RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 8: 1024-1028 (August 2020)

Study of *Leptospira* Infection in Buffaloes through Molecular and Bacteriological Techniques

Pande Dushyant, Khan Wiqar, Chaudhari Sandeep, Shinde Shilpshri, Patil Archana, Likhite Amrut, Allai Rakesh[1]

10.18805/ijar.B-3860

ABSTRACT

In India buffaloes are reared for milk, meat and draft purposes. The economic importance of buffalo rearing in Nagpur is more than cow and contribute major share to the milk production of the district. Apart from its zoonotic implications; Leptospirosis is one of the major causes of abortion, still births, infertility, repeat breeding, decreased milk production, mastitis in ruminants. Considering its endemic nature in India, the present study is aimed at estimating the prevalence of leptospirosis in buffaloes and to establish the possible route of transmission through related canine, rodent and environment. A total of 621(299 each blood and sera and 23 urine) samples collected from 196 apparently healthy and 103 clinically suspected buffaloes and blood samples of 21 stray dogs along with 27 environmental samples were subjected to PCR targeting sec Y gene (202bp) and 16SrRNA gene (331bp).A total of 92 samples including 53 blood samples (buffalo= 45, rodent= 5, canine= 3), 30 buffalo sera samples, four urine samples (buffalo=2 and canine=2) and five environmental samples comprising of 3 water and 2 feed, were processed for isolation of *Leptospira* in the EMJH medium. Out of 196 blood samples of apparently healthy buffaloes a total of 27 (13.77%) animals were found positive for 16S rRNA gene and 3 (1.53%) for *sec Y* gene. Similarly, 17.47% (18/103) animals were positive for 16S rRNA gene while 7 (6.79%) for *sec Y* gene. A total of 30 (15.30%) apparently healthy animals were positive for either 16SrRNA gene or *secY* gene. Whereas 25 (24.27%) samples from suspected animals were positive for either of the gene. All the urine samples from buffaloes, dogs and environmental samples were negative for presence of both the genes. All the samples processed for isolation of organism turned negative. The accurate and rapid diagnosis of leptospirosis is important and beneficial for surveillance and investigation of transmission dynamics of the disease. Even though the active infection can be readily diagnosed through blood samples it is essential to screen each sample by various tests for proper understandings about the disease distribution.

Key words: Buffalo, Canine, Isolation, Leptospirosis, PCR.

INTRODUCTION

In India buffaloes are reared for milk, meat and draft purposes. The total buffalo population in India is 108.7 million which is 21.23% of the total livestock population of country. Buffalo population in Maharashtra is 5.59 million and Nagpur contributes about 1.5% to the total buffalo population of state. The buffaloes in Nagpur district are primarily reared for milk since the buffalo milk fetches higher price than cow. Therefore, the economic importance of buffalo rearing is more and contribute major share to the milk production of the district. Infertility, sterility render the animal uneconomical for rearing leading to culling for slaughter causing huge economic loses to the livestock farmers. Leptospirosis is an important bacterial zoonotic disease that infects humans and wide range of domestic animals. The disease is globally distributed and fatal in humans and has public health significance. (Selvaraj *et al*., 2010 and Ayral *et al*., 2014). Leptospirosis is one of the major causes of abortion, still births, infertility, repeat breeding, failure to thrive, decreased milk production, mastitis in ruminants and great economic loss along with zoonotic infections globally (Costa *et al*., 2015 and Balamurugan *et al*., 2016). In India, the prevalence of Leptospirosis in farm animals including buffaloes in many states has been reported earlier (Varma *et al*., 2001; Sharma *et al.,* 2003; Sivaseelan *et al*., 2003; Balakrishnan *et al*., 2011 and Patel *et al.,* 2017). The endemicity of Leptospira in India since has been reported in 20th century (Chowdry *et al*., 1903, Wooly *et al*., 1911). Tamil Nadu, Andhra Pradesh, Kerala, Maharashtra, Gujarat and Andaman and Nicobar Islands are endemic zones for Leptospirosis (Sharma *et al*., 2003; Balamurugan *et al*., 2013; Patel *et al*., 2014; Balakrishnan *et al*., 2015; Pandian *et al*., 2015 and Balamurugan *et al*., 2017). Contact with infected animals, their urine and tissues, contaminated wet environment, occupational and recreational exposures to the water bodies contaminated with *Leptospira* are the risk factors for the

Department of Veterinary Public Health and Epidemiology Nagpur Veterinary College, Nagpur-440 006, Maharashtra, India.
[1]Department of Animal Reproduction, Gynaecology and Obstetrics, College of Veterinary and Animal Sciences, Udgir-413 517, Maharashtra, India.

