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Contagious agalactia in small ruminants from Maharashtra

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Contagious agalactia (CA) and contagious caprine pleuropneumonia (CCPP) are the principle mycoplasmoses in small ruminants. CA caused by *Mycoplasma agalactiae* is a serious, economically important and OIE listed disease syndrome (OIE 2021) in sheep and goat. It was the first mycoplasmosis of small ruminants for which clinical descriptions and microbiological findings were described (Bergonier *et al.* 1997). This pathogen is transmitted through oral, respiratory and mammary route. The clinical signs of CA include mastitis which can involve 60–80% of lactating females, followed by arthritis, keratoconjunctivitis and abortion in less than 10% of affected animals (Bergonier *et al.* 1997, Kumar *et al.* 2014, Yatoo *et al.* 2018). Clinical signs can be seen at various stages during the evolution of the disease, not necessarily in the same animal but in individuals in the affected flock or herd.

Mycoplasma agalactiae (Ma) is the main cause of the CA in sheep and goats, however *M. capricolum* subsp. *capricolum* (Mcc)*, M. mycoides* subsp. *capri* (Mmc) and *M. putrefaciens* can produce a similar pattern of disease, more often in goats, which may be accompanied by pneumonia. Clinical signs produced by infections of Mcc, Mmc and *M. putrefaciens* (Mp) are sufficiently similar to be considered indistinguishable from CA caused by Ma. Moreover, mastitis and arthritis is seen in Mp infection in goats, which is very similar to that caused by Ma, Mmc and Mcc (Rodriguez *et al.* 1994). Further, the consensus of the working group on CA of the EC COST2 Action 826 on ruminant mycoplasmoses, it has been suggested that all four mycoplasmas should be considered as causal agents of contagious agalactia (OIE 2018).

Though Mcc, Mmc and Mp have been added to the etiology of CA; they can occasionally only cause similar clinical syndrome exclusively in goats. Also, *M. mycoides* group, *M. m. capri* and *M. c. capricolum*, are more often isolated from pneumonic goats (De La Fe *et al.* 2005) or from polyarthritic kids (Agnello *et al.* 2012) and only rarely reported in sheep in some areas (Gómez-Martín *et al.* 2013). Migliore *et al.* (2021) stated that only *M. agalactiae* is recognized nationally and internationally, and subject to animal disease regulations covering CA. They further opined that, classical contagious agalactia should be diagnosed and confirmed only when *M. agalactiae* is detected either by isolation or molecular methods. The prevention and control of CA can be achieved through both live attenuated and inactivated vaccines (Kumar *et al.* 2014, Mogos *et al.* 2021), however vaccination against CA is not practiced in India.

The disease has been reported in many parts of the world, most notably in the Mediterranean Basin and many Asian countries like Iraq, the United Arab Emirates, Iran, Afghanistan, Pakistan, Nepal, the People's Republic of China, Mongolia and Indonesia (Campos *et al. 2009*, Kumar *et al.* 2014, Yatoo *et al.* 2018, Jay and Tardy 2019). However, there are no confirmed reports of CA in Maharashtra state of India.

Considering the above facts, the present investigation confirming contagious agalactia based on clinical signs, detection of *M. agalactiae* by PCR and nucleotide sequencing, and response to specific treatment in sheep and goat flocks from Maharashtra state of India adds to the knowledge of epidemiology of disease and supports statement of Migliore *et al.* (2021) regarding *M. agalactiae* infection as the sole cause of contagious agalactia.

The flocks from Satara and Pune districts of Maharashtra State of India consisting both sheep and goats were involved in the present investigation. After obtaining history, visits were arranged at respective flocks and information on flock size, duration of illness, clinical signs, morbidity, mortality, history of vaccination and other managemental practices was recorded. The milk samples from mastitic animals and articular exudate from arthritic animals were collected aseptically into sterile containers for molecular diagnosis and stored at -20°C until further use.

