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Isolation of *mycoplasma* species from respiratory infections of sheep and goats

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Abstract
The present study is carried out on isolation of *Mycoplasma* species from respiratory infections of sheep and Goats because Mycoplasma is also one of the major etiological agent in the production of respiratory infections which produces economic losses to small-scale farmers. 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. Isolation carried out on PPLO broth and PPLO agar. Positive samples produce color change in broth from brown to yellow and production of two different types of colonies on agar. One type is typical fried egg colony and other type is nipple-like with prominent tip. Conformation also done by PCR targeting *Mycoplasma* genus yielding 280 bp product size.

Keywords: Isolation, *mycoplasma*, respiratory infections, sheep and goats

1. Introduction

Small ruminants rearing is considered as most affordable and plays a pivotal role in the livelihood of landless, small and marginal farmers by generating revenue and employment. Sheep and goats provides source of income with help of its utilities like wool, meat, milk, leather and manure. In spite of their adaptability to various weather conditions, sheep and goat rearing is facing diversified problems including infectious and non-infectious diseases, poor manage mental practices and under feeding. The death rate in small ruminants was more due to respiratory and digestive diseases in contrast to other diseases (Karna *et al.*, 2020) [6]. The most common problem associated with respiratory diseases of all ages in sheep and goats is pneumonia. Among various infectious diseases, Mycoplasmal infections are highly pathogenic and causes significant morbidity and mortality in sheep and goats. Mycoplasma spp. can cause respiratory, arthritis, mastitis, genital and conjunctival infections. Mycoplasmal infections are difficult to diagnose by the clinical signs and post-mortem lesions alone and they are not always pathognomonic. Isolation and identification of mycoplasmas 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 Churchward, 2012) [14]. The present study mainly focus on the isolation and characterization of various Mycoplasmal species based on colony morphology.

2. Materials and Methods

2.1 Collection of samples

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 in PPLO broth and incubated under anaerobic conditions maintaining 5% CO_2 for 7-10 days.

2.2.1 Preparation of PPLO (*Mycoplasma*) broth

PPLO (*Mycoplasma*) broth has been prepared according to the manufacturer guidelines (Hi media). To a conical flask containing 70 ml double distilled water, 2.1gm of PPLO broth base media was added. Then medium was sterilized at 15 LBS pressure at 121 °C for 15 min.

One vial (30 ml) of *Mycoplasma* enrichment supplement (Horse serum 20 ml, Yeast extract 25% w/v solution10 ml, Thallus acetate 25 mg and Penicillin G 20,000 IU) was added to sterilized media after media got cool down to 45 °C. The suspension was mixed well and transferred aseptically into autoclaved micro centrifuge tubes, stored at 4°C for future use.

2.2.2 Preparation of PPLO (*Mycoplasma*) agar
PPLO (*Mycoplasma*) agar was prepared according to the manufacturer guidelines (Hi media). To a conical flask containing 70ml double distilled water, 1.4gm of PPLO broth base media was added. Then medium was sterilized at 15 LBS pressure at 121°C for 15min. One vial (30 ml) of *Mycoplasma* enrichment supplement (Horse serum 20 ml, Yeast extract 25%w/v solution10 ml, Thallus acetate 25 mg and Penicillin G 20,000 IU) was added to sterilized media after media got cool down to 45 °C. The suspension was mixed well and poured into petriplates, allowed to solidify and stored at 4 °C for future use.

2.3 Inoculation
Sheep and goats suspected for Mycoplasmosis, samples (Nasal swabs, lung tissue and pleural fluid) were collected and incubated at 37 °C in CO_2 incubator maintaining 5% CO_2 for 10 days. The incubated microcentrifuge tubes were observed daily for mass turbidity and change in colour. The samples identified positive were sub-cultured on PPLO agar media by spreading whereas for lungs, a direct impression with the cut surface was made onto PPLO agar without spreading. The sub-cultured PPLO agar plates were incubated at 37°C in CO_2 incubator maintaining 5% CO_2 for 2 weeks.

