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Detection of mycoplasma in cases of bovine mastitis refractory to antibiotic treatment in northern Kerala#

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Abstract

Mastitis is the inflammation of the mammary gland parenchyma and many microorganisms, mostly bacteria are associated with the condition. A study was conducted to assess whether mycoplasma is associated with cases of mastitis that are refractory to antibiotic treatment as the condition caused by the microbe is reported to be difficult to treat. A total of 71 milk samples were collected from antibiotic-resistant mastitic cows in the northern districts of Kerala. Total DNA was extracted from all the samples by phenol-chloroform method with prior sample lysis and subjected to Mycoplasma spp. and Mycoplasma bovis specific Polymerase Chain Reaction (PCR) and the amplicons obtained were sequenced. Mycoplasma spp. was detected in two (2.81 per cent) samples and no samples were positive for M. bovis. Sequencing of PCR amplicons revealed the presence of Mycoplasma hyorhinis in one of the samples. The identity of the Mycoplasma spp. in the other sample could not be conclusively proved as the nucleotide sequence obtained matched with that of M. bovis, M. arginini, and uncultured Mycoplasma spp. detected in nasal swabs of animals. Both the milk samples from which mycoplasma was detected were positive for multidrug-resistant gram positive and gram-negative bacteria and from one sample fungi could be isolated. From the results obtained, though mycoplasma was detected, it could not be proven that it is the sole cause of the condition. Also, the presence of mycoplasma is not wide spread in cases of refractory mastitis in northern Kerala.

Keywords: *Mastitis, Mycoplasma, multi-drug-resistant bacteria, PCR*

Mastitis is defined as inflammation of the mammary gland parenchyma. Bovine mastitis is caused mostly by bacteria, though fungi and to a lesser extent virus can also act as causative agents of the condition. Combined infections with bacteria and other groups of organisms can also lead to mastitis. *Mycoplasma* spp. have been linked to several diseases in cattle, including otitis media, urogenital tract inflammation, arthritis, pneumonia, and mastitis (Maunsell *et al*., 2011, Gourlay and Howard, 1979). *Mycoplasma bovis* (*M. bovis*) is reported to be the most common causative agent of mycoplasma mastitis. Other species that are associated with the condition include *Mycoplasma californicum, Mycoplasma canadense,* and *Mycoplasma bovigenitalium.* The onset of the condition is rapid, and the source of infection is often endogenous and follow outbreaks of respiratory tract infection in heifers or cows (Wieland, 2024). Liu *et al*.

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(2020) reported that mastitis caused by *M. bovis* is on the rise and since the pathogen lacks a cell wall the range of possible treatments for the condition is limited and that it is resistant to several classes of antibiotics. The present study was carried out to detect whether mycoplasma are responsible for cases of bovine mastitis refractory to antibiotic treatment in northern Kerala.

A total of 71 milk samples were collected in sterile vials from various farms and households in northern districts of Kerala. All samples were collected from cases of bovine mastitis that were refractory to antibiotic treatment. Samples were stored and transported to laboratory at 4 °C. The total DNA was extracted from the samples after a centrifugation, and lysis step. The milk samples were subjected to centrifugation at 8,000 rpm for 20 min and the fat layer was pipetted out. Pipetted out 400 µL of the milk sample along with the pellet if any and 400 µL of lysis buffer containing 10per cent SDS, 0.5M EDTA, 17.5per cent NaCl and 10M NaOH (Kumar and Mugunthan, 2018) was added and vortexed. Subsequent steps to extract total DNA were carried out as described by Ocak *et al*. (2023). Briefly, 300 µL of tris saturated phenol, 300 µL of chloroform/isoamyl alcohol (49/1) were added to the lysate and vortexed. To this 100 µL of 3M sodium acetate was added and vortexed for 15 sec. The suspension was centrifuged at 12,000 rpm for 10 min. Subsequently, 700 µL of the clear aqueous phase was pipetted out to a new microcentrifuge tube and to this 700 µL of cold isopropyl alcohol was added and mixed by inversion. The tube was kept at -80 °C for one hour for the precipitation of DNA. After thawing, the sample was centrifuged at 12,000 rpm for 10 min. The supernatant was discarded and the pellet was washed in 300 µL of 70per cent ethanol at 12,000 rpm for 3 min. The pellet was dried and resuspended in 30 µL of nuclease free water (NFW) and stored at -20 °C. The extracted DNA was subjected to polymerase chain reaction (PCR) for detection of *Mycoplasma* spp. and *M. bovis* by using the primers reported by Botes *et al*. (2005) and Ahmed *et al*. (2023) respectively, both targeting the 16S rRNA region of the bacteria. The specific amplicons generated were sequenced and analyzed by Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

Of the samples tested, specific amplicons of 270 bp were detected in two (2.81%) of the samples in *Mycoplasma* spp. PCR (Fig. 1). None of the samples were positive in specific PCR for *M. bovis*. In BLAST analysis, it was revealed that the sequence obtained from one sample was having same identity percentage to sequences of *M. bovis, M. arginini,* and *Mycoplasma* spp. detected from nasal swabs of animals. The sequence from the other sample was that of *Mycoplasma hyorhinis*. In one of the samples, Gram negative bacilli could be isolated and in the other, both Gram positive cocci and Gram-negative bacilli were isolated. The Gram-negative bacilli were sensitive to enrofloxacin and resistant to gentamicin, ceftriaxone-tazobactam, amoxicillin-sulbactam, oxytetracycline, cefaperazone and ceftriaxone. In the other sample, the bacteria were sensitive to ceftriaxone-tazobactam, gentamicin, oxytetracycline, cefaperazone and resistant to amoxicillin-sulbactam, enrofloxacin and ceftriaxone. Thus, both the samples contained multi-drug resistant (MDR) bacteria and fungi was isolated from one of the samples.

