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Molecular Characterization and Sero-epidemiological Study of Leptospirosis in Cattle of Nagpur and Surrounding Regions

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A B S T R A C T

A zoonotic disease Leptospirosis is caused by pathogen of the genus *Leptospira* and it is an emerging global public health problem. In the present study a total of 266 cattle blood and sera samples were collected from the towns and villages of Nagpur, Wardha, Bhandara Gadchiroli and Durg districts during December 2017 to 2018 and tested for the presence of *Leptospira*. These samples were from randomly selected herds with history of repeated breeding, abortion, reproductive disorders, etc. also including some apparently healthy animals. The presence of leptospiral DNA in blood sample was assayed by PCR amplification of *rrs* (16S rRNA) gene. Antibodies against *Leptospira* serovars were tested using an enzyme-linked immunosorbent assay (ELISA) and microscopic agglutination test (MAT). Out of 266 blood samples, in 33 samples leptospires DNA was identified with the frequency of 12.40%. A total of 53 cattle sera were positive in commercial *Leptospira* Bovine Hardjo ELISA kit indicated 19.92% seroprevalence. In the MAT analysis 120 samples revealed presence of different serovars with the seropositivity 45.11%. The study supports the probable role of cattle in maintaining *Leptospira* Hardjo along with some other serovars and warrants an intensive control and surveillance programme for reducing leptospirosis in cattle.

Introduction

Leptospirosis is among the fastest globally re-emerging anthropozoonosis caused by pathogenic bacteria of the genus *Leptospira*. The disease affects a variety of domestic animals *viz.* cattle, buffalo, sheep, goat, horse and swine which results into heavy economic losses to the farming community (Srivastava, 2008). It is more predominate in tropical countries having high rain fall, humidity, presence of marshy land and paddy grown

area (Favero *et al.,* 2017). Infection in humans usually results from direct or indirect exposure to the urine of infected animals or healthy carriers, aborted fetus and uterine discharges which may contaminate soil, pasture, drinking water and feed is the main source of infection (Adugna, 2016, Favero *et al.,* 2017). Extreme weather events such as cyclones and floods, increased rainfall associated with global warming are considered as the factors for the increased incidence of leptospirosis in animals and humans (Lau *et al.,* 2010).

Infection of *Leptospira* organism in cattle can be divided into two groups of strain; stain adapted to bovine and strain adapted to other domestic or wild animal. The serovar mainly associated with cattle is serovar Hardjo however; serovars Pomona, Icterohaemorrhagiae Hebdomadis, Australis, Bankinang and Grippotyphosa can also be associated to bovine leptospirosis (Balamurugan *et al.,* 2018). *Leptospira interrogans* serovar Hardjo type Hardjobovis is the primary cause of acute and chronic leptospirosis in cattle, and in addition causes persistent infection of kidneys and female reproductive tract (Morey *et al.,* 2006). In bovines, clinical signs are usually mild or inapparent when infected by host-adapted serovars. However, for infections with non-host-adapted serovars, clinical symptoms may range from mild to severe (Lilenbaum and Martins, 2014). The disease in bovine is responsible for direct or indirect economic losses, which include costs of abortion, stillbirth, infertility, failure to thrive, reduced productivity and decreased milk yield (Quinn *et al.,* 1994). The economic losses to the farming community is also due to associated veterinary costs in domestic and commercial livestock, with potential for malnutrition and impoverishment amongst individuals and communities dependent on animal sources of protein, especially in subsistence economies (Srivastava, 2008). As per the studies conducted in different parts of the world, the serovar responsible for reproductive losses in case of bovines depends on the type of serovar endemic in that region since leptospiral antibodies may present in the serum for considerable period of time after infection, which indicates the present or part exposure to the leptospiral antigen (Balamurgan *et al.,* 2013). The early identification of carrier animals and information on the shedding state are crucial to prevent the spread of leptospiral infection to other animals and humans. By considering the all these facts the aim of the present study was to characterize *Leptospira* organism by molecular and serological techniques in the Nagpur and surrounding regions.

