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Molecular Diagnosis and Management of Leptospiral Mastitis

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ABSTRACT

Background: Bovine Leptospirosis is one of the leading causes of abortion, infertility, stillbirths and sudden drop in milk production to complete agalactia in cattle and buffaloes. An outbreak in water buffaloes with the history of sudden appearance of bloody/ pinkish milk from all four quarters and decrease in milk production to complete agalactia was investigated in village Baghot, Mahendergarh, Haryana, India.

Methods: A total of fifty eight (n=58) biological samples (milk=38, blood=11 and urine =9) samples) from 17 buffaloes were collected from Baghot and its neighbouring villages of district Mahendergarh, Haryana, India. All collected samples were processed for molecular diagnosis using pathogenic Leptospira specific TaqMan probe based real-time PCR and nucleotide sequencing.

Result: The pathogenic *Leptospira* spp. was detected in real time PCR from milk samples of 6 animals (35.2%) out of 17 buffaloes. None of the blood and urine sample was found positive for pathogenic leptosipra spp. PCR amplified product were further sequenced and confirmed pathogenic *Leptospira* spp. The affected animals in the village were treated with parenteral administration of Streptopenicillin, Tranexamic acid and Ascorbic acid for 3-5 days, in recommended doses. Treated animals responded positively to the therapeutic regimen. Furthermore, animal owners and veterinary staff were instructed to take protective measures to ensure no further spread to animals and humans.

Key words: Blood in milk, Bovine leptospirosis, *lip*L32, TaqMan probe, Zoonoses.

INTRODUCTION

Leptospirosis is a zoonotic disease of worldwide importance affecting all mammals with different clinical presentations. It is most commonly seen in cattle, buffaloes, swine, dogs and horses. Leptospirosis is endemic in Southeast Asia (Ahmed *et al.*, 2012). In bovine population, the disease has been reported in India and abroad (Pande *et al.*, 2020).

Leptospira have been categorized into saprophytic and pathogenic groups. All the pathogenic *Leptospira* belonged to *L. interrogans* group. The clinical presentation of the disease depends upon the age, physiological status of host species and pathogenicity of strain and serovar of *Leptospira* spp. (Evangelista and Coburn, 2010). In cattle, leptospirosis is clinically characterized by abortion, infertility, stillbirths and sudden drop in milk production to complete agalactia. In lactating cows, the infection is associated with presence of blood-tinged milk to agalactia (Bolin, 2005). Leptospiral “milk drop syndrome” or agalactia in cattle usually associated with serovar *Hardjo*. Agalactia lasts for 2 to 10 days after which milk production returns to normal, with or without antibiotic treatment (Little, 1986).

The disease is endemic in India and has also been serologically reported in cattle from Haryana (Balamurugan *et al.*, 2016). Most of the work on *Leptospira* in India is done using serological tests such as MAT or ELISA (Balamurgan *et al.*, 2013). In India, serological and molecular tests have been employed to detect pathogenic leptospires primarily using outer membrane protein (OMP) genes such as *lipL21* and *lipL32* (Cheema *et al.*, 2007; Natarajaseenivasan *et al.*, 2011; Balakrishnan and Meenambigai, 2016).

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The present study was aimed to diagnose *leptospirosis* in clinical samples (milk, blood and urine) from buffaloes and to decide line of treatment to control the disease.

MATERIALS AND METHODS

Disease epidemiology

The cases of disease from neighbouring villages started appearing in Disease Investigation Laboratory, Mahendergarh, Haryana. Over the period of four months, a total of 158 lactating cattle (n= 86) and buffaloes (n=72) reported to have signs of blood in milk. A sudden outbreak of the disease

was reported in the month of April, 2017 at village Baghot (28.4224°N, 76.2678°E), Mahendergarh. At the time of disease investigation, seventeen affected animals were present in the village along with the history of abortion in one buffalo during early pregnancy.

The lactating water buffalo population of village Baghot, district Mahendragarh, Haryana, Northern India (Fig 1) often drank water from a common pond which stores canal and waste water from village. The buffalo population of the village was reared in an unorganized fashion with owners having 1-3 buffalo per house. Milking was done manually as every house had one or two animals. The animals were fed with dry fodder, concentrate and greens depending upon the availability.

