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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; SP-12(8): 300-304
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www.thepharmajournal.com
Received: 26-05-2023
Accepted: 01-07-2023

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Molecular detection of Non-*Mycoplasma* mycoides cluster organisms in respiratory infections of sheep and goats

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Abstract
Infections of respiratory tract were one of the main problems in sheep and goats farming. Causing production and economic losses to farmers. *Mycoplasma* is one of etiological agent. Among all species, not only *Mycoplasma* mycoides cluster group but also non cluster organisms play important role in production of disease. In the present study a total of 188 *Mycoplasma* suspected samples, 95 (44 nasal swabs, 49 lung tissues and 2 pleural fluids) from sheep and 93 (56 nasal swabs, 33 lung tissues and 4 pleural fluids) from goats were collected. Molecular detection of genus *Mycoplasma*, *Mycoplasma ovipneumoniae* and *Mycoplasma agalactiae* was carried out by PCR targeting 16S*r*RNA yielding 280 bp, 361 bp and 360 bp product. The overall incidence of genus *Mycoplasma* was found to be 68.42% in sheep and 55.91% in goats. The overall incidence of *Mycoplasma ovipneumoniae* and *Mycoplasma agalactiae* was found to be 32.63% & 12.63% in sheep and17.2% &13.97% in goats.

Keywords: *Mycoplasma agalactiae,* molecular detection, pleural fluids

Introduction

Mycoplasmosis is the term used to describe diseases caused by *Mycoplasma spp.* which impose serious constraints on sheep and goat production because of high mortalities, ill thrift, a substantial reduction in meat, milk, and wool yield in Africa, Europe, Middle East, Australia (Cordy, 1984) [1], Asia (Belton, 1990) [2] and North America(Ruffin, 2001) [3].The etiological agents of *Mycoplasma*l pneumonia in goats include *M. mycoides* subsp. *mycoides* large colony (MmmLC), *M. mycoides* subsp. *capri* (*Mcc*), *M. capricolum* subsp. *capricolum* (*Mcc*), *M. capricolum* subsp. *capripneumoniae*, *M. arginini*, *M. bovis*, *M. agalactiae* (*Ma*) and *M. ovipneumoniae* (DaMassa *et al*.,1983) [4], while in sheep *Mycoplasma*l pneumonia have been associated with *M. ovipneumoniae, M. agalactiae, M. capricolum* subsp. *capricolum*(*Mcc*)*, M. mycoides* subsp. *mycoides* large colony (Mmm LC) and *M. arginini* (Chaturvedi *et al*., 1992) [5]. *Mycoplasma ovipneumoniae* is known to play an important role in the etiology of chronic, non-progressive, atypical pneumonia of sheep, goats, and bighorn sheep is a predisposing factor for bacterial or viral infections (Ayling *et al*.,2007; Nicholas *et al.*,2008, b).Gil *et al*. (1999) [6, 7, 8] reported that *Mycoplasma agalactiae* is the classical etiological agent of contagious agalactia. *M. agalactiae* is supposed to be the major pathogen that accounts for 90% of outbreaks of contagious agalactiae syndrome in goats (Bergonier *et al.,*1997) [9], and almost 100% in sheep (Lambert 1987) [10].
Common clinical symptoms include fever, anorexia, lethargy, and unwillingness to follow the herd, followed by the clinical symptoms depending upon the involvement of various organs such as mammary glands, lungs, genitalia, joints, and conjunctiva. Rare abortions in pregnant animals have also been reported (Srivastava, 1982; Kizil & Ozdemir, 2006 and Gil *et al.* 2003) [11, 12, 13]. Halium *et al.* (2019) [14] examined living animals clinically and noticed that animals were suffered from different signs of respiratory manifestations including fever of 40-42°C with depression, nasal and ocular discharges (mucoid, mucopurulent, and purulent), cough, and abnormal chest sound on auscultation. By considering the above conditions the present focus on the molecular detection of non-cluster organisms in respiratory infections of sheep and goats.

