

Original Research Article

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Isolation and Molecular Characterization of *Mycoplasma* Isolates from Pneumonic Sheep and Goats in Andhra Pradesh

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ABSTRACT

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In the present study, attempts for the isolation of *Mycoplasma* organisms were carried out in PPLO broth and PPLO agar medium. Out of 253 sheep and 112 goat nasal swab samples inoculated in PPLO broth, 27 sheep and 14 goat samples showed colour change in broth. Upon sub-culturing of 27 sheep and 14 goat broth nasal swab samples on PPLO agar, 17 sheep and 9 goat samples produced colonies. Positive samples produced yellow color change to broth medium with mild turbidity after 48 hrs of incubation at 37°C in CO₂ incubator maintaining 5% CO₂. After 7th day of incubation on PPLO agar, the agar plates showed fried egg colonies and granular colonies when examined under 10X magnification of microscope. Further *Mycoplasma* isolation was confirmed by PCR targeting 16S rRNA gene of genus *Mycoplasma* and *Mycoplasma ovipneumoniae*.

Introduction

Mycoplasmas are the smallest prokaryotic cells capable of self-replication and are pleomorphic organisms ranging from spherical (0.3 to 0.9 picomole in diameter) to filamentous (up to 1.0 picomole long). Mycoplasmas lack cell wall and have flexible triple layered cell membrane which allows them to pass through bacterial membrane filters of pore size 0.22 to 0.45 μm (Quinn *et al.*, 1994). The *Mycoplasma* genome consists of small circular double stranded DNA

molecule of 580-2220 kb size. These are largely extracellular parasites on mucous membranes of animals (Razin *et al.*, 1998). Mycoplasmas are fastidious in nature, hence it is very difficult to grow *Mycoplasma* on ordinary laboratory media. Sterol (cholesterol) and fatty acids are essential components for the growth of *Mycoplasma spp.* Therefore, some special media are being used for the growth and isolation of different pathogenic *Mycoplasma spp.* *Mycoplasma* can grow on media enriched with some special components like beef heart infusion,

peptone, yeast extract and animal serum @ 5-20% with various supplements (Thiaucourt *et al.*, 1992; Nicholas, 2002). Pleuro pneumonia like organism (PPLO) broth and modified Hay media are commonly used for the isolation and culturing of various *Mycoplasma spp.* by many researchers (Noah *et al.*, 2011; Ongor *et al.*, 2011; Sadique *et al.*, 2012; Kabir and Bari, 2015). Chakraborty *et al.*, (2014) suggested adding thallium acetate, fluconazole and penicillin in the media to prevent bacterial and fungal contamination. The present study aimed at isolation and identification of Mycoplasmas by culturing nasal swabs and lung samples collected from sheep and goats with respiratory symptoms on PPLO broth and PPLO agar plates.

Materials and Methods

Sample collection

Nasal swabs from 253 sheep and 112 goats showing respiratory symptoms of nasal discharge, coughing and sneezing were collected and this was transferred into a sterile micro centrifuge tube containing PPLO broth and brought to the laboratory over ice maintaining cold chain. The samples were immediately incubated at 37°C in CO₂ incubator maintaining 5% CO₂ for 48 hours.

Preparation of PPLO (*Mycoplasma*) broth

PPLO (*Mycoplasma*) broth was prepared as per the manufacturer guidelines. 2.1gm of PPLO broth base media was added in 70 ml of double distilled water in a conical flask. Then medium was autoclaved at 15 lbs pressure at 121°C for 15min. After autoclaving the media was allowed to cool down to 45°C and then one vial (20 ml) of mycoplasma enrichment supplement was added mixed well and dispensed aseptically into sterile microcentrifuge tubes and stored at 4°C for further use.

