitory, mild respiratory affections (APHIS, 2015). Stressors such as shipping, weather, unbalanced rations, sub-clinically infected sheep may suffer from acute pneumonia (Nicholas et al., 2008). Solo, it isn't usually highly pathogenic, but make sheep highly susceptible to other respiratory microbes by interfering with regular respiratory tract ciliary activity and by quelling lymphocytes (Niang et al., 1998b; Nicholas et

al., 2008; Shahzad et al., 2010). It is documented that *M. arginini* isn't the main causative agent of pneumonia but the condition can be worsened in the presence of other microorganisms (Nicholas et al., 2008). Mycoplasma isolation rate in our study (37 %) is close to the 36 % reported by Güler (1993). In the present study, most of the isolates were identified as *M. ovipneumoniae,* and that agrees with Sheehan et al. (2007). These results elucidate that the former is more persistent than the latter in our geographic area. H_2S increases the bacterial resistance to oxidative stress and antibiotics by a dual mechanism of suppressing the DNA-damaging Fenton reaction via Fe_2+ sequestration and animating the major antioxidant enzymes catalase and SOD. This cytoprotective mechanism of H_2S parallels that of NO (Gusarov et al., 2009), suggesting that bacteria capable to produce both gases may benefit from their synergistic action.

In agreement with Gusarov et al. (2009), these results demonstrate the dependent, and specific antagonistic effects of NO and H_2S against antibiotics. Impressively, in contrary to bNOS, which is present in only a few Gram-positive species, H_2S enzymes are essentially universal. Because endogenous H_2S diminishes the effectiveness of many clinically used antibiotics, the inhibition of this gas keeper should be considered as an increment therapy against many pathogens.

The same as nitric oxide, H_2S, formerly known to be a toxic gas, is now considered as a signaling molecule related to constructive functions in mammals from vaso-relaxation, cardio-protection, and neurotransmission to anti-inflammatory action in the gastrointestinal tract (Gadalla and Snyder, 2010; Kimura, 2010; Wang, 2010). Three H_2S generating enzymes have been recognized in mammals: Cystathionine b-synthase (CBS), cystathionine g-lyase (CSE) and 3-mercaptopyruvate sulfur-transferase (3MST). CBS and CSE produce H_2S predominantly from L-cysteine (Cys). 3MST does so via the intermediate synthesis of 3-mercaptopyruvate produced by cysteine aminotransferase (CAT), which is inhibited by aspartate (Asp) competition for Cys on CAT. The bacterial combating for iron is often accompanied by hemolysis. This is a convenient effect since the bacteria gain access to many nutrients that are released from the lysed RBCs.

Most importantly, they gain access to iron-bound inside the RBCs hemoglobin molecules. *In vivo*, oxidized hemoglobin has a lowered binding affinity towards oxygen and also the release of oxygen is hindered. However, this process is reversible. Like oxidation, hemoglobin can also be sulfonated, resulting in so-called sulfhemoglobin (sulfHb), which appears as a greenish-brownish discoloration of blood (Chatfield and La Mar, 1992). As a result, hemoglobin loses its ability to bind oxygen in a non-reversible manner. Both, the formation of sulfHb and metHb are forms of alpha hemolysis. Secretion of H_2O_2 or H_2S is the prime cause for bacterial oxidation or sulfenylation of hemoglobin. Alpha-hemolysis following hydrogen peroxide production is used for typing of bacterial species and is typically seen in *S. pneumoniae* and *S. mutans* (Duane et al., 1993). Hemoglobin alteration and hemolysis as result of hydrogen sulfide production has been studied in several oral pathogens. Among them, the cystalysin of *Treponema denticola*, *E. coli* and its hemoxidative and hemolytic activity have been elaborately studied (Chu et al., 1995, 1997). The production of hydrogen sulfide is a prevalent feature of oral pathogenic bacteria and responsible for periodontal diseases and oral malodor (Song et al., 2021). The genera *Fusobacterium*, *Prevotella* and *Porphyromonas* are amongst the predominant H_2S producers (Basic et al., 2017). Hemolytic activity associated with the production of H_2S has not only been reported in *T. denticola*, but also for *Fusobacterium nucleatum*, *Streptococcus anginosus*, *Streptococcus intermedius*, *Prevotella intermedia*, *M. pneumonia*, *Mycoplasma arginini* and *M. bovis* (Großhennig et al., 2016; Abdelazeem et al., 2020). Shatalin et al. (2011) announced that putative cystathionine b-synthase, cystathionine g-lyase, or 3-mercaptopyruvate sulfur transferase suppression in *B. anthracis*, *P. aeruginosa*, *S. aureus* and *E. coli* repress H_2S production, requisition these pathogens highly sensitive to a multitude of antibiotics. Exogenous H_2S restrains this effect. The resistance mechanism of gas-mediated antibiotic count on alleviation of oxidative stress enjoined by antibiotics. Our study provides prudence into the mechanism responsible for *M. arginini* and *M. ovipneumoniae* host cell cytotoxicity, distinctly elucidating that although H_2S production and hemolysis are not relevant (0.26) yet, H_2S and antibiotic resistance for oxytetracycline was relevant (0.76). On the other hand, the irrelevance between catalase and biofilm formation (-0,27) as summarized in our study bring to light quite clearly that the presence of catalase has a beneficial impact on biofilm formation (Simmons and Dybvig, 2015).