Materials and Methods
Collection of samples

A total of 188 *Mycoplasma* suspected samples, 95 (44 nasal swabs, 49 lung tissues and 2pleural fluids) from sheep and 93 (56 nasal swabs, 33 lung tissues and 4 pleural fluids) from goats were collected in PPLO broth and incubated under anaerobic conditions maintaining 5% CO_2 for7-10days.

DNA Extraction from PPLO inoculated with Nasal swabs and Pleural fluids

Extraction of DNA from PPLO broth inoculated with nasal swabs and pleural fluids were carried out as per the method described by Liu *et al*. (2001) [15].After incubation for 7-10 days in PPLO broth the clinical material present on the swab was extracted by gentle overtaxing of micro centrifuge tube and then the swabs were removed from the tubes. Later, these samples were subjected for centrifugation at 13,000 rpm for 10 min in refrigerated centrifuge. The cell pellet obtained was washed twice with 1 ml of PBS and suspended in a final volume of 100 μl of PBS. The cell suspension was boiled at 95°C for 10 min in water bath and immediately chilled on ice. After cooling, the lysate was centrifuged at 13,000 rpm for 2 min in a refrigerated centrifuge. 2µl of supernatant was used as DNA sample.

DNA Extraction from lung tissues

Extraction of DNA from lung tissue was carried out as per the method described by Manimaran and Singh (2017) [16]. Two grams of tissue was taken from each sample and was homogenized by adding 2 ml of TE buffer in mortar and pestle. The homogenized suspension was transferred to micro centrifuge tubes and allowed to settle for 10 min. The supernatant was collected and centrifuged at 13,000 rpm for 10 min in refrigerated centrifuge. The pellet was dissolved in 100 μl of TE buffer and then boiled for 10 min. immediately, the samples were chilled on ice. After cooling, the lysate was again centrifuged at 13,000 rpm for 2 min in a refrigerated centrifuge.2µl of supernatant was used as DNA sample.

PCR for detection of 16S*r*RNA gene of genus *Mycoplasma*

PCR for detection of 16S*r*RNA gene of genus *Mycoplasma* was carried out as per the method of Cetinkya *et al.* (2009) [17]. Details of the primer sequence and PCR conditions are enlisted in Table 1 and 2.

PCR for detection of 16S*r*RNA gene of Spp. *Mycoplasma ovipneumoniae*

PCR was carried out for detection of 16S *r*RNA gene of Spp. *Mycoplasma ovipneumoniae* as per the method of McAuliffe *et al.* (2003) [18]. Details of the primer sequence and PCR conditions are enlisted in Table 1 and 2.

PCR for detection of 16S*r*RNA gene of Spp. *Mycoplasma agalactia*

PCR was carried out for detection of 16S *r*RNA gene of Spp. *Mycoplasma agalactia* as per the method of Halium *et al.*(2019) [14]. Details of the primer sequence and PCR conditions are enlisted inTable1 and 2.

Results

Screening of samples by PCR for detecting genus *Mycoplasma* by targeting 16S rRNA

Out of44 sheep and 56 goat nasal swab DNA samples screened, 33 samples from sheep and 36 samples from goat were positive for genus *Mycoplasma* yielding 280 bp product. Similarly out of49 sheep and 33 goat lung tissue DNA samples screened, 32 samples from sheep and 15 samples from goat were found to be positive for genus *Mycoplasma* yielding 280 bp product (Fig.1). The PCR product of two representative positive samples of genus *Mycoplasma* collected from sheep (MS15) and goat (MG15) were sequenced. The same sequences were submitted to Gene bank and allotted with Accession number OP925812 and OP926019. The obtained nucleotide sequences were verified by NCBI-BLAST and found that nucleotide sequences showed 99.57 to 96.54% homology with *M. ovipneumoniae* and *M. conjunctivae* strains.

