AN OUTBREAK OF CONTAGIOUS AGALACTIA IN SHEEP

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The present study was aimed for the confirmatory diagnosis of contagious agalactia syndrome in sheep herds belonging to Ibrahimpatnam and Mylavaram mandals of Krishna district, Andhra Pradesh. A total of 37 clinical samples were collected from the affected sheep with a history of keratoconjunctivitis, corneal opacity, arthritis, anorexia and mastitis without any reports of mortality. The symptoms of the affected sheep were preliminarily suggestive of Mycoplasmal infections. An attempt was made for confirming the organism based on genotypic characterization by employing molecular based technique, polymerase chain reaction using mycoplasma group specific primers for a quick diagnosis. All the 37 samples were processed for DNA extraction by employing high salt DNA extraction method and phenol chloroform method. Amplification of the mycoplasma gene produced an amplicon product size of 270bp indicating 100% positivity to *Mycoplasma* infection among the herd.

Key words: Keratoconjunctivitis, Mycoplasma, Polymerase chain reaction, Small ruminants, Tetracyclines

Mycoplasmas are the smallest self-replicating bacteria capable of cell-free existence. They are more closely related to the Gram-positive bacterial group. It is an atypical bacterium belonging to the class of *Mollicutes* and is devoid of cell wall around their cell membrane. In small ruminants, these bacterial infections are associated with predisposing factors like primitive herding practices, inefficiency of

antimicrobial therapies and adoption of very few prophylactic measures (Gil *et al.*, 1999). Mycoplasma infections affect primarily the mammary glands, joints and eyes causing severe infections in small ruminants that result in economic loss to the shepherds due to high morbidity rather than mortality (Lambert, 1987). The incubation period of the mycoplasma infection in goats and sheep is one to eight

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weeks (Azevedo *et al.*, 2006). In some animals, the clinical symptoms include fever, anorexia, lethargy and aversion to follow the herd (Kizil and Ozdemir, 2006). Mycoplasma infections are present all over the continents especially in Europe, Asia and Africa, mainly in the countries around the Mediterranean (Azevedo *et al.*, 2006; Ekwu *et al.*, 2001; Fasanya *et al.*, 1987; Real *et al.*, 1994; Singh *et al.*, 2004). The mycoplasma infections localize in the mammary glands, eye, joints, lungs and cause local inflammation in the respective area. Pneumonia and abortions are characteristic of chronic mycoplasma infections in small ruminants. In the present study, few sheep were investigated for mycoplasmal infections as they were showing symptoms of corneal opacity, arthritis, anorexia and mastitis.

The affected sheep (n = 37) were showing clinical signs like keratoconjunctivitis, corneal opacity, arthritis, anorexia and clots in milk without any reports of mortality. A total of thirty seven samples pertaining to different specimens were collected from Ibrahimpatnam and Mylavaram mandal of Krishna district, Andhra Pradesh and brought to NTR CVSc., Gannavaram in transport medium like phosphate buffer saline placed on ice. The list of clinical samples is listed in Table 1.

The DNA was extracted from all the 37 samples by employing two methods, viz. high salt method and phenol-chloroform method.

The procedure of Aravindakshan *et al.* (1997) was followed. Briefly, 1mL culture was centrifuged at $5000 \times g$ for 10 min at 4°C. The pellets were then re-suspended in 1mL of PBS and mixed properly and later centrifuged at $5000 \times g$ 10 min at 4°C. The pellets were then re-suspended in 100 µL of TKM-I solution, a low salt buffer [TrisHCl (10mM) pH 7.6, KCl (10mM), MgCl₂ (10mM), EDTA (2mM)] and centrifuged at $5000 \times g$ 10 min at 4°C. The pellet was re-suspended in 100 µL of TKM-II solution, a high salt buffer [TrisHCl (10mM) pH 7.6, KCl (10mM), MgCl₂ (10mM), EDTA (2mM) NaCl (0.4 M)] and incubated for 15 min at 37°C. To the above suspension 50 µL of SDS-Sodium Dodecyl Sulphate, 10% plus 250 µL of 6M NaCl was added and mixed thoroughly and centrifuged at $10,000 \times g$ for 5 min at 4°C. The supernatant was collected into an eppendorf tube and absolute alcohol was added to double the quantity of supernatant obtained and centrifuged at $10,000 \times g$ for 5 min at 4°C. The pellet was re-suspended in 75% ethyl alcohol and centrifuged at $10,000 \times g$ for 5 min at 4°C. The process

Table 1. List of clinical samples

Species	Milk	Joint exudates	Nasal swabs	Corneal swabs	Total
Ovine	13	9	6	9	37

was done twice to remove any impurity. Finally, the pellet was dissolved in 50 μ L of Milli Q water and stored at -80°C until further use.

The DNA samples for PCR were prepared by phenol-chloroform method (Azevedo *et al.*, 2006). One milliliter culture was centrifuged at $20,000 \times g$ for 20 min at 4°C . The pellets were then re-suspended in 400 μ L of lysis buffer [Dextrose-EDTA buffer, pH 8.4, plus 30 μ L of proteinase K (240 $\mu\text{g}/\text{mL}$) plus 30 μ L of SDS-Sodium dodecyl sulphate, 10%], and incubated for 30 min at 50°C . After fast cooling, it was carried out double extraction with 500 μ L of phenol (pH 7.8) followed by one extraction with 500 μ L of chloroform and addition of 100 μ L of ethanol to the final aqueous phase and stored overnight at -20°C . Samples were centrifuged at $20,000 \times g$ for 10 min

at 4°C and the pellets were re-suspended in 100 μ L TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and placed in -80°C until further use.

The nucleotide sequence of primers used in the genus specific PCR assay are as follows. Forward primers 5'-GGGAGCAAACAGGATTAGATACCCT-3'; and Reverse primers 5'TGCACCATCTGTCACTCTGTAAACCTC-3' (Ossewaarde *et al.*, 1996; Timenetsky *et al.*, 2006). The PCR was carried out as described by Azevedo *et al.* (2006) with slight modifications.

The PCR reaction was conducted using thermocycler (Eppendorf PTC-100, MJ Research). The reaction volume was 20 μ L, with 3 μ L of Milli Q water, 1 μ L (20 pm/mL concentration) of each forward and

Fig. 1. An amplicon size of 270bp produced from the DNA extracted by high salt method (1, 2, 3) and phenol chloroform method (5, 6, 7) by representative samples

reverse primer, 5µL DNA extracts. The PCR was performed by initial denaturation at 94°C for 5min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 1min, and final extension at 72°C for 4min. The PCR product was analysed using 1.5% agarose gel. Bands were run and analysed on 2% agarose stained with ethidium bromide (0.5 µg/mL) and visualized under ultra-violet light in a Gel Doc (Bio Rad, US). The yield of DNA extracted from high salt and phenol chloroform method was varying from 80-220 ng/µL concentration. An amplicon size of 270bp was produced with the *Mycoplasma* group specific primers in all the 37 clinical samples indicating that the animals were succumbed to contagious agalactia syndrome.

Mycoplasma agalactiae is one of the predominant etiological factors responsible for contagious agalactia syndrome. However, other species like *M. capricolum* subsp. *capricolum*, *M. putrefaciens*, *M. mycoides* subsp. *capri* or *M. mycoides* subsp. *mycoides* large colony (LC) type can also cause this disease. With an aim of confirming the contagious agalactia syndrome in the affected sheep herd, the samples were processed for DNA extraction and subjected for the highly specific molecular technique i.e. polymerase chain reaction (Lambert, 1987). The Genus specific primers for *Mycoplasma* produced a PCR product size of 270bp from all the clinical samples

indicating that all the sheep were affected and confirming that the organism for the disease is *Mycoplasma*. Earlier, similar results were reported by Ossewaarde *et al.* (1996) and Timenetsky *et al.* (2006). *Mycoplasma* is responsible for severe economic loss in livestock. Nonetheless, it is one of the most neglected diseases. *Mycoplasma* is highly resistant to the room temperature, thus supporting its rapid spread of the infection among the flock (Kumar *et al.*, 2014). The high morbidity of the entire flock results in severe economic losses thus a quicker diagnosis is essential. Isolation of the mycoplasmal organisms was not carried out in the laboratory as these are very fast contaminating organisms and it is also difficult to differentiate on morphological and biochemical tests. Many tests have been employed for the diagnosis of Mycoplasmal organisms of which PCR is one of the most sensitive and faster tests to confirm. PCR can detect the organism even after storage of the samples at -20°C for 24 months (Tola *et al.*, 1996; Tola *et al.*, 1997).

Most of the diseases in small ruminants are mainly due to Mycoplasmal infections that contribute to nearly 90% in causing contagious agalactia syndrome (Verbisck-Bucker *et al.*, 2008). The infected sheep were treated with tetracyclines (2.5 mg/kg body weight) for a period of 5 days. The prognosis was good after administration of the drugs and affected sheep recovered slowly back to normalcy.

In conclusion, contagious agalactia can be controlled on following good hygienic precautions and avoiding the predisposing factors to a lesser extent. Segregation, quarantine procedures, healthy sanitary conditions like removal of bedding materials, disposing of left over feed and disinfection of the premises may contribute

for the healthy status of the sheep affected with mycoplasmal infections.

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