2.4 Confirmation of *Mycoplasma colonies* by PCR
DNA extracted from *Mycoplasma* suspected colonies by using the standard method described by Liu *et al.* (2001) [11] targeting genus-specific primers (GPO3F-5'TGGGGAGCAAACAGGATTAGATACC-3'; MGSO-5'TGCACCATCTGTCA CTCTGTTA ACCTC-3') yielding 280 bp product size (Cetinkaya *et al.*, 2009) [2].

3. Results
3.1 Growth in Mycoplasmal broth (PPLO broth)
Positive samples for *Mycoplasma* in PPLO broth showed little cloudiness with yellow colour change after 7-10 days of incubation indicating significant growth of organisms (Fig 1). The growth was usually noticed at the bottom of the tubes. When the tubes were agitated, the growth was noticed like swirl.

3.2 Growth on Mycoplasma agar (PPLO agar)
Isolates starts by production of colonies on PPLO agar after seven days of inoculation under anaerobic conditions. Colonies were identified after 10th days of inoculation. Agar plates were examined under low and high power lens in the microscope to characterize the type and characteristics of different *Mycoplasma* species. Two types of *Mycoplasma* colonies were identified. One type was typical fried egg colony (Fig 2) and other type was having nipple like with prominent tip (Fig 3).

3.3 Cultural confirmation of *Mycoplasma* using PCR
Out of 100 nasal swabs (44 sheep and 56 goat) inoculated in PPLO broth, 12 sheep and 15 goat nasal swabs produced colour change to yellow in broth after one week of incubation (Fig 4). After sub-culturing of 12 sheep and 15 goat broth nasal swabs onto agar plate, 4 sheep and 6 goat samples showed colonies. DNA extracted from these 4 sheep and 6 goat nasal swab samples when subjected to PCR targeting 16S *r*RNA of genus *Mycoplasma* yielded 280 bp product indicating the presence of *Mycoplasma.*
Similarly, out of 49 sheep and 33 goat lung tissues collected, a small portion of lung tissues from 14 sheep and 11 goats inoculated into PPLO broth. Out of which 10 sheep and 7 goat tissues produced colour change. Upon sub-culturing of 10 sheep and 7 goat broth samples onto PPLO agar, 6 sheep and 4 goat samples produced colonies after 10 days of incubation. DNA extracted from these 6 sheep and 4 goat lung tissue samples when subjected to PCR targeting 16S *r*RNA of genus *Mycoplasma* yielded 280 bp product indicating the presence of *Mycoplasma.*
Out of 2 sheep and 4 goat pleural fluids collected, only one goat sample showed a color change in the broth after 7 days of incubation (Fig 4). DNA extracted from the pleural fluid sample when subjected to PCR targeting 16S *r*RNA of genus *Mycoplasma* yielded 280 bp product indicating the presence of *Mycoplasma.*