PCR amplification of 16S*r*RNA gene of *Mycoplasma ovipneumoniae* from genus *Mycoplasma* positive samples

Out of 33 sheep and 36 goat nasal swab DNA positive samples, 16 samples from sheep and 16 samples from goat were positive for *Mycoplasma ovipneumoniae* yielding 361 bp product. Out of 32 sheep and 15 goat lung tissue DNA positive samples, 15 samples from sheep were positive for *Mycoplasma ovipneumoniae* yielding 361 bp product (Fig.2). None of goat lung tissue and pleural fluid found positive. The PCR product of one representative positive sample of *sps. Mycoplasma ovipneumoniae* collected from sheep lung tissue AP MOVIT1 was sequenced. The same sequence was submitted to Gene Bank and allotted Accession number OQ148368.The obtained nucleotide sequence was verified by NCBI-BLAST and found that sequences showed 99.56 to 95.32% homology with *Mycoplasma ovipneumoniae* strains (Fig.3).

PCR amplification of 16S*r*RNA gene of *Mycoplasma agalactiae* from genus *Mycoplasma* positive samples

Out of 33 sheep and 36 goat nasal swab DNA positive samples 4 samples from goat were positive for *Mycoplasma agalactia* yielding 360 bp product (Fig.4) and none of sheep nasal swab found positive. Similarly out of 19 sheep and 15 goat lung tissue DNA positive samples, 12 samples from sheep and 9 samples from goat were positive for *Mycoplasma agalactiae* yielding 360 bp product. None of pleural fluids found positive.

Discussion

The small ruminant rearing is facing diversified problems due to infectious and noninfectious diseases, poor managemental practices and underfeeding. *Mycoplasmosis* in small ruminants is multi systemic disease collectively caused by various pathogenic *Mycoplasma* spp. Many species of *Mycoplasma* like *Mycoplasma mycoides* cluster (*Mccp*, *Mcc*and *Mmc*) and non-cluster pathogenic species (*Mycoplasma agalactiae*, *Mycoplasma putrefaciens*, *Mycoplasma ovipneumoniae, and Mycoplasma arginini*) (Thiaucourt *et al.,* 2000) [19] infecting different systems of the host.

Isolation and identification of *Mycoplasma*s from clinical samples is the confirmatory test for diagnosis. The isolation of the organism is very difficult, owing to its nutritional fastidiousness and osmotic fragility (Nicholas and Church ward, 2012). The species identification of *Mycoplasma* isolates by biochemical and serological methods is also complicated, since the members share many antigenic and genomic features (Thiaucourt and Bolske, 1996) [21]. Therefore, there is a need for sound advanced diagnostic approaches for early and rapid detection of disease. Molecular technique like Polymerase Chain Reaction (PCR) offers a

rapid and sensitive method for the diagnosis of the disease.

In the present study, samples which were genus *Mycoplasma* positive also screened for *Mycoplasma ovipneumoniae and Mcoplasma agalactiae* which are commonly associated with respiratory infections of sheep and goat. (Nicholas *et al.*, 2008; Egwu *et al*. 2012; DaMassa 1983; Kizil and Ozdemir 2006) [7, 22, 4, 22, 12].

In the present study, standardized protocol was used for screening of *Mycoplasma ovipneumoniae* PCR targeting 16S *r*RNA gene. Out of 65 genus positive sheep samples (33 nasal swabs and 32 lung tissues), 31 samples (16 nasal swabs and 15 lung tissues) and similarly in goats, out of 52 genus positive goat samples (36 nasal swabs, 15 lung tissues and 1 pleural fluid), 16 samples nasal swabs were found positive for *Mycoplasma ovipneumonia*e.

The overall incidence of *Mycoplasma ovipneumoniae* in sheep and goats was found to be 32.63% and 17.2%. The present study reported that the incidence of *my coplasama ovipneumoniae* in respiratory infections was more in sheep compared to goats. The results were in accordance with the studies conducted by Ongor *et al*. (2011) [23] in Turkey. In their studies, the prevalence of *Mycoplasma ovipneumoniae* in sheep were 29.5% (13/44) and in goat were 8.1% (56/692). Mounika yadav *et al.* (2020) [24] from AP reported the occurrence of *Mycoplasma ovipneumoniae* in sheep and goats was 68.4% and 50% which was high compared to our present study. Sheehan *et al.* (2007) [25] detected 90% incidence in sheep which was very high compared to our present study. Cheng *et al.* (2015) [26] from China identified 28.89% positivity from sheep which were in accordance tour present investigation. Other researcher Daee *et al*. (2020) [27] from Iran reported only 4% incidence of *M ovipneumoniae* which was very much lower than our present investigation. Yang *et al*. (2010) [28] from China in their studies conducted on goat lung tissues reported that 75 percent of samples were positive for *Mycoplasma ovipneumoniae.*