For centuries and in contrast to mammal-derived H_2S, bacteria derived H_2S has been considered to be only a byproduct of sulfur metabolism, without specific physiological function in non-sulfur microorganisms. It has been concerned with the role played by these enzymes (Gusarov et al., 2009). Considering some functional correspondence between mammalian gasotransmitters, (Gadalla and Snyder, 2010; Kimura, 2010; Wang, 2010; Shatalin et al., 2011) supposed that bacterial H_2S may, be cytoprotective. Furthermore, not too much is known about the metabolic pathways involving H_2S in mesophilic bacteria (Shatalin et al., 2011). The virulence role played by hydrogen peroxide production in pathogenic

Mycoplasma species is still debatable (Szczepanek et al., 2014; Schumacher et al., 2019), *M. arginini* and *M. ovipneumoniae* could also be under the same conclusion and the role of H_2S production in their pathogenesis needs more *in vivo* studies.

Some *Mycoplasma* species such as *M. genitalium* and *M. pneumoniae* lack *xer*-like site-specific recombinase genes, supposedly due to reductive evolution of their minimum genomes (Himmelreich et al., 1997) and thus has no need to Xer-like recombinase for resolving their dimer chromosomes during cell division. Recchia and Sherratt (1999); Glew et al. (2002); Chopra-Dewasthaly et al. (2017) supposed that Vpma antigenic divergence is fundamental for survival and continuation inside the immunocompetent host. Despite that Xer1 is substantial for causing Vpma variation in vitro, it is not considered as a virulence factor as alternative Xer1-independent mechanisms operate in vivo, probably under the selection pressure of the immune response induced by the host. This study highlights new aspects of mycoplasma antigenic variation systems, including the expression mechanisms regulated by host factors (Chopra-Dewasthaly et al., 2017). The data introduced by Chopra-Dewasthaly et al. (2017) summarized that in spite of not being a virulence factor of *M. agalactiae*, Vpma phase variation of Xer1 recombinase might critically have impact on its surveillance during natural infections, a situation that could explain the absence of the *xer* gene in our *M. arginini* and *M. ovipneumoniae* isolates.

CONCLUSIONS AND RECOMMENDATIONS

The scenario of globalization and regulations concerned with international trades, continuous monitoring of enlisted diseases is obligatory. Consequently, a standard protocol for sampling, isolation, and identification processes is a must. Effective control of the respiratory diseases of sheep, through accurate diagnosis, and detailed genetic analysis of the causative agents is needed. Detection of mycoplasma's DNA originated from recovered microbes of the nasal cavities of sheep marks carriage and possibly shedding, but may be without an active infection. Antibiotic treatment may help tentatively, but response likely varies depending on the strain or/and species of mycoplasma involved. More in *vivo* studies should be done for determination of the role of H_2S production in the pathogenesis of *M. arginini* and *M. ovipneumoniae*. These potent virulence factors could not be used in drug therapy and vaccine design because H_2S produced by *M. arginini* was host associative.