Materials and Methods

Details of samples

A total of 266 cattle blood and sera samples were collected during the period from December 2017 to December 2018 from Nagpur, Wardha, Bhandara, Gadchiroli and Durg districts. The region of sample collection is located in eastern region of the Indian state of Maharashtra (Nagpur Division). The latitude and longitude of the region is 21° 9′ 36″ N, 79° 4′ 48″ E. The area is characterized by a tropical climate with average temperature of 39.22^0C. During monsoon, it is very humid. The samples collected were from randomly selected herds with history of repeated breeding, abortion, reproductive disorders, etc. including some apparently healthy animals. No animal had history of earlier vaccination.

Molecular characterization

DNA isolation from whole blood of animal samples was carried out as per the method of Martin *et al.,* (2004) with slight modification.

All DNA extracted from the blood samples tested by PCR amplification using oligonucleotide primers (Lepto A. 5′-GGC GGC GCG TCT TAA ACA TG-3′) (Lepto B. 5′-TTC CCC CCA TTG AGC AAG ATT-3′) to amplify a 331 base pair fragment of *rrs* (16S rRNA) gene common to all *Leptospires* (pathogenic and nonpathogenic) as described by Merien *et al.*, (1992). The PCR was carried out in a PCR tube (0.2ml) as per the protocol described by Merien *et al.*, (1992) with modifications. A 25 µl reaction volume consisting of 2 µl of template DNA added to a tube containing 12.5 µl of 2X Master Mix, 1 µl of each primer and 8.5µl of nuclease free water. Amplification was performed in a thermal cycler (Applied Biosystems, USA) with initial denaturation at 94°Ċ for 3 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 60°Ċ for 30 sec, extension at 72°C for 40 sec and then a final extension at 72°C for 10 min. Negative (no DNA template) and positive (DNA from *Leptospira interrogans*) controls were also performed and aliquots were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide and images were obtained by UV transillumination.

Enzyme Linked Immunosorbent Assay (ELISA)

A commercial indirect ELISA kit (PrioCHECK™ L. hardjo Ab Strip Kit) was used for the detection of antibodies against antibodies against *Leptospira interrogans* serovar Hardjo in serum. Indirect ELISA was performed as per the protocol outlined in the user's manual. The optical density of microwells was read using a micro plate reader (Thermo Scientific™) at a wavelength of 450. ELISA optical density (OD) readings were transformed to serum/positive percentage (PP) according to specific equation cited by manufacture.

Microscopic Agglutination Test (MAT)

All the sera samples from animals were subjected to MAT at Indian Council of Agricultural Research-National Institute of Veterinary Epidemiology and Disease Informatics (ICAR-NIVEDI), Bengaluru. Serological tests and leptospira culture protocol in this study were based on the standard methodology using a panel of 18 reference serovars. The panel of antigens included *L. interrogans* serovars Australis (Aus), Bankinang (Aut), Canicola (Can), Sejroe (Sej), Hebdomadis (Heb), Icterohaemorrhagiae (Ict), Pyrogenes (Pyr), Kaup (Kau), Pomona (Pom), Hurstbridge (Hus), Javanica (Jav), Panama (Pan), Copenhageni (Cop), Bataviae (Bat), Djasiman (Dja), Shermani (She) and Grippotyphosa (Grippo). A MAT titre of 1:40 or above was taken as a positive reactor.