Sample collection and processing

A total of fifty eight biological samples (milk=38, blood=11 and urine=9) samples) from 17 buffaloes were collected from of village Baghot, Mahendergarh, Haryana, India. Water sample from near about pond was also collected. Re-sampling of milk samples was also done after one week of treatment.

Collection of milk samples

Milk samples were collected from the affected buffaloes with clinical signs and history of blood in milk by hand-milking after proper cleaning of udder by buffalo owners. The milk samples (1 ml) were processed after storing overnight at 4°C. Centrifugation was done 10,000×g for 3 min. The upper fatty layer was then removed using a sterile inoculation loop/paper strip and the supernatant was discarded. The pellet that was stored at -20°C till further use in the Department of Animal Biotechnology, LUVAS, Hisar.

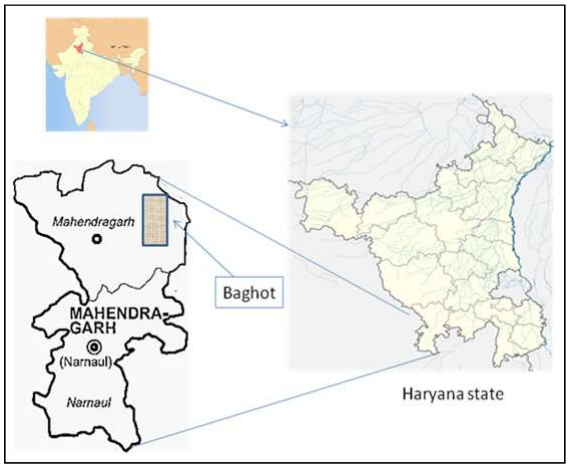


Fig 1: A map of study area of Baghot village in Mahendergarh district of Haryana state of India.

Collection of urine samples

Mid-stream urine samples (approximately 30 ml) were collected from suspected buffaloes into sterile plastic vials and transported to the laboratory at 4°C. Samples were immediately centrifuged at 5000×g for 15 min., the pellets were resuspended in 1 ml of phosphate buffered saline (PBS). The solution was then transferred into a clean 1.5 ml microcentrifuge tube and centrifuged at 8000×g for 5 min. The supernatant was discarded and pellets were resuspended in PBS and stored at 20°C until used for testing (Baquero *et al.*, 2010).

Collection of blood samples

The blood (5 ml) of affected buffaloes were collected from jugular vein in EDTA vials and stored at 4°C till further use.

Collection of water sample from pond

The water sample was collected in sterile vial from the nearby common pond of the village. The sample was kept at 4°C before DNA isolation.

Isolation of DNA

DNA from collected samples (milk, blood, urine and pond water) was extracted by using QIAamp DNA Blood Mini Kit (Qiagen, USA) as per manufacturer's protocol. The concentration and purity of the extracted DNA was measured spectrophotometrically (BIO-RAD, India) by measuring the wavelength at A_{260} and A_{280} and their purity was assessed by taking the 260/280 ratio (Sambrook and Russel, 2001).

Primers and probe

The primers and TaqMan probe (ABI, USA) used in the study in the real-time PCR and conventional PCR for detection of pathogenic *Leptospira* spp. and nucleotide sequencing, respectively, are shown in Table 1.

Real-time PCR (qPCR)

The extracted genomic DNA was subjected to real-time PCR for amplification of *Leptospira* spp. specific pathogenic gene (*Lip L* 32). The real-time PCR assay was performed on Step One Plus (ABI, USA) real-time PCR system in a total reaction volume of 25 µl consisting of 12.5 µl of 2X TaqMan Universal Master Mix (ABI, USA), 1 µl (0.8 µM) of

Table 1: List of primers and probe used in PCR and qPCR analysis.

Targeted gene	Primers/ probe	Nucleotide sequences (5′....3′)	Reference	Amplicon size (bp)
Lip L 32(qPCR)	Forward	AAGCATTACCGCTTGTGGTG	Stoddard *et al.*, 2009	108
	Probe	NED –AAAGCCAGGACAAGCGCCG-NFQ		
	Reverse	GAACTCCCATTTCAGCGATT		
16S rRNA	Forward (LPW57)	AGTTTGATCCTGGCTCAG	Woo *et al.*, 2001	1352
	Reverse (LPW58)	AGGCCCGGGAACGTATTCAC		

each forward primer, reverse primer and probe and 2 µl of extracted DNA as template. The amplification and fluorescence detection was performed using the thermal conditions as: preheating at 50°C for 2 min., initial denaturation at 95°C for 10 min., 40 cycles of denaturation at 95°C for 15 sec. and annealing and extension at 60°C for 1 min.