NOVELTY STATEMENT

Our study distinctly elucidates that; although H_2S production and hemolysis are not relevant yet, H_2S and antibiotic resistance for oxytetracycline was relevant. On the other hand, the irrelevance between catalase and biofilm formation clears that the presence of catalase has a beneficial impact on biofilm formation. The interactions between haemolysis/tulathromycin demonstrated significance: between oxytetracycline/H2S; tylosin/streptomycin; lincomycin/florfenicol; and between lincomycin/oxytetracycline. The *xer* virulence gene, the QRDR genes *par*C, *par*E and *gyr*A and the Macrolide and Lincomycin resistance genes; *rplD* and *rplV* genes; were undetected. As a result, Although Haemolysin, catalase and hydrogen sulphide production represent a unique potential phenotypic virulence determinants, yet, they couldn't be used as candidates for drug therapy and vaccine design, as H_2S production by *M. arginini* is host dependent.

AUTHOR'S CONTRIBUTION

MO data curation, Investigation. KMO supervision, data curation, writing original draft, review and editing. MH supervision, data curation, writing original draft, review and editing. MAEM Supervision. JAA data analysis, data interpretation. AS data curation. OLF data analysis, data interpretation. HND data curation, investigation and review final version.

Conflict of interest

The authors have declared no conflict of interest.

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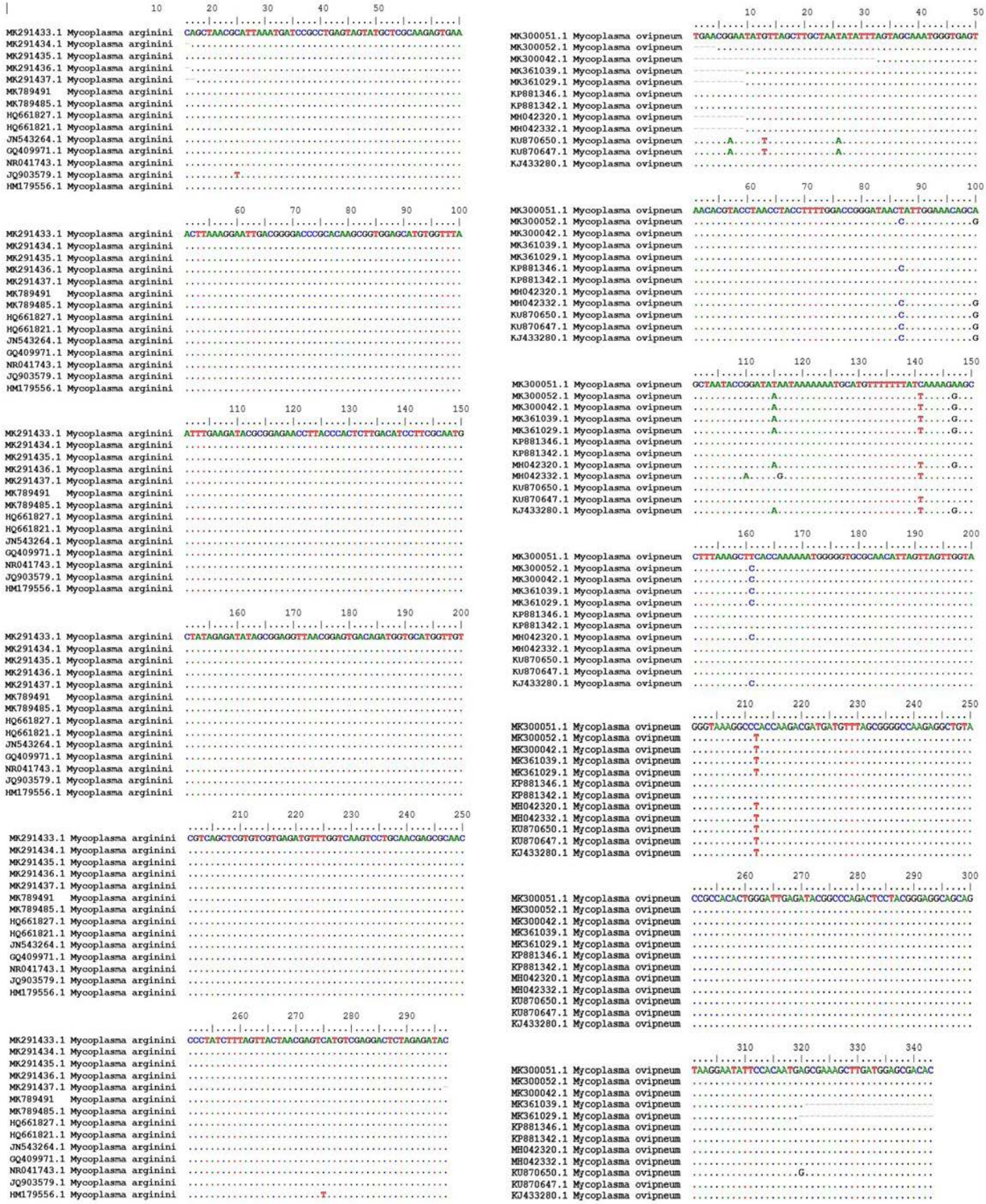
Supplementary Table S1: Oligonucleotide primers used for detection of *Mycoplasma* species, virulence genes, and Quinolones, Macrolide and Lincosamide resistance (QRDR) genes.