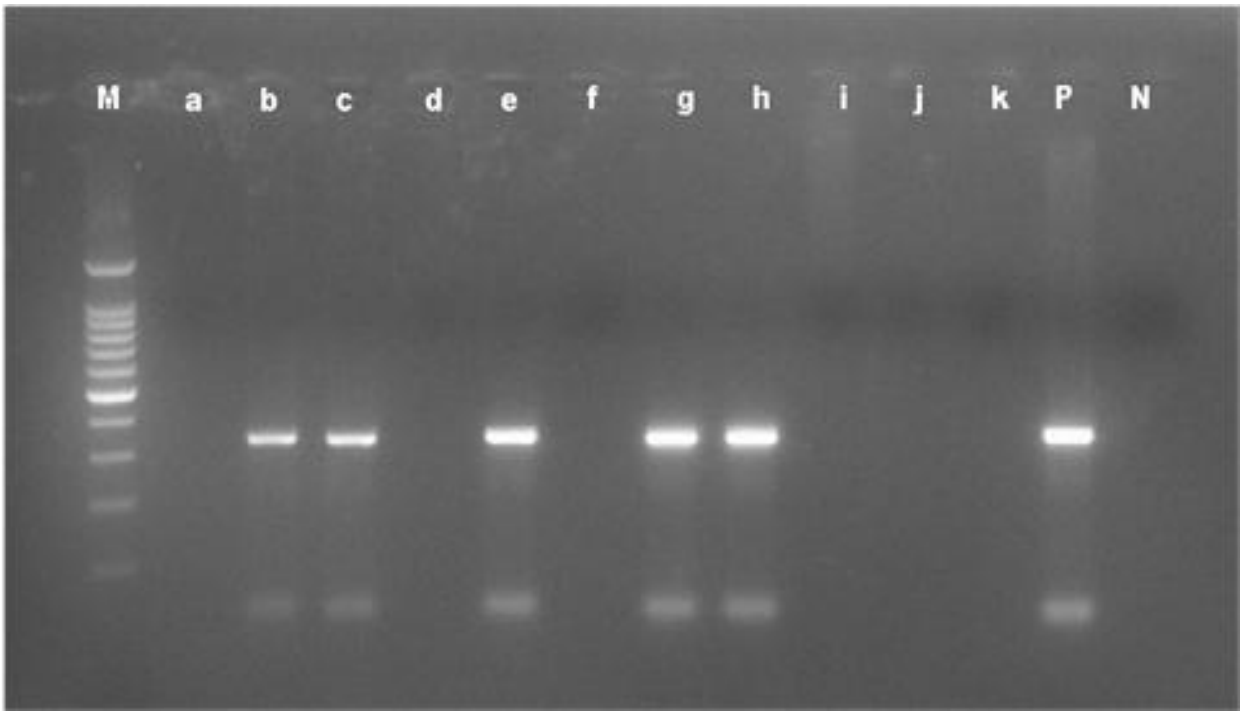
Results and Discussion

The PCR assay used in the present study was genus specific and detected all leptospiral serovars. The results of this study showed a high frequency of *Leptospira* spp. in the blood samples of cattle. The results of study showed that out of 266 blood samples, 331 bp fragment of *rrs* gene was amplified in 33 (12.40%) samples (Fig. 1). The results of the study revealed the direct detection of *Leptospira* spp. in the blood of cattle by PCR is useful in rapid identification of carrier animals. PCR has the advantage that it does not require the isolation of the organism and detects DNA from both viable and nonviable organisms (Noubade *et al.*, 2002). The finding of the study supported the findings of Cheema *et al.*, (2007), Jafari *et al.*, (2011) and Patel *et al.*, (2017) who too carried out the investigation on field samples.