Amplification of 16S rRNA gene and sequencing

The extracted DNA was also used in conventional PCR for amplification of 16S rRNA gene of *Leptospira* spp. The PCR assay was performed in a total reaction volume of 50 µl consisting of 25 µl of 2X High Fidelity Phusion Master Mix (NEB, UK), 2 µl each of forward and reverse primer, 2 µl of DMSO and 12 µl of extracted DNA as template. The cyclic conditions used for amplification in PCR were: initial denaturation at 98°C for 45 sec., 35 cycles of denaturation at 98°C for 15 sec., annealing at 60°C for 30 sec. and elongation at 72°C for 30 sec. followed by final elongation at 72°C for 5 min. The PCR products were run on 1.5% agarose gel containing 0.5 µg of ethidium bromide/ml and were visualized under gel documentation system (BIO-RAD, India). The PCR products were purified using QIAGEN gel extraction kit (USA) as per manufacturer's protocol and the purified PCR products were subjected to sequencing by dideoxy chain termination method in ABI 3130 XL sequencer (ABI, USA) using BigDye terminator chemistry.

Phylogenetic analysis

Homologous sequences of region of interest in the *Leptospira* spp. were retrieved from the GenBank database through a BLAST search. This search was performed on the NCBI web server (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by submitting the nucleotide sequences obtained from our field samples as queries against the refseq genomic and refseq RNA databases. Then, we manually selected representative *Leptospira* spp. and serovar sequences of pathogenic [*Leptospira kirschneri* (KP114456), *Leptospira copenhageni* (CP020414), *Leptospira borgpetersenii* (AY887899)], intermediate pathogenic [*Leptospira fainei* (AY631885), *Leptospira inadai* (AY631896), *Leptospira broomii* (AY796065)] and non-pathogenic [*Leptospira vanthielli* (AY631897), *Leptospira terpstrae* (AY631888), *Leptospira biflexa* (AY631876), *Leptospira wolbachii* (AY631879)] *Leptospira* spp. from the BLAST results and conducted multiple sequence alignment using BioEdit software. The phylogenetic tree in this study was inferred by the neighbor-joining method implemented in MEGA 6. The multiple sequence alignment of DNA sequences was performed by BioEdit software.

Therapeutic management

After collection of samples, all the affected animals in the village were treated with the parenteral administration of Inj. Streptopenicillin, Inj. Tranexamic acid and Inj. Ascorbic acid in recommended doses by intramuscular route for 3-5 days depending on the clinical recovery in individual cases.

Results and Discussion

Disease epidemiology and sample collection

Over the period of four months (starting from 01.04.2017 to 30.08.2017), a total of 158 lactating cattle (no= 86) and buffaloes (n=72) reported to have signs of blood in milk (Fig 2a and 2b). In both, cattle and buffaloes, one quarter was usually affected. The number of affected animals and their affected quarters had been tabulated (Table 2).

Real-time PCR

Out of the fifty eight biological samples from 17 animals, only fourteen (n=14) milk samples were found positive in real-time PCR. These fourteen milk samples were from six (n=6) buffaloes hence, the per cent prevalence was 35.2% (6/17). The cycle threshold (Ct) of the positive samples was ranged from 26 to 34. None of the blood, urine and pond water samples was found positive in real-time PCR (Fig 3).

The per cent prevalence (35.2%) in present investigation of a suspected outbreak of bovine leptospirosis was comparatively higher than a recent molecular study carried out on the cattle and buffalo population of Gampaha district of Srilanka (Denipitiya *et al.*, 2017) and different districts of Karnatka state, India, (Shivaraj, 2014) in which only 10% and 7.36% of the animals were found positive by real-time PCR and conventional PCR, respectively. The possible reason of higher positivity of samples towards

Table 2: No. of affected animals (cow and buffaloes) and affected quarters.

No. of affected animals (Cow)	No. of quarters affected	No. of affected animals (Buffaloes)	No. of quarters affected
54	1	57	1
14	2	2	2
4	3	0	3
14	4	13	4
86		72	



Fig 2A: Blood-tinged milk collected from affected buffaloes.

leptospirosis between these studies might be attributed to the fact that the sample collection in study performed by Shivaraj (2014) was done randomly from aborted animals without consideration of the clinical signs of leptospirosis, whereas in the present study, samples were taken from the animals that were suspected of having leptospirosis with clinical manifestations in the form of blood-tinged milk and history of abortion.