Preparation of PPLO (*Mycoplasma*) agar

PPLO (*Mycoplasma*) agar was prepared as per the manufacturer guidelines. 3.6 gm of mycoplasma agar base along with 0.8 gm of agar was dissolved in 70 ml of double distilled water in a conical flask. Then medium was autoclaved at 15 lbs pressure at 121°C for 15 min. After autoclaving the media was allowed to cool down to 45°C and then one vial (20ml) of Mycoplasma enrichment supplement was added and mixed well. Then the media was poured into Petri plates and were stored at 4°C after setting.

Inoculation

Nasal swabs and lung tissue samples collected from sheep and goats suspected for Mycoplasmosis in PPLO broth medium were incubated at 37°C in CO₂ incubator maintaining 5% CO₂ for 2 days. The incubated microcentrifuge tubes were examined daily for presence of mass turbidity and change in color. The positive growths were sub-cultured on PPLO agar media by spreading a few drops of sample or by making a direct impression with the cut surface of lung lesion without spreading. After sub-culturing onto PPLO agar medium, the Petri plates were incubated at 37°C in CO₂ incubator maintaining 5% CO₂ for 10-15 days.

PCR for detection of genus *Mycoplasma* and *Mycoplasma ovipneumonia*

PCR for detection of 16S rRNA gene of genus *Mycoplasma* and *Mycoplasma ovipneumonia* was carried out as per the method of Cetinkya *et al.*, (2009) and McAuliffe *et al.*, (2003) respectively.

Primers

The oligonucleotide primers were procured from Sigma Aldrich India Pvt. Ltd.,

Bangalore. Details of the primer sequence are enlisted in Table 1 & 2. Working solutions were prepared from original stock (100 pmol/μl) in nuclease free water at 10 pmol/μl. The reaction mixture consists of Taq buffer A (10X) -2.5μl, MgCl₂ (25mM) - 1.5μl, dNTP mix (10mM)- 0.5μl, Taq DNA Polymerase (1U/μl)- 1.0μl, Forward primer (10 pmol/μl) - 1.0μl, Reverse primer (10 pmol/μl) - 1.0μl, Template DNA - 5.0μl, Nuclease free water - 12.5μl. The tubes were then spun for 10 sec and PCR was carried out in Thermal cycler (Eppendorf Pvt. Ltd., Hamburg, Germany). Cyclic conditions used for amplification of *Mycoplasma* and *Mycoplasma ovipneumoniae* are given in detail in Table 3 & 4.

Agarose gel electrophoresis of PCR product in 2% agarose

Amplified products were analysed by agarose gel electrophoresis in 2% agarose gels in TBE buffer (appendix) containing ethidium bromide at 60 volts for 90 min to detect PCR amplified product of genus *Mycoplasma* and *Mycoplasma ovipneumoniae* targeting 16S

rRNA. The gels were viewed under UV trans-illuminator and photographed with gel documentation system (AlphaInnotech, Alphaimager HP).

Results and Discussion

Out of 253 sheep and 112 goat nasal swab samples inoculated in PPLO broth, 27 sheep and 14 goat samples showed colour change in broth. Upon sub-culturing of 27 sheep and 14 goat broth nasal swab samples on PPLO agar, 17 sheep and 9 goat samples produced colonies.

Growth in mycoplasmal (PPLO) broth

Mycoplasma suspected samples from sheep and goat were inoculated in PPLO broth and positive samples produced mild turbidity with yellow colour change in broth medium after 48hr of incubation indicating multiplication of organisms (Fig. 1). The growth was generally seen at the bottom of the tubes and produced a whirlpool like structure when the tubes were slightly agitated.