In present study *Leptospira* spp. serovar Hardjo is present with a prevalence of 19.92%

(53/266) in cattle despite the lack of reports on clinical cases of the disease. It is therefore possible that losses from leptospirosis in the cattle population in this region may be underestimated as there is evidence from a number of countries including India that serovar Hardjo continues to cause substantial reproductive losses in cattle through abortion and infertility. In similar studies by using the same *Leptospira* Bovine Hardjo kit, the different rate of seroprevalence was reported for leptospirosis in various Indian states such as Maharashtra (30.40%), Gujarat (13.50%), Punjab (3.70%), Tamil Nadu (50%), Haryana (4.46%), Telangana (4%), Jharkhand (10%), Karnataka (16.66%) and Chhattisgarh (23.68%) (Balmurugan *et al.,* 2016). Moreover, in some of the countries also serovar hardjo was found to be most prevalent i.e. in Arizona, USA (Songer *et al.,* 1983), Malaysia (El Jalii, 2008), Turkish (Kocabiyik and Cetin, 2004), Iraq (Al-Badrawi *et al.,* 2010) and Brazil (Mineiro *et al.,* 2011). Besides being an important cause of bovine abortion, reduced fertility and agalactia, serovar Hardjo also poses a potential zoonotic threat to humans who are exposed to infected cattle (Balmurugan *et al.,* 2016).

Fig.1 Agarose gel PCR products



Lane M: 100 bp DNA Ladder
Lane P: Positive control (331 bp),
Lane N: Negative control,
Lane a, d, f, i, j, k: Negative samples
Lane b, c, e, g, h: Positive samples

In cattle out of 266 sera screened, 120 sera were positive for with one or more serovars with an overall seroprevalence rate of 45.11%. The highest seropositivity was recorded against serovar Panama (30.83%) followed by Hebdomadis (25.83%), Javanica (25%), Icterohaemorrhagiae (18.33%), Djasiman (17.5%), Sejroe (12.5%), Bataviae (10.83%), Hurstbridge (10%), Australis (8.33%), Automnalis (8.33%), Copenhageni

(7.5%), Kaup (7.5%), Pomona (7.5%), Canicola (5.83%), Shermani (5.43%), Grippotyphosa (5%) and Pyrogenes (4.16%). The higher prevalence of these serovars in this study could be explained by the fact that the cattle had close contact with the reservoirs of this serovar. In addition, the longer immune response induced by this serovar and the higher frequency of new infections with this serovar may account for the observed results, as suggested by Guitin *et al.,* 2001. This study supports that bovines may have a role in maintaining different serovars, apart from being a well-known reservoir for Hardjo serovar in cattle in study region. The available literatures did not reveal any such studies in this region. In India a number of different serovars had been reported from time to time from the different states such as serovars Pomona, Hebdomadis, Medanensis, Hardjo, Andamana and Saxkoebing from Andhra Pradesh (Mrunalini *et al.,* 2000), Grippotyphosa, Pomona and Icterohemorhagiae from Andaman and Nicobar (Varma *et al.,* 2001), Hardjo, Bataviae, Canicola, and Australis from West Bengal (Mandal *et al.,* 2008), Icterohaemorrhagiae and Grippotyphosa from Uttar Pradesh (Sachan *et al.,* 2011) and Pomona in cattle of various South Gujarat district (Patel *et al.,* 2014) during different survey. Several factors such as herd size, co-grazing with infected cattle, access to contaminated water sources, use of infected bulls, inadequate husbandry practices, and replacement with animals from other farms have been found to be associated with leptospiral infections in cattle (Lilenbaum and Santos, 1996; Guitian *et al.,* 2001; Aslantas and Ozdemir, 2005).

In conclusion, in agreement with similar studies, the results obtained from the present study revealed that direct detection of *Leptospira* spp. in the blood samples of carriers by PCR is useful in rapid identification of carrier animals. Significant prevalence of *Leptospira* Hardjo serovar in the organised dairy farms of this region proves the endemicity of this serovar in dairy cattle. High prevalence of different leptospiral serovars in apparently healthy bovine in this region indicates the presence of the agent in the environment which may be a potential zoonotic risk to animal hadlers, milkers, and other domestic species in the farm. This study warrants the need for continuous monitoring of *Leptospira* burden in animals and human in close proximity to each other to combat the zoonotic infection.

Conflict of interest

No conflicts of interests are declared by authors for the contents in the manuscript.

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