As bovine display intermittent shedding of leptospires and the affected animals were treated by administering antibiotics by the local veterinarian decreasing the load of bacteria in the clinical samples, we can predict that our qPCR data represent an under-representation of prevalence of leptospirosis in that particular region. This finding is in agreement with Denipitiya *et al.* (2017).

Phylogenetic analysis

The PCR results for 16S rRNA amplification showed amplification of 1343 bp of expected amplicons on agarose gel electropherogram. The sequence obtained was aligned with different *Leptospira* sequences. The BLAST analysis of sequence of 16S rRNA revealed 99.85% identity within the members of pathogenic clad, however, 95.25% with members of intermediate pathogenic clad and only 89.32% along with members of non-pathogenic clad. The highest per cent identity (99.85%) of obtained sequence was observed with *Leptospira interrogans* serovar *Kirschneri* and *Copenhageni*. The genetic analysis of leptospires is very important to establish the genetic relatedness and evolutionary variation among the different *Leptospira* spp. prevalent in a geographical area (Shivaraj, 2014). Sequence analysis of 16S rRNA gene is extensively used for molecular detection or taxonomic analysis of different bacterial species. In this study, 16S rRNA gene-based primers were used and they generated the desired product. The BLAST analysis shows 99.85% similarities with published sequences of pathogenic leptospires of India and other global isolates. These results are in accordance with the previous studies done by Shivaraj (2014).

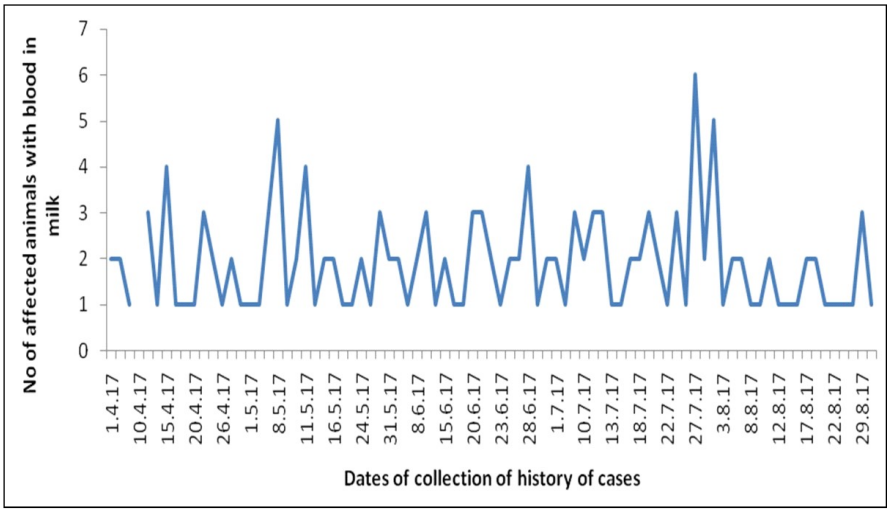


Fig 2B: Temporal distribution of cases of Leptospiral milk drop syndrome.

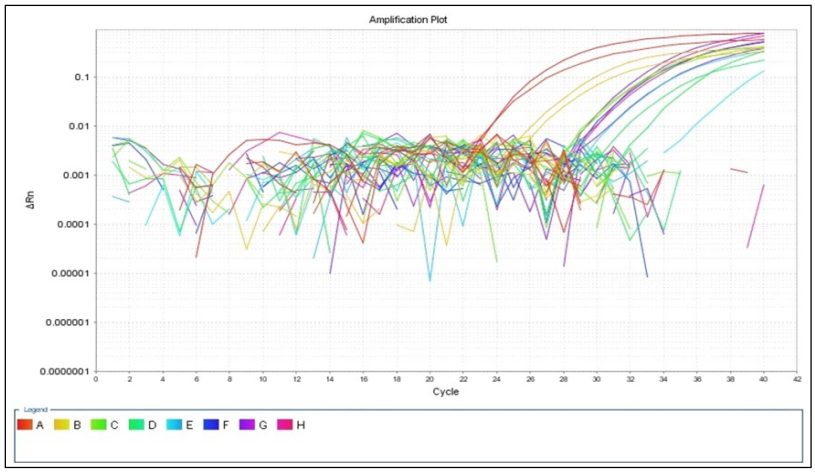


Fig 3: Linear amplification curve showing detection of pathogenic *Leptospira* spp. from haemolactic milk using Qpcr.