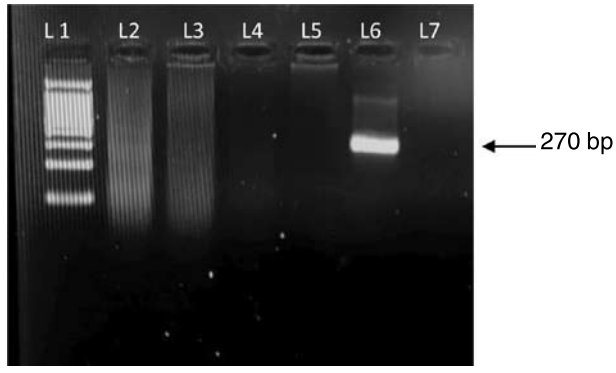

Fig. 1. Agarose gel showing 270 bp amplicons generated by PCR targeting 16S rRNA region of *Mycoplasma* spp.

(Lane 1 – 100 bp DNA marker, Lanes 2, 3, 4, 5 – Negative samples, Lane 6 – Positive sample, Lane 7 – No template control)

Mastitis is marked by pathological alterations in the glandular tissues as well as physical, chemical, and typically bacteriological changes in milk (Radostits *et al*., 2000). Among dairy producers, mastitis continues to be a major cause of economic loss even though great technological advances have been made (Fetrow *et al*., 2001). The condition is usually caused by bacteria. *M. bovis*, are considered as important causative agents of mycoplasma mastitis. The important peculiarity of mycoplasma mastitis is that it is refractory to treatment (Britten, 2012). There is growing levels of antimicrobial resistance among wild-type *M. bovis* isolates and lack of cell walls in mycoplasmas enable them to be intrinsically resistant to beta-lactam antibiotics that are widely used in dairy farms (Gelgie *et al*., 2024).

In a study conducted by Kurt and Eşki (2021), the predominant pathogen isolates recovered from milk from cows with clinical mastitis were fungi (21.9%), *Escherichia coli* (19.9%) and *Staphylococcus aureus* (13.7%), followed by *Mycoplasma bovis* (8.2%) and *Streptococcus uberis* (6.8%). Ampicillin, imipenem, chloramphenicol and streptomycin were the least effective antimicrobial agents, while the most effective agents were florfenicol, amikacin and kanamycin/cefalexin.

In the present study, on BLAST analysis, one of the sequences obtained matched with that of *M. bovis, M. arginini,* and uncultured *Mycoplasma spp.* detected in nasal swabs of animals. However, all samples were negative in PCR carried out to detect *M. bovis*. In a study carried out by Junqueira *et al*. (2020) in Brazil, clinical mastitis milk samples were cultured on Hayflick agar and incubated in a microaerophilic atmosphere. Also, the presence of *M. bovis*

in the samples was detected by PCR. *Mycoplasma* spp. was isolated in two per cent of the milk samples, and *M. bovis* was detected in three per cent of the milk samples by PCR. Sachse *et al.* (2010) reported that in a farm in southern Germany housing 1800 cows, unresponsive mastitis was observed. *Mycoplasma bovis* was detected in 20 per cent of milk samples from the affected cows. They also reported that one year later, 16 per cent of the cows were still excreting the bacteria, although clinical signs had disappeared.

While *M. bovis* is a known pathogen causing mycoplasma mastitis, there are no documented cases of *M. arginini*-induced mastitis. However, the latter is the most frequently isolated mycoplasma from some farms (Gioia *et al*., 2021) and this species is commonly found in bulk milk that comes from herds that have mastitis of various causes (Fox *et al*., 2005; Higuchi *et al*., 2011; Justice-Allen *et al*., 2011). Stipkovits *et al*. (2013) concluded that *M. arginini* infection does not cause evident mastitis characterised by a decrease in milk production and an increase of somatic cell count. However, it predisposes animals to infection with *Streptococcus dysgalactiae*, leading to clinical mastitis.

In the second sample, the mycoplasma detected was found to be *M. hyorhinis*. The bacteria is considered as a commensal microorganism of the upper respiratory tract and tonsils of pigs (Switzer *et al*., 1955). It has also been detected from pneumonic lungs in fattening pigs (Luehrs *et al*., 2017). However, to the best of the knowledge of the authors, there are no reports that suggest the organism to be a causative agent of mastitis.

In the present study, both the samples from which mycoplasma was detected were also positive for MDR bacteria and in one sample, fungi could also be isolated. Hence, from the results of this study, it is not possible to conclusively prove whether mycoplasma is responsible for the unfavourable treatment response as MDR bacteria and fungi can cause a similar outcome. Also, *Mycoplasma* spp. was detected in only two samples which indicates that they are not present to a great extent in samples from cases of refractory mastitis. Further research utilising a larger number of samples have to be undertaken to assess the actual prevalence and role of the pathogen in refractory mastitis in Kerala.

Summary

The study could detect and characterise mycoplasma in a few cases of mastitis refractory to antibiotic treatment. However, its role in causation of mastitis could not be conclusively proven as other pathogen(s) that could be the cause of the condition were also detected in the samples. From the results, it seems that mycoplasma is not commonly associated with cases of bovine mastitis refractory to treatment in northern Kerala.

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Conflict of interest

No conflict of interest in this study

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