Table.1 Primers used for detection of 16S rRNA gene of genus *Mycoplasma*

Primers	Primer Name	Nucleotide Sequence	Amplicon Size
<i>Mycoplasma</i> genus specific primer (Cetinkya et al., 2009)	GPO3F	5'-TGGGGAGCAAACAGGATTAGA TACC-3'	280 bp
	MGSO	5'-TGCACCATCTGTCACTCTGTAA CCTC-3'	

Table.2 Primers used for detection of *Mycoplasma ovipneumoniae*

Primers	Primer Name	Nucleotide Sequence	Amplicon Size
<i>Mycoplasma ovipneumoniae</i> (McAuliffe et al., 2003)	LMF1	5'-TGAACGGAATATGTTAGCTT-3'	361 bp
	LMR1	5'-GACTTCATCCTGCACTCTGT-3'	

Table.3 Cyclic conditions used for amplification of 16S rRNA gene of genus *Mycoplasma*

S.No	Temperature (°C)	Duration	No. of cycles
1.	94 (initial denaturation)	4 min	1
2.	94 (denaturation)	30 sec	35
3.	56 (annealing)	30 sec	
4.	72 (extension)	30 sec	
5.	72 (final extension)	10 min	1

Table.4 Cyclic conditions for amplification of *Mycoplasma ovipneumoniae*

S.No	Temperature (°C)	Duration	No. of cycles
1.	94 (initial denaturation)	5 min	1
2.	94 (denaturation)	30 sec	30
3.	55 (annealing)	30 sec	
4.	72 (extension)	30 sec	
5.	72 (final extension)	7 min	1

Fig.1 Colour change in PPLO broth inoculated with nasal swab after 48 h of incubation



Fig.2 Typical fried egg colony of *Mycoplasma*

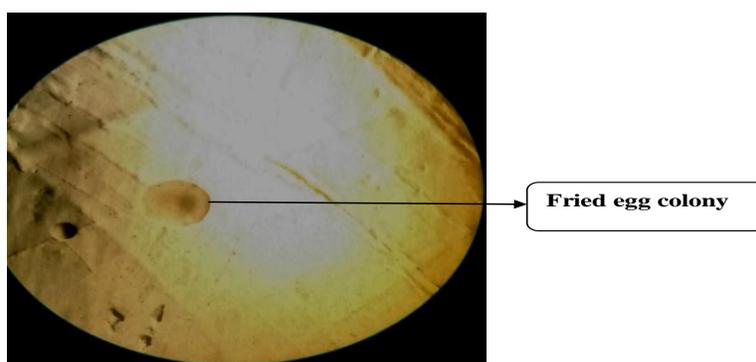


Fig.3 Granular colonies of *Mycoplasma ovipneumoniae*

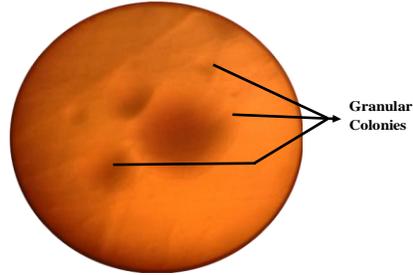
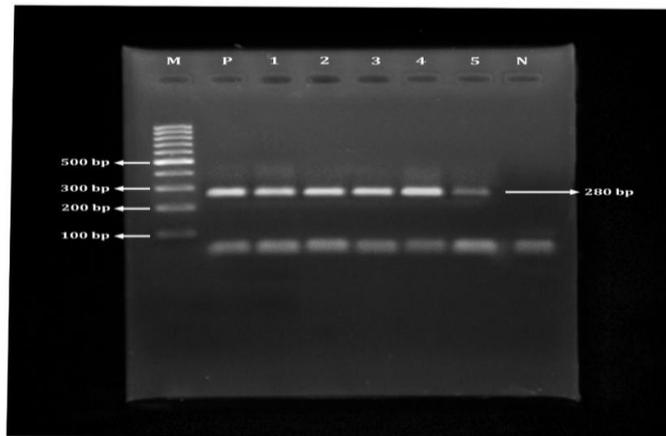
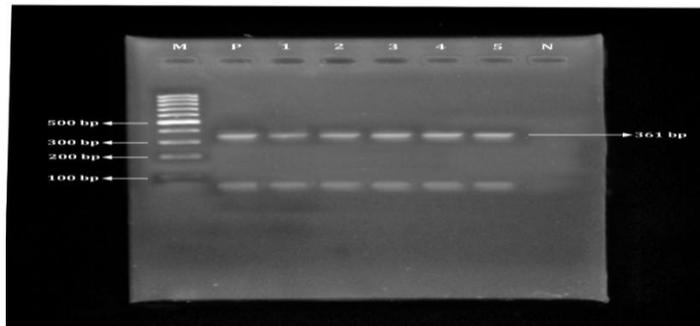