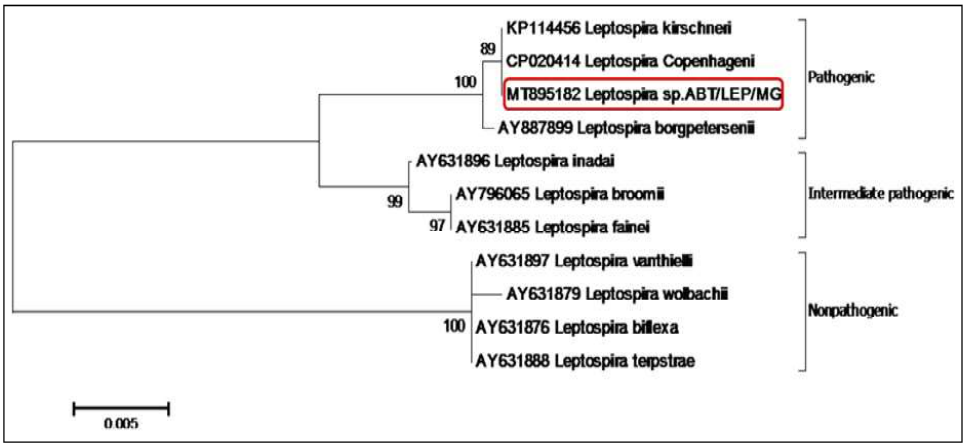


Fig 4: 16S rRNA gene sequence based molecular phylogeny analysis.

The obtained DNA sequences have been deposited in the GenBank database with the accession number MT895182. The Neighbour-Joining phylogenetic tree was constructed using pathogenic, intermediate and non-pathogenic strains of *Leptospira* spp. and the obtained sequence was clustered in pathogenic clad (Fig 4). The positions of insertions or deletions, as well as differing and mixed bases were analysed using multiple sequence alignment tool which includes the positions that differentiate the species within the three *Leptospira* clads of phylogenetic tree. Only two dissimilar bases *i.e.*, at position 95 (C to T) and 210 (A to G) were observed in the present sequence as compared to other sequences of pathogenic *Leptospira* in the phylogenetic tree. The presence of pathogenic leptospiral DNA was confirmed in milk of outbreak affected animals by qPCR and sequencing.

Therapeutic management

Affected animals in the village were successfully treated with the parenteral administration of Inj. Streptopenicillin as antibiotic of choice in leptospirosis, Inj. Tranexamic acid as antifibrinolytic agent and Inj. Ascorbic acid as antioxidant in recommended doses by intramuscular route for 3-5 days depending on the clinical recovery in individual cases. Treated animals responded positively to the therapeutic management. Treated animals either have been recovered completely or in the various stages of recovery uneventfully at the time of next visit. All the recovered animals have gained its previous milk yield except the one which was in late pregnancy. So, it can be stated that confirmatory molecular diagnosis and proper line of treatment may lead to uneventful recovery in buffaloes affected with leptospiral milk drop syndrome with clinical signs in the form of blood in milk. This is also helpful in reducing the chances of further spread of the disease to other animals as well as zoonotic transmission to the humans.

CONCLUSION

In conclusion, clinical signs, response to treatment regimen and molecular diagnosis confirmed leptospirosis or Bovine leptospiral milk drop syndrome in buffaloes. To the best of our knowledge this is the first report of leptospiral mastitis in buffalo in India. The prompt detection by molecular diagnosis will help to minimize this zoonotic disease. The early identification of carrier animals and information regarding the shedding state of leptospires is crucial to prevent the spread of leptospiral infection to other dairy animals as well as to humans. The rapid, reliable, sensitive, specific and easy to perform molecular assays are urgently needed for epidemiological surveillance and early diagnosis of *Leptospira* spp. so that such an outbreak can be easily detected and controlled properly on time to avoid heavy losses to livestock sector and public health.

Conflict of interest

All authors decalre that they have no conflict of interest.

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