For confirmation of *Mycoplasma ovipneumoniae* representative sample sequenced for 16S *r*RNA and verified by NCBI-BLAST. The obtained nucleotide sequences showed 99.56 to 95.32% homology with *Mycoplasma ovipneumoniae* strains. The phylogenetic analysis of present sheep isolate of OQ148368 segregated into a distinct cluster with clade formed by MK138543.1, goat isolate and MK182761.1, sheep isolate of Andhra Pradesh submitted by Mounika Yadav *et al.* (2020) [24].

In the present study PCR targeting 16S*r*RNA gene was standardized for detection of *Mycoplasma agalactiae.* Out of 65 genus positive sheep samples (33 nasal swabs and32 lung tissues), 12 lung tissues and out of 52 genus positive goat samples (36 nasal swabs, 15 lung tissues and 1 pleural fluid), 13(4 nasal swabs and 9 lung tissues) samples121 were found positive for *Mycoplasma agalactiae* in the present investigation. The overall incidence of *Mycoplasma agalactiae* in sheep and goats was found tobe 12.63% and 13.97%. The present study reported that the incidence of in respiratory infections was more in goats compared to sheep. Reji *et al.* (2018) [29] in a case study conducted at Kerala reported the incidence of *Mycoplasma agalactiae* in nasal discharges of goats. Azevedo *et al*. (2006) [30] from Brazil isolated *M. agalactiae* in nasal swabs of sheep and goats. According to Loria *et al*. (1999) [7] from UK in his study noticed that occurrence of *M. agalactiae* is occasionally found in lung tissues but in our present investigation percentage of incidence of *Mycoplasma agalactiae* was more in lung tissues. Filioussis *et al*. (2022) [32] from Switzerland, detected My coplasama *agalactiae* in composite milk samples and stated it mainly infects goats. In our studies we recorded higher incidence of *M. agalactia* in respiratory infections in goats.

Conclusion

The present study was carried out in animals showing respiratory symptoms likes increase in temperature from 41 to 43°C, dyspnea, coughing, sneezing, purulent and mucopurulent nasal discharges for the detection of non *My coplasma mycoides* cluster organisms in sheep and goats. The results shows that there was more incidence of *Mycoplasma ovipneumoniae*. In our studies we recorded higher incidence of *M. agalactia* in respiratory infections in goats. We were going to concluded that along with cluster group, non-cluster organisms also plays important role in manifestation of respiratory infections. So proper managemental practices, deworming and treatment reduces production losses.