Corresponding Author: Shinde Shilpshri, Department of Veterinary Public Health and Epidemiology Nagpur Veterinary College, Nagpur-440 006, Maharashtra, India. Email: shilpi_shri5@rediffmail.com

How to cite this article: Dushyant, P., Wiqar, K., Sandeep, C., Shilpshri, S., Archana, P., Amrut, L. and Rakesh, A. (2020). Study of *Leptospira* Infection in Buffaloes through Molecular and Bacteriological Techniques. Asian Journals of Dairy and Food Research. 54(8): 1024-1028.

Submitted: 11-06-2019 **Accepted:** 13-12-2019 **Published:** 17-02-2020

disease (Hoyos *et al*., 2017). Information on the current status of prevalence of leptospirosis among buffaloes in Nagpur district is inadequate. The present study is aimed at estimating the prevalence of leptospirosis in buffalos employing molecular and bacteriological techniques and to establish the possible route of transmission from the vicinity of the farms.

MATERIALS AND METHODS

Sample collection

A total of 621 (299 blood, 299 sera and 23 urine) samples collected from 196 apparently healthy and 103 clinically suspected buffaloes and blood samples of 21 stray dogs from the vicinity of the farms were collected in vacutainers with EDTA and clot activator. Urine samples from 10 dogs were also collected. The environmental samples consisting of soil (04), water (16) and feed (07) were also collected from the dairies in sterile containers for molecular and bacteriological processing.

DNA extraction

DNA was extracted from the blood, urine and environmental samples as described by Martin *et al*. (2001); Ghatak *et al*. (2013) and Wilson *et al*. (1997) respectively. DNA from soil sample was extracted by Zymo spin fecal DNA extraction kit. Leptospira 16SrRNA gene (331 bp) was targeted to differentiate between the pathogenic and saprophytic species of the *Leptospira* with primers LeptoA-F GGCGGCGCGTCTTAAACATG and LeptoB-R TTCCC CCCATTGAGCAAGATT of 331bp (Merien *et al*., 1992).For detection of pathogenic *Leptospira secY* gene (202bp) of *Leptospira* spp. was targeted with the primers Lepto1-F GCGATTCAGTTTAATCCTGC and Lepto2-R GAGTTA GAGCTCAAATCTAAG of 202 bp (Salgado *et al*., 2015). The electrophoresis was performed by 1.5% agarose gel electrophoresis containing Ethidium bromide (0.5µg/ml) at 100 volts and the gel was visualized under UV trans-illuminator using Gel Documentation System (BIORAD).

Isolation of *Leptospira*

A total of 92 samples including 53 blood samples (buffalo= 45, rodent= 5, canine= 3), 30 buffaloes sera samples, four urine samples (buffalo=2 and canine=2) and five environmental samples comprising of 3 water and 2 feed, were processed for isolation of *Leptospira* in the Ellinghousan MacClugh. Johnson and Harris medium as described by Balamurugan *et al.* (2013). The blood, sera, urine and environmental samples were aseptically inoculated in the 8ml sterile (EMJH) medium containing rabbit sera, β-lactamase, 5-fluoro Uracil and sodium polyanethole sulphonic acid sodium and incubated at 28°C for one week. The incubated samples were regularly (weekly) examined under dark field microscope for development of Dinger's disk and cork screw shaped motility of the spiral organisms.

RESULTS AND DISCUSSION

Molecular Detection

The prevalence data of healthy and suspected animals from Nagpur region obtained by PCR targeting 331bp16*S rRNA* gene and 202 bp *SecY* gene is summarized in Table 1.

