

Original Research Article

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Polymerase Chain Reaction in the Diagnosis of Spontaneous Leptospirosis in Bovines

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ABSTRACT

Keywords

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Accurate and prompt diagnosis of leptospiral infection in bovine is difficult due to limitation of current procedures. The present study describes the application of polymerase chain reaction (PCR) for detection of leptospiral deoxyribonucleic acid (DNA) in blood and urine samples in the spontaneous cases of bovine leptospirosis. In most of studies leptospire DNA detected by PCR in experimentally induced infection so in present study trying to evaluate this assay in naturally infected cattle and buffalo. A total of 120 blood and 304 urine samples were collected from cattle and buffaloes. These samples were subjected to PCR using two sets of primers i.e. G1/G2 and LipL32 and it revealed that 9/120 blood and 33/304 urine samples were found to be positive. The results suggested that PCR could be an excellent approach for diagnosis of Leptospirosis in field samples also. The specificity of primers was checked by using reference pathogenic serovar of *Leptospira*.

Introduction

Leptospirosis is an important zoonotic infection of worldwide distribution and is caused by pathogenic spirochetes of the genus *Leptospira*. In bovines course of the disease ranges from acute to chronic form. The acute form is manifested by fever, hematuria, hemoglobinuria, meningitis and death, while the chronic form is characterised by various reproductive expressions like irregular estrus, repeat breeding, birth of weak calves, abortion, mummification of foetuse, retention of fetal membranes, mastitis, etc. depending upon serovar involved and susceptibility of animals (Ellis, 1994). Clinically bovine

leptospirosis is difficult to diagnose because these expressions are nonspecific and may be easily confused with other febrile ailments (Ellis, 1984). Mostly the disease occurs in subclinical or chronic form. Various methods like serology (MAT), dark-field microscopy (DFM), microbial culture, immune based and molecular tools (PCR, qPCR) are used to diagnose leptospirosis these days (Turner, 1970; Szeredi and Haake, 2006; Sreekumari *et al.*, 2010). Each of these methods has their merits and demerits but the molecular tools have been considered to be the most precise and quick diagnostic method and have been

used on blood and urine samples. In Indian literature there is paucity of information on the application of molecular tools like PCR and qPCR in diagnosis of bovine Leptospirosis (Meenambigai *et al.*, 2011). As such the aim of this investigation is to study the suitability of PCR in detecting leptospiral DNA in clinical samples like urine and blood in naturally infected bovine leptospirosis cases.

Materials and Methods

A total of 120 blood and 304 urine samples were collected from bovines (cattle and buffaloes) belonging to individual households, Panjarapol, slaughter house (only buffaloes) and cases presented at TVCC, College of Veterinary Science & A. H., Navsari.

Collection of urine samples

Midstream urine samples (30-50 ml) were collected from apparently healthy and sick cattle (N=232) and buffaloes (N=72) in plastic containers after cleaning the vulvar and perianal areas whose serum/blood samples were already collected.

All the urine samples were placed in ice container and transported to laboratory as soon as possible.

Sample preparation

In laboratory urine samples were immediately filtered using 0.45micrometer pore size filters (Pall Life Science) in sterile 50 ml conical centrifuge tubes.

The filtrate was centrifuged at 7800 rpm for 20 minutes at 4⁰C in a refrigerated centrifuge. After discarding the supernatant the pellet was vortex and transferred to sterile 1.5 ml microcentrifuge tube and resuspended in phosphate-buffered saline (pH 7.2).

Genomic DNA isolation from urine samples

There suspended pellets were used for DNA extraction using the method described by Ambily *et al.*, (2012) with required modifications. Briefly, resuspended pellet samples were centrifuged at 14000 rpm for 15 minutes at 4⁰C. The supernatant was discarded and 1ml PBS (7.2 pH) was added to the pellet and centrifuged at 14000 rpm for 10 minutes at 4⁰C. This step was repeated twice, supernatant was discarded and 0