References	PCR conditions	Amplicon size	Sequence	Target gene
van Kuppeveld *et al.* (1992)	94°C 5min. (94°C 45s, 55°C 60 sec, 72°C 1 min) x 40, 72°C 10 min	275bp	GPO3F (5'-TGGGGAGCAAACAGGATTAGATACC-3') MGSO 5'-TGCACCATCTGTCACTCTGTTAACCTC-3')	*Mycoplasma* group specific
Vahid *et al.* (2009)	94°C 3min. (94°C 45s, 60°C 60 sec, 72°C 1 min) x 32, 72°C 10 min	326bp	(5'-TGATCATTAGTCGGTGGAGAGTTC-3') (5'-TATCTCTAGAGTCCTCGACATGACTC-3')	*M. arginini*
McAuliffe *et al.* (2003)	94°C 5min (94°C for 30 sec, 55°C for 30 sec., and 72°C for 30 sec.) x 30, 72°C for 3 min.	361bp	LMFI (5'-TGAACGGAATATGTTAGCTT-3') LMRI (5'-GACTTCATCCTGCACTCTGT-3')	*M. ovipneumoniae*
Bashiruddin *et al.* (1994)	94°C 5min. (94°C 45s, 50°C 60 sec, 72°C 1 min) x 30, 72°C 10 min	1500bp	(5'-TAG AGG TAC TTT AGA TAC TCA AGG-3') (5'-GAT ATC TAA AGG TGA TGGT-3')	*M. mycoides* cluster
Woubit *et al.* (2004)	94°C 2min. (94°C 45s, 47°C 15 sec, 72°C 15 sec.) x 30, 72°C 10 min	316bp	(5'-ATCATTTTTAATCCCTTCAAG-3') (5'-TACTATGAGTAATTATAATATATGCAA-3')	*M. capricolum* subspecies *capripneumoniae* (Mccp)
Oliveira (2008)	94°C 5min. (94°C 60s, 57°C 60 sec, 68°C 1 min) x 35, 72°C 10 min	360bp	(5'-CCT TTT AGA TTG GGA TAG CGG ATG-3') (5'- CCG TCA AGG TAG CGT CAT TTC CTA C-3')	*M. agalactiae*
	Fluoroquinolones resistance genes			
Tatay-Dualde *et al.* (2017)	93°C 5min. (93°C 45s, 57.5°C 45seg, 72°C 1 min) x 30, 72°C 10 min	729	(5'-CACATCAACCTTCCAACCAA-3') (5'-GACGTCGGCATCAGTCATAA-3')	*gyrB*
	93°C 5min. (93°C 45s, 57.5°C 45seg, 72°C 1 min) x 30, 72°C 10 min	605	(5'-TGATGGTCTTAAACCTGTGCAA-3') (5'-TGTTGGAAAATCTGGTCCTTG-3')	*ParC*
	93°C 5min. (93°C 45s, 56°C 45seg, 72°C 50 seg) x 30, 72°C 10 min	828	(5'-GGCACACCTGAGGCTAAGAG-3') (5'-TATCGCCCATCAGTGTTGAA-3')	*ParE*
	Macrolide resistance genes			
Prats-van der Ham *et al.* (2017)	3 min at 94°C, 30 cycles of 94°C for 45 sec ,annealing at (57.5 °C) for 45 sec ,extension at 72°C for 1min , and a final cycle of 10 min at 72°C	848	(5'-CGTTTTTGAGCGCGTTATTA-3') (5'-GGCCATTCCATATTCAGTGC-3')	23S rRNA of domain 2 (23S rRNA O2D2)
	3 min at 94°C, 30 cycles of 94°C for 45sec, annealing (58.5 °C) for 45 sec ,extension at 72°C for 1min , and a final cycle of 10 min at 72°C	752	(5'-TCTCTGCTAAACCGCAAGGT-3') (5'-CCATTCGCCGCTATGATATT-3')	23S rRNA of domain 5 (23S rRNA O2D5)
	3 min at 94°C, 30 cycles of 94°C for 45 sec, annealing at (56.9°C) for 45 sec ,extension at 72°C for 1min , and a final cycle of 10 min at 72°C	816	(5'-AACCAAGAAATCTACTTCAACTGC-3') (5'-AACAGCTTTGCTTTGTGCT-3')	***rplD***
	3 min at 94°C, 30 cycles of 94°C for 45 sec ,annealing at (57.5°C) for 45 sec ,extension at 72°C for 1min , and a final cycle of 10 min at 72°C	345	(5'-CTCAGGTCATGGTGCTGAAA-3') (5'-CGGAAGCCATTTGGATTAAC-3')	***rplV***
	Virulence gene			
Chopra-Dewasthaly *et al.* (2008)	95°C for 7 min, 30 cycles of 95°C for 43 sec, annealing at 56°C for 43 sec and extension at 72°C for 43 sec with the final extension step at 72°C for 10 min	513	(5'-GCTAGGTCTAGATAGAGTGATATACGACAC-3') (5'-TACTGTGGTACCTAGACTATTGATGCTTAC-3')	Xer