4. Discussion
Sheep and goat were dependable source of income and plays a tremendous role in rural economy especially for landless, small and marginal farmers by providing meat, wool, and milk. In developing countries, the majority of farmers generate their income from livestock (Abbas *et al.*, 2013) [1]. The isolation of *Mycoplasma* organisms were carried out in PPLO broth and PPLO agar medium in the current investigation. Sheep and goats suspected for Mycoplasmosis, samples (Nasal swabs, lung tissue and pleural fluid) were collected and incubated at 37 °C in CO_2 incubator maintaining 5% CO_2 for 7-10 days in PPLO broth. Similar conditions required for the growth of *Mycoplasma* were also reported by (Ezzi *et al.*, 2007; Ley and Yoder, 1997; Harasawa *et al.*, 2004) [4, 10, 5]. Positive samples for *Mycoplasma* in PPLO broth showed little cloudiness with yellow color change after 7-10 days of incubation. The findings were in agreement with the earlier reports of Kumar *et al.* (2013) [9] where in, it was noticed the change in color of PPLO broth with haziness after 2 days of incubation in his studies. Kumar *et al.* (2013) [9] 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 72hr of incubation, PPLO broth was streaked across the surface of PPLO agar plate and incubated at 37 °C with 5% CO_2 in CO_2 incubator for 2 weeks. After 10 days of incubation, the agar plates were examined under low power and high dry objective microscope for colony morphology. In the present study, two types of colonies were observed. One type of colonies were circular, convex giving fried egg appearance with central prominent tip darker than periphery coinciding with *M. agalactiae colonies*. Similar findings were observed by Srivastava (1982) [15] in his study identified colonies with dark centered appearing a typical fried-egg appearance and illustrated this event as "film and spot" and Kumar *et al.* (2014) [7] reported after 48 hrs of incubation on PPLO agar plate, it produced small size, circular, convex colonies with central part of colonies darker than the periphery. Other type of colonies was granular without a central nipple. McAuliffe *et al.* (2006) [12] in his studies observed colonies with a fried egg appearance typical for more *Mycoplasmas* and centerless granular colonies were

seen on solid medium after 4 to 6 days of incubation and were identified in DGGE in mixed culture as *Mycoplasma ovipneumoniae*. Daee *et al.* (2020) [3] in his observation identified *Mycoplasma* growth such as cloudiness in the *Mycoplasma* broth and *Mycoplasma* colonies on the agar plates *and* Mounika Yadav *et al.* (2020) [13] also identified center less granular colonies on PPLO agar after 7 days of incubation. For further confirmation, DNA was extracted from colonies by boiling and snap chilling method was subjected for PCR amplification using *Mycoplasma* genus specific primers. Out of 44 sheep and 56 goat nasal swabs, 12 sheep and 15 goat samples produced colonies after 10 days of incubation on PPLO agar. The PCR amplification of these samples yielded 280 bp product in all 17 sheep and 9 goat nasal swabs tested which indicates the presence of *Mycoplasma*. In the present study molecular detection of *Mycoplasma from* direct field samples was higher compared to detection of *Mycoplasma* from colonies. Similar findings of higher *Mycoplasma* detection by PCR compared to isolation was observed by Kumar *et al.* (2011) [8] and Mounika Yadav, *et al.* (2020) [13].

5. Conclusion

Fig 1: Color change in PPLO broth inoculated with nasal swab after 7days of incubation