Table 1: Different sets of Primers used

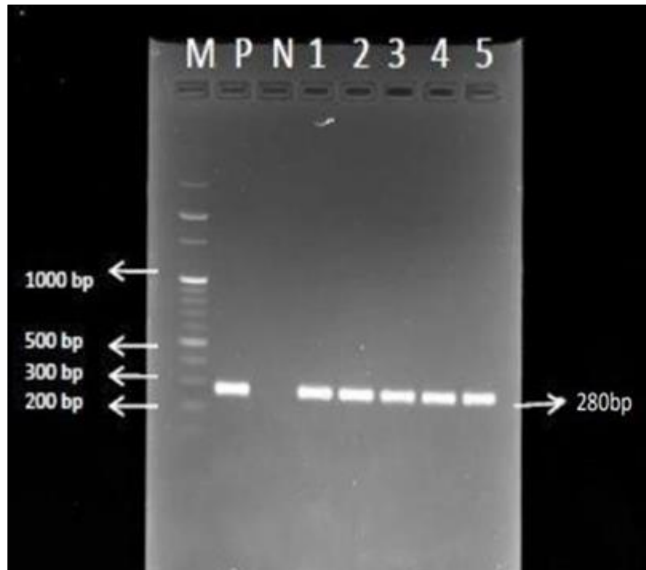
Primers	Gene	Primer Name	Nucleotide Sequence	Amplicon Size
Mycoplasma genus specific primer (Cetinkaya *et al*., 2009) [17]	16S *r*RNA	GPO3F	5'TGGGGAGCAAACAGGATTAGA TACC-3'	280 bp
		MGSO	5'TGCACCATCTGTCACTCTGTTAA CCTC-3'	
Mycoplasma ovipneumoniae McAuliffe *et al.* (2003)	16S *r*RNA	LMF1	5'TGAACGGAATATGTTAGCTT-3'	361bp
		LMR1	5'GACTTCATCCTGCACTCTGT-3'	
Mycoplasma agalactiae (Halium *et al*., 2019) [14].	16S *r*RNA	Mga- F	5'-CCT TTT AGA TTG GGA TAG CGG ATG -3'	360bp
		Mga-R	5'- CCG TCA AGGGTAG CGT CATTTC CTA C 3'	

Table 2: Cyclic conditions for amplification

Sr. No	Species	Intial denaturation (°C)	Denaturation (°C)	Annealing (°C)	Extension (°C)	Final Extension (°C)	No of cycles
1.	*Mycoplasma genus*	94-4 min	94 - 30 sec	56 -30sec	72 -30sec	72-10min	35
2.	*Mycoplasma ovipneumoniae*	94-5 min	94-30sec	55-30sec	72-30 sec	72- 7 min	30
3.	*Mycoplasma agalactiae*	94-4min	94-30 sec	60-1min	72-30 sec	72-10 min	35

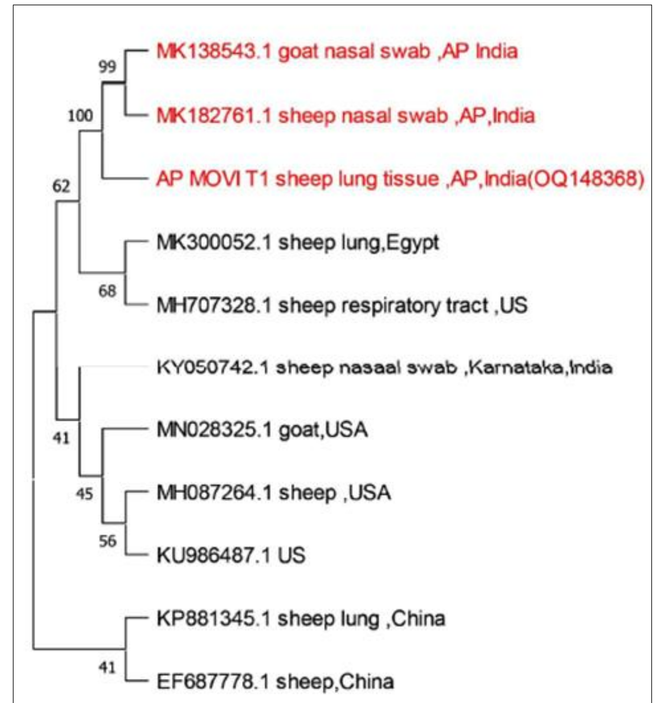
Table 3: Overall incidence of non-*Mycoplasma* mycoides cluster organisms

Sr. No	Bacterial species detected	Sheep			Goat		
		Total no of samples collected	Total tested positive	Percentage (%) of Incidence	Total no of samples collected	Total tested positive	Percentage (%) of Incidence
1.	Genus *Mycoplasma*		65	68.42		52	55.91
2.	*Mycoplasma ovipneumoniae*	95	31	32.63	93	16	17.20
3.	*Mycoplasma agalactiae*		12	12.63		13	13.97