Out of 196 apparently healthy buffaloes a total of 27 (13.77%) were found positive for 16S rRNA gene and 3(1.53%) for *secY* gene. Similarly, of the 103 suspected animals; 17.47% (18) were positive for 16S rRNA gene followed by 7(6.79%) for *secY* gene. It is observed that a total of 30(15.30%) apparently healthy animals were positive for either 16SrRNA gene or *secY* gene. Whereas 25(24.27%) from suspected animals were positive for either of the genes. Comparatively more number of animals 27(13.77%) and 18(17.48%) were positive for 16S rRNA gene from apparently healthy and suspected category respectively. High prevalence of 16srRNA gene is because 16srRNA gene (331 bp) reveals both saprophytic and pathogenic species. The *secY* gene (202 bp) was found in 3(1.53%) and 7(6.80%) apparently healthy and suspected animal samples respectively revealing the presence of pathogenic strains of *Leptospira* in both the categories. All the urine samples from buffalos, dogs and environmental samples were negative for presence of both the genes.

Isolation

A total of 92 samples including 53 blood samples (buffalo= 45, rodent= 5, canine= 3), 30 buffalo sera samples, four urine samples (buffalo=2 and canine=2) and five environmental samples comprising of 3 water and 2 feed, *Leptospira* in the EMJH medium revealed no growth of leptospiral organisms after incubation of 4-5 weeks.

The findings of the present study were in concurrence with the findings of the studies conducted in the other parts of the world and reported the prevalence of leptospirosis in the range of 16.66% - 42.85% by PCR targeting the 16S rRNA gene in buffaloes (Marianelli *et al*., 2007, Balamurugan *et al.,* 2013, Balakrishnan *et al.,* 2014, Shafighi *et al.,* 2014). The present study highlights the wide spread nature of leptospirosis in buffaloes of peri urban region of Nagpur. Further studies employing, pulsed field gel electrophoresis,

Table 1: Prevalence of *Leptospira* in healthy and suspected buffaloes.

Total No. of blood sample (299)		No. of apparently healthy animals positive		No. of suspected animals positive		No. of urine samples positive	
Apparently healthy	Suspected	16S rRNA	*secY*	16S rRNA	*secY*	16S rRNA	*secY*
196	103	27 (13.77%)	3(1.53%)	18(17.47%)	7 (6.79%)	0	0

hybridization techniques and pathological studies are required to identify the species prevalence as well as to know the pathogenic nature.

The detection of 16SrRNA (331 bp) in present study in both apparently healthy and suspected cases reveals the presence of saprophytic and also pathogenic *Leptospira* in the blood of animals. Merien *et al.* (1992) also reported the use of 16S rRNA gene as an epidemiological marker for differentiating infected species in leptospirosis suspected humans and animals. The present study reported detection of *secY* (202 bp) housekeeping gene, in apparently healthy (1.53%) and suspected (6.80%) animals which indicated the presence of pathogenic *Leptospira* species in both categories revealing the carrier status of healthy animals.

None of the urine samples (23) collected from the suspected animals was positive for the presence of both genes i.e.16SrRNA & *sec Y*. Low urinary PCR positivity was reported in cattle (4.02%) and buffalo (4.8%) targeting 16srRNA and lipL32 gene respectively (Burhan *et al.,* 2000 and Hazikolaei *et al*., 2016). However, Hamond *et al.* (2015) reported overall 32.4% PCR prevalence in livestock (cattle, horses, goats & pigs) of Brazil with reproductive problems which includes 21.6% prevalence in cattle by urinary PCR. The variation in the Urinary PCR results including the negative results of urine samples of suspected animals may be due to fact that results of PCR vary according to the species of animals and samples used for screening (Marianelli *et al.,* 2007).

All the 27 environmental samples (soil: n = 04; water: n = 16 and feed: n = 07) processed in the present study were negative for presence of both the genes. The findings of present study are in contrast with the findings of Ganoza *et al*. (2006) and Ridzlan *et al*. (2010) who reported 67.9% and 66.67% prevalence of leptospires in water and soil samples from Iquitos and Malaysia respectively. Similarly, Rawlin *et al*. (2014) also reported 62.5% prevalence of pathogenic leptospiral DNA in surface water samples collected from St. Kitts. Appropriate conditions such as pH of water, temperature, characteristics of the water or soil and availability of wild animals which act as reservoirs help the leptospires to sustain in the environment. Heavy rainfall could facilitate the spread of the organism and contaminate the environment (Ridzlan *et al.,* 2010). Therefore, information about the circulating serovars, their maintenance hosts and changes in the climatic and environmental conditions is essential for understanding the epidemiology of leptospirosis in a region (Rawlin *et al*., 2014).