Fig.4 Amplification of 16S rRNA gene of genus *Mycoplasma* from clinical cases of respiratory infections in sheep and goats



LaneM : Ladder (100 bp)
LaneP : Positive Control
Lane1,2,3,4 & 5: Field samples positive for 16S rRNA gene of genus *Mycoplasma*
LaneN : Negative Control

Fig.5 Amplification of 16S rRNA gene PCR for of *Mycoplasma ovipneumoniae* from clinical cases of respiratory infections in sheep and goats



Lane M : Ladder (100 bp)
Lane P : Positive Control
Lane 1,2,3,4 & 5 : Field samples positive for 16S rRNA gene *Mycoplasma ovipneumoniae*
LaneN : Negative Control

Growth on mycoplasmal (PPLO) agar

The suspected growths of *Mycoplasma* from broth culture were inoculated on to PPLO agar plates. Isolates started producing colonies three to four days after inoculation on *Mycoplasma* agar. Colonies were observed after 7th day of incubation. Petri plates were observed under microscope (10X) to study the colony characteristics of various *Mycoplasma* isolates. *Mycoplasma* colonies exhibited a typical fried-egg appearance (Fig. 2). Along with the fried egg colonies, granular colonies of *Mycoplasma* without central nipple (Fig. 3) was observed which may be suggestive of *Mycoplasma ovipneumoniae* colonies.

Confirmation of Mycoplasmal colonies by PCR targeting 16sRNA gene of genus *Mycoplasma* and *Mycoplasma ovipneumonia*

DNA extracted from colonies were subjected to PCR for confirmation. Out of 17 sheep and 9 goat samples which produced colonies on PPLO agar, all yielded 280bp long sequence of 16S *rRNA* gene of *Mycoplasma* (Fig. 4). Only 4 sheep and 1 goat sample colonies yielded 361bp long sequence of 16S *rRNA* gene of *Mycoplasma ovipneumoniae* (Fig. 5).

In the present study, attempts for the isolation of *Mycoplasma* organisms was carried out in PPLO broth and PPLO agar medium. After 48hr of incubation, the positive samples produced yellow colour change to broth medium with mild turbidity. The findings were in accordance with the earlier reports of Kumar *et al.*, (2012) wherein, his studies observed change in colour of PPLO broth with mild turbidity after 48 hr of incubation. Kumar *et al.*, (2013) in their studies reported that out of 171 clinical samples, 45 samples showed fine turbidity and PH shift (acidic) imparting a yellow colour to medium within 3 to 10 days indicating *Mycoplasma* growth.

After 72 hr of incubation, PPLO broth was streaked across the surface of PPLO agar plate and incubated at 37°C with 5% CO₂ in CO₂ incubator for 10-15 days. After 7th day of incubation, the agar plates were examined under 10X magnification of microscope for colony morphology. In the present study, two types of colonies were observed. One type of colonies was circular, convex giving fried egg appearance with central part of colonies darker than periphery. Other type of colonies were, granular without central nipple. Similar findings were observed by Kumar *et al.*, (2012) after 48 hrs of incubation on PPLO agar plate, it produced small size, circular, convex colonies with central part of colonies darker than periphery. McAuliffe *et al.*, (2005) in his studies observed colonies with fried egg appearance typical for many mycoplasma and center less granular colonies were seen on solid medium after 4 to 6 days of incubation and were identified by DGGE in mixed culture as *Mycoplasma ovipneumoniae*.

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