Supplementary Table S2: Investigation of phenotypic antibacterial resistance, phenotypic virulence traits, virulence and Fluoroquinolone Resistance genes in *M. arginini* and *ovipneumoniae* species isolated from pneumonic sheep lungs.

***Mycoplasma* species**	**Antibiotics**							**Phenotypic virulence traits**				**Virulence gene**	**QRDR antibiotic resistance genes**			**Macrolide and Lincomycin resistance genes**		**Source**
	Danofloxacin	Tulathromycin	Tylosin	Streptomycin	Lincomycin	Florfenicol	Oxytetracycline	Hemolysis	Catalase	H_2S	Biofilm	Xer	*par*C	*par*E	*gyr*A	*rpl*D	*rpl*V	
arginini MK291435	0.25	2	1	1	4	4	2	-	-	-	0.272	-	-	-	-	-	-	Lung
arginini MK291436	0.5	2	1	2	2	4	4	-	-	+	0.250	-	-	-	-	-	-	Lung
arginini MK291437	2	1	2	1	2	2	4	+	+	+	0.216	-	-	-	-	-	-	Lung
arginini	0.25	0.5	2	2	2	2	0.25	+	+	-	0.262	-		-	-	-	-	Lung
argininii	0.125	1	1	1	1	4	2	+	+	+	0.564		-		-	-	-	Lung
arginini	0.25	1	1	2	2	2	1	+	-	-	1.099	-	-	-	-	-	-	Lung
arginini	1	0.125	2	2	2	4	0.25	+	-	-	1.153	-	-	-	-	-	-	Lung
arginini	2	1	0.5	2	4	4	4	+	-	+	0.347	-	-	-	-	-	-	Lung
arginini	2	2	0.5	2	4	4	4	+	+	+	0.340	-	-	-	-	-	-	Lung
arginini MK291433	0.5	1	2	2	1	2	4	+	-	+	0.187	-	-	-	-	-	-	Nasal swab
arginini MK291434	1	2	2	2	2	2	2	-	+	-	0.187	-	-	-	-	-	-	Nasal swab
arginini	2	2	2	0.5	2	4	2	-	+	-	0.292	-	-	-	--	-	-	Nasal swab
arginini	1	2	0.25	1	2	2	1	+	-	-	0.259	-	-	-	-	-	-	Nasal swab
arginini	1	1	2	1	4	4	2	+	+	-	0.279	-	-	-	--	-	-	Nasal swab
ovipneumoniae MK300051	0.5	0.5	4	4	8	8	4	+	-	+	0.371	-	-	-	-	-	-	Lung
ovipneumoniae MK300052	0.5	1	2	4	4	8	2	+	-	-	0.347	-	-	-	-	-	-	Lung
ovipneumoniae MK300042	1	1	2	2	4	4	2	-	-	-	0.365	-	-	-	-	-	-	Lung
ovipneumoniae MK361039	0.25	0.5	0.5	1	8	16	4	+	+	+	0.400	-	-	-	-	--	-	Lung
ovipneumoniae MK361029	1	1	2	2	8	8	4	+	+	-	0.356	-	-	-	-	-	-	Lung



Supplementary Figure S1: Sequence results submitted to the Genbank.