In leptospirosis bacteria are found in blood in first few days followed by immune phase which is characterized by the appearance of antibodies and clearance of leptospires from the blood stream. Isolation of organisms takes weeks and antibodies can be detected in the blood after one week, under such circumstances PCR of the blood samples can rapidly confirms the diagnosis of disease in early phases (with in first 2 weeks). However due to presence of very small amount of *Leptospira* in blood very sensitive diagnostic tests are required (Bourhy *et al.,* 2011).

The role of *Leptospira* in abortions and reproductive disorders has already been established (Balamurugan *et al.,* 2013). Further, the infected cattle are known to be maintenance host resulting in the infection or may be clinically normal but harbour the infection and acts as potential source of infection (Higgins *et al.,* 1980 and Balamurugan *et al.,* 2013). The present study was successful in diagnosing the infection in apparently healthy buffaloes in the Nagpur region highlighting the carrier status of these animals.

Transmission of the infection among the maintenance host (which is largely asymptomatic) is efficient and they also transmit the infection to other animals, humans get infection either directly through the infected animals or indirectly through contaminated environmental sources (Balamurugan *et al.,* 2013). The findings of the present study do not establish a direct correlation among the reservoir hosts and environment due to variations in topographic and climatic conditions, pH and the moisture conditions and water holding capacity of soil. Water logging condition of the field enhances the growth of leptospires along with salt concentration. Alkalinity of soil with pH 7.2-7.6 plays a key role for free growing organisms. Hygienic conditions and management practices of the farm reduces the chances of spill over of infection to environment. Again, the time of exposure with the reservoir hosts and surface water, considering wallowing habit of buffaloes are the probable reasons for *Leptospira* infection (Saito *et al.*2014). Further, temperature, pH of soil and water, changes in water quality and quantity due to variations in precipitation and temperature might also affect the rate of transmission (Rawlin *et al*., 2014).

The present study reports negative results for isolation of Leptospira. The other studies conducted in different part of the world reported isolation of Leptospira from domestic animals and rodents in the range of 2 to 26% (Koizumi *et al* 2009; Suepaul *et al* 2010; Benacer *et al* 2013; Director *et al* 2014 and Kurilung *et al* 2017). The findings of the present study are in concurrence with Vasconellos *et al* (2001), Harkin *et al* (2003); Fearnely *et al* (2008) and Fornazari *et al* (2012).

PCR positive and culturally negative results have also been reported by Boonslip *et al*. (2011) and Thaipadunpanit *et al*. (2011). There are several explanations for PCR positive but culture negative results. The animal might have received the antimicrobial treatment, but the drug may not have cleared the non-viable organism by the times blood samples were taken. *Leptospira* spp. might also perish in the blood collection tube prior to the laboratory culture due to fluctuations in the ambient temperature. Timing of sampling after the onset of symptoms is also important. (Boonslip *et al*., 2011).

The isolation of *Leptospira* depends upon the clinical material and stage of infection. During the leptospiraemia

phase (1-10 days) the isolation may be successful from blood, liver, spleen and kidney. The samples collected in the present study were from peri urban and urban areas of Nagpur city. The samples were collected based upon the clinical signs and at the time of sample collection the stage of infection in buffaloes is quite difficult to note. There is also the possibility of samples getting contaminated and due slow growth rate of the organism their growth is overwhelmed by contaminants leading to negative results.

Additionally, the success of isolation is influenced by various factors such as number of organisms per inoculation, type of media used, type of specimen, phase of infection and contamination of samples especially urine. The difficulty in isolation of *Leptospira* and slow growth of this fastidious organism made the culture technique time consuming and with low sensitivity (Bourhy *et al*., 2011; Suepaul *et al*., 2010; Benacer *et al*., 2013 and Kurilung *et al*., 2017).

CONCLUSION

Leptospirosis is globally widespread bacterial zoonoses. The disease in endemic in many states of India. The accurate and rapid diagnosis of leptospirosis is important and beneficial for surveillance and investigation of transmission dynamics of the disease. Even though the active infection can be readily diagnosed through blood samples it is essential to screen sample by various tests for proper understandings about the disease distribution.

ACKNOWLEDGEMENT

The authors would like to thank to Niche Area of Excellence as 'Centre for Zoonoses' funded by Indian Council of Agricultural Research, New Delhi.

CONFLICT OF INTEREST: None

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