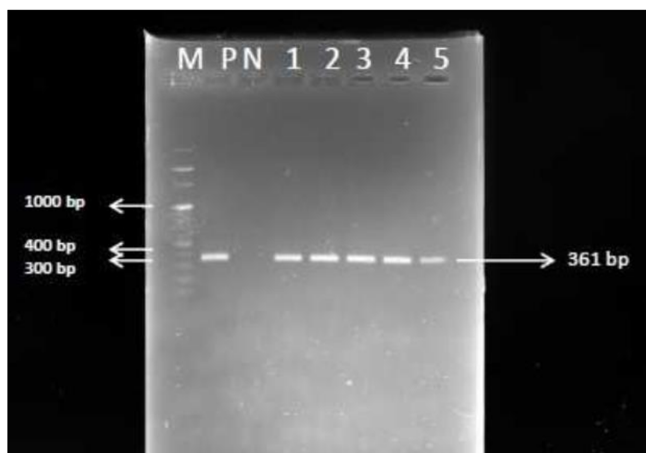
Lane M: Ladder (100 bp)
Lane P: Positive Control
Lane N: Negative Control
Lane 1-2: Nasal swabs positive for 16S rRNA gene of genus *Mycoplasma*
Lane 3-4: Lung tissues positive for 16S rRNA gene of genus *Mycoplasma*
Lane 5: Pleural fluid positive for 16S rRNA gene of genus Mycoplasm

Fig 1: Amplification of 16S *r*RNA gene of genus *Mycoplasma* from Nasal swabs, Lung tissues and Pleural fluid samples



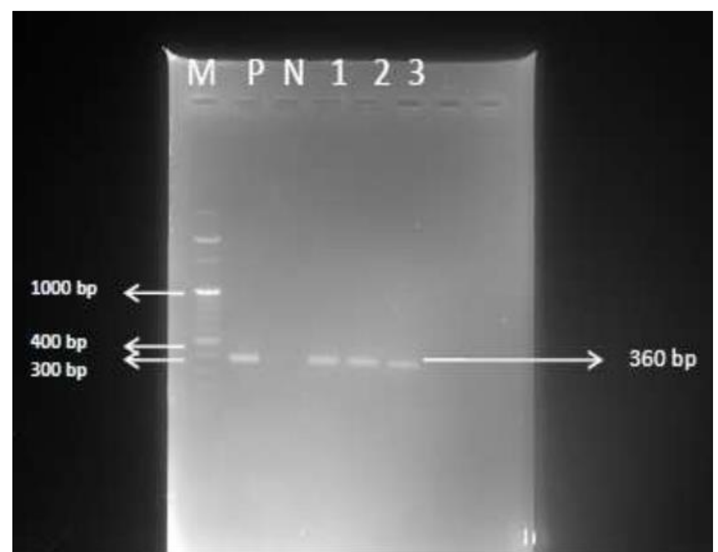
The phylogenetic tree was constructed using the MEGA version 11.0 by the Maximum Likelihood with 1000 bootstrap replicates using Kimura 2-parameter model

Fig 3: Phylogenetic analysis of *spp. Mycoplasma ovipneumoniae* sequences with other reference sequences available in NCBI database**.**



Lane M: Ladder (100 bp)
Lane P: Positive Control
Lane N: Negative Control
Lane 1, 2, 3: Nasal swabs positive for 16S *r*RNA gene spp. *Mycoplasma ovipneumoniae*
Lane 4, 5: Lung tissues positive for 16S *r*RNA gene spp. *Mycoplasma ovipneumoniae*

Fig 2: Amplification of 16S *r*RNA gene PCR of spp. *Mycoplasma ovipneumoniae* from positive culture DNA and lung tissues in sheep and goats



Lane M: Ladder (100 bp)
Lane P: Positive Control
Lane N: Negative Control **Lane 1, 2, 3:** Field samples positive for 16S *r*RNA gene spp. *Mycoplasma agalactiae*

Fig 4: Amplification of 16S *r*RNA gene PCR for of spp. *Mycoplasma agalactiae* from clinical cases of respiratory infections in sheep and goats

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