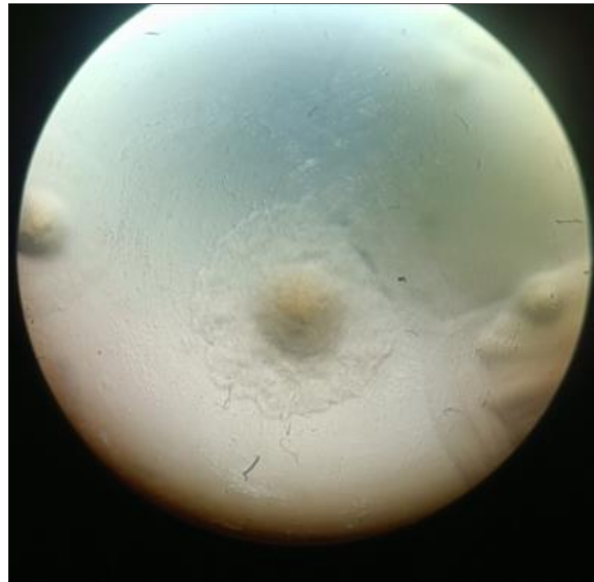

Fig 2: Typical fried egg colonies- *M. Ovipneumoniae*

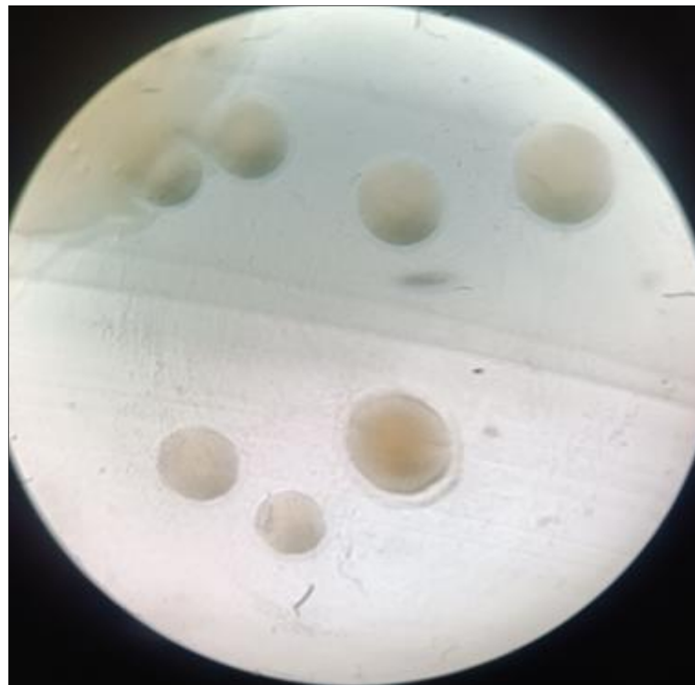

Fig 3: Fried egg colonies-prominent tip *M. Agalactiae*

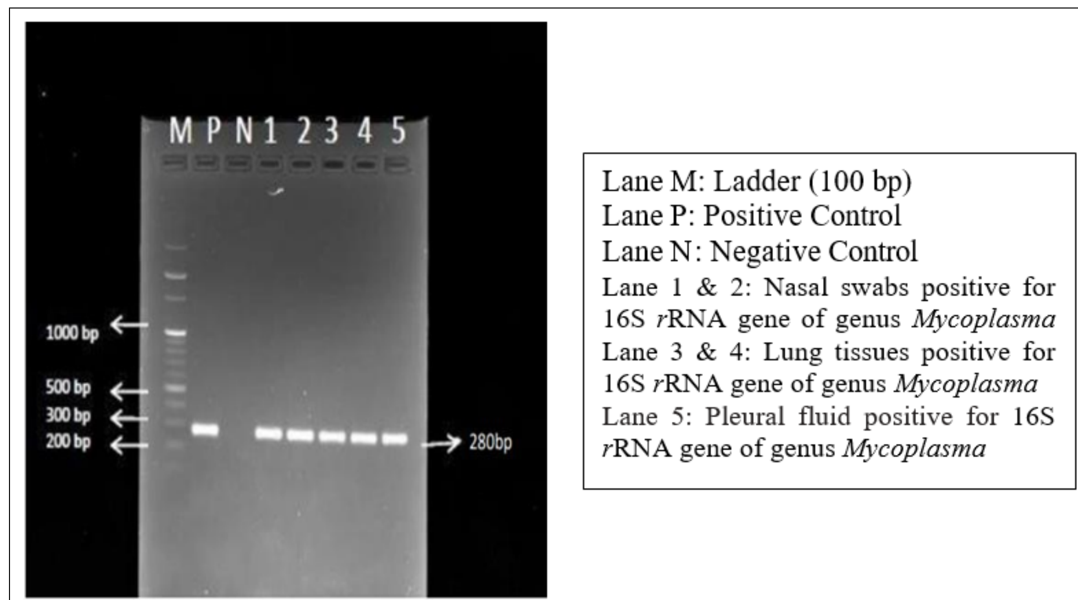

Fig 4: Amplification of 16S *r*RNA gene of genus *Mycoplasma* from Nasal swabs, Lung tissues and Pleural fluid samples

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