The DNA from samples (mastitic milk and articular exudate) was extracted by using phenol chloroform method as described by Sambrook *et al.* (1989). The extracted DNA sample was stored in -20°C until use. Thereafter, the DNA samples were tested with group/genus specific PCR to detect 16S rRNA gene of *Mycoplasma* spp using forward

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primer 5'-TGGGGAGCAAACAGGATTAGATACC-3' and reverse primer: 5'-TGCACCATCTGTCAC TCTGTTAACCTC-3' as described by Centikaya *et al.* (2009). The PCR was performed in 25 µl reaction volume comprising 2.5 µL 10× PCR master mix, 1 µL $MgCl_2$, 2 µL dNTPs , 1 µL forward primer, 1 µL reverse primer, 5 µL template DNA, 1.25 U taq polymerase and 12.38 µL nuclease free water. PCR amplification proceeded with an initial denaturation step of 94°C for 4 min followed by 25 cycles at 94°C for 15 sec (denaturation), 53°C for 15 sec (annealing), 72°C for 15 sec (extension), and final extension of 72°C for 5 min. Amplicons (278 bp size) were visualized via electrophoresis on a 2.0% agarose gel and documented with a gel documentation system.

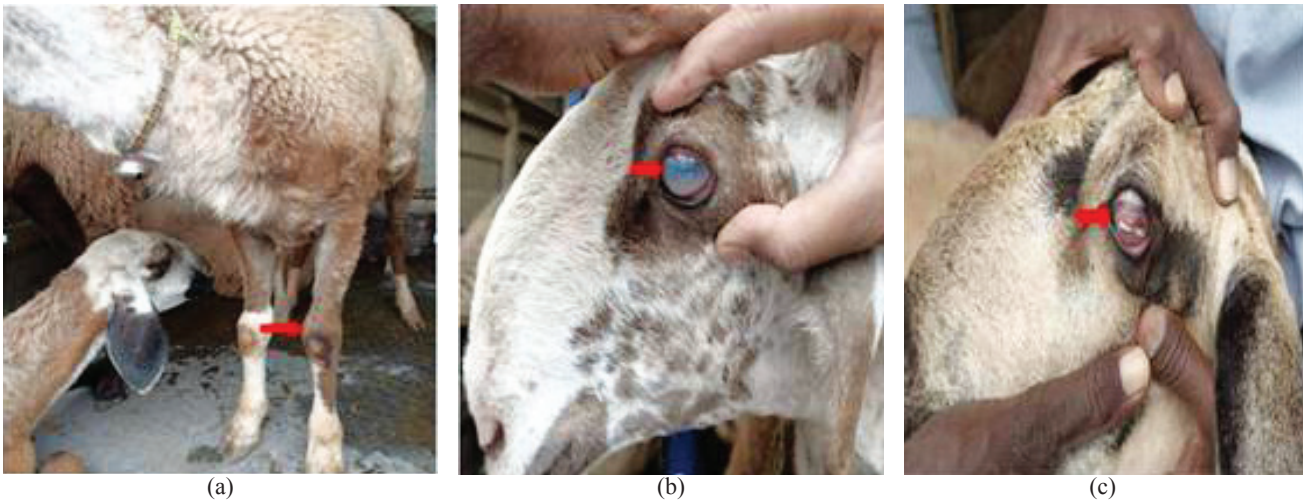
The PCR products from flock located in Khandala Tehsil of Satara district was subjected to nucleotide sequencing. PCR products (278 bp) were gel purified using PureLinkTM Quick Gel extraction kit (Invitrogen, Carlsbad, CA). The purified PCR products were sequenced in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (NimaGen BV, The Netherlands). Finally, the obtained nucleotide sequences were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/) to confirm whether the sequences represented *M. agalactiae*.

Clinical findings: Total 3 flocks i.e. 2 from Satara and 1 from Pune district of Maharashtra State of India consisting both sheep and goats were involved in the present investigation. The outbreaks were recorded during January 2021 to August 2021. The flock size ranged from 70 to 150. Morbidity in the flocks from Khandala and Wai Tehsils of Satara district was 55 and 70 %, respectively. The morbidity in flock from Pune district was 42%. Total 4.5, 7 and 3% mortality was recorded in the flocks from Khandala, Wai Tehsils of Satara district and Pune, respectively.

Clinically, anorexia, reluctance to follow the herd, lethargy and fever were the first clinical signs noted. Later, mastitis (Fig. 1) in more than 55-65% lactating animals in herd was noted to be most important clinical sign. Hypogalactia and subsequent agalactia were also characteristic findings. Arthritis (Fig. 2) involving knee or hock joints either unilaterally or bilaterally causing lameness was also noted in 35-48% animals in all three flocks. Keratoconjunctivitis leading to corneal opacity (Fig. 2) and in severe cases abscessation and loss of lens tissue (Fig. 2) was also one of the important clinical signs. There was partial to complete loss of vision. Occasional abortions were also recorded. Bergonier *et al.* (1997), OIE (2018) and Migliore *et al.* (2021) also described similar clinical signs in contagious agalactia. In India, more recently, Kumari and Pushpa (2019) also reported similar clinical signs in sheep herds belonging to Krishna district of Andhra Pradesh wherein they confirmed involvement of genus *Mycoplasma* by PCR but have not identified mycoplasmal species.

Fig. 1. Sheep showing signs of mastitis as evident through change in colour and consistency of milk.

The sheep and goats were reared together in all three



(a) (b) (c)

Fig. 2. (a) Bilateral swelling of knee joint in affected sheep; (b) Keratoconjunctivitis leading to corneal opacity; (c) Severe abscessation and loss of lens tissue in affected eye causing blindness.

flocks and both were found to be affected with clinical signs mentioned above. None of the clinical case from both sheep as well as goat revealed pneumonia, as evident through absence of signs like abnormal nasal discharge, coughing, tachypnea and abnormal sounds on auscultation. The X-ray examination of the thoracic cavity in above clinical cases did not reveal lesions of pneumonia.

The ailing animals were treated with Inj. Tylosin tartrate (Ventycin, Venworld) @ 10 mg per kg body weight, Inj. Meloxicam and Paracetamol (Melonex plus, Intas Pharmaceutical Ltd.) @ 2-3 ml /33 kg B.W. and Inj. Vitamin AD_3E (Vetade, Zydus AHLD) @ 2 ml per adult sheep/goat for consecutive 4 days. The signs of recovery started third day onwards and after a week of treatment, there was complete recovery in 85, 91 and 90% animals in three different flocks as above, respectively. The efficacy of Tylosin in treatment of mycoplasmal infections in small ruminant has also been reported by Jay and Tardy (2019), Dabbir (2020) and Parray *et al.* (2022).

Detection of Mycoplasma *spp by PCR:* The DNA was extracted from milk and articular exudate samples collected from different flocks and stored at –20° C until use. Further, the DNA was subjected to group specific PCR to detect 16S rRNA gene of *Mycoplasma* spp and revealed band of expected 278 bp size in agarose gel electrophoresis (Fig. 3) indicating positive reaction.

Sequencing and BLAST analysis: The PCR products were subjected to sequencing and nucleotide sequences of field sample were subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/) to establish identity of pathogen based on GenBank accessions. The sequence obtained had 100% identity with 16S rRNA gene of *Mycoplasma agalactiae* isolate JF4428 (Accession No. LT578418.1). The sequence had max score 479, total score 958, query cover 94% and E value 6e-131. Thus the mycoplasma present in our filed samples was confirmed to be *M. agalactiae* (Fig. 4).

In the present investigation, *Mycoplasma* spp was detected in mastitic milk and articular exudate samples by PCR and *M. agalactiae* was identified based on the sequencing. Mattson *et al.* (1991) and Cockrevski *et al.* (2001) described use of gene probes, complementary to segments of chromosomal DNA or 16S ribosomal RNA (rRNA) to differentiate between closely related species.

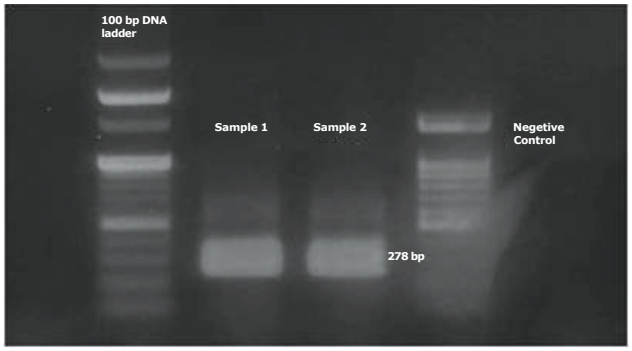


Fig. 3. PCR amplification of genus specific 16S rRNA gene *Mycoplasma* spp (278 bp product).

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479 bits(259)      6e-131()     259/259(100%)      0/259(0%)      Plus/Plus

Query  16     ACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATCATTAGTTGATGGGGAAC  75
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89146  ACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGATCATTAGTTGATGGGGAAC  89205

Query  76     TCATCGACGCAGCTAACGCATTAAATGATCCGCCTGAGTAGTACGTTCGCAAGAATAAAA  135
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89206  TCATCGACGCAGCTAACGCATTAAATGATCCGCCTGAGTAGTACGTTCGCAAGAATAAAA  89265

Query  136    CTTAAAGGAATTGACGGGGATCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATA  195
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89266  CTTAAAGGAATTGACGGGGATCCGCACAAGCGGTGGAGCATGTGGTTTAATTTGAAGATA  89325

Query  196    CGCGTAGAACCTTACCCACTCTTGACATCTTCTGCAAAGCTATGGAGACATAGTGGAGGT  255
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  89326  CGCGTAGAACCTTACCCACTCTTGACATCTTCTGCAAAGCTATGGAGACATAGTGGAGGT  89385

Query  256    TAACAGAATGACAGATGGT  274
              |||||||||||||||||||
Sbjct  89386  TAACAGAATGACAGATGGT  89404
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Fig. 4. Nucleotide sequence alignment of filed *M. agalactiae* (Query) and subject *Mycoplasma agalactiae* JF4428 (Subject).

Similarly, Dedieu *et al.* (1995) and Tola *et al.* (1995) also described that detection of *M. agalactiae* in milk samples of affected animals by DNA extraction and subsequent PCR to be faster than cultural isolation of the organism and has reduced the time required for diagnosis from days to hours.

Though Rodriguez *et al.* (1994) and OIE (2018) stated that the clinical signs of infections caused by Mcc, Mmc and Mp are sufficiently similar to be considered indistinguishable from contagious agalactia caused by *Ma*; the findings of our study reports detection of only *Mycoplasma agalactiae* from clinical cases of contagious agalactia by most sensitive and specific PCR and sequencing. On similar lines, Migliore *et al.* (2021) opined that, classical contagious agalactia should only be diagnosed and confirmed when *M. agalactiae* is detected either by isolation or molecular methods. Hence, our findings support opinion of Migliore *et al.* (2021).

To conclude, the present investigation confirms contagious agalactia based on clinical signs, detection of *M. agalactiae* by PCR, nucleotide sequencing and provides information on response to specific treatment in sheep and goat flocks from Maharashtra state of India. Also this paper reports, the sequence information of 16S rRNA gene of *Mycoplasma agalactiae* for the first time from Maharashtra state of India. Being first report from Maharashtra state, these findings adds to the knowledge of epidemiology of disease in the country.

SUMMARY

Mycoplasma agalactiae causes contagious agalactia, a serious, economically important and OIE listed disease syndrome, in sheep and goats. There are no reports of this disease from Maharashtra state of India. The aim of the present investigation was to record epidemiological information and to detect *M. agalactiae* in clinical samples from natural outbreaks of contagious agalactia. Clinically, anorexia, reluctance to follow the herd, lethargy and fever were the initial clinical signs noted. Later, mastitis, arthritis and keratoconjunctivitis were noted as important clinical signs in both sheep and goats. However, all signs were not present in every case. DNA was extracted from mastitic milk and articular exudate collected from suspected cases and PCR was performed using primers specific for

16S rRNA gene of *Mycoplasma* spp. The agarose gel electrophoresis of PCR product revealed the presence of bands of expected size (278 bp) indicating positive amplification. The nucleotide sequence analysis of PCR amplicons showed 100% identity with 16S rRNA gene of *Mycoplasma agalactiae* isolates JF4428 (Accession No. LT578418.1). The outbreaks of contagious agalactia were confirmed for the first time in Maharashtra state of India and carries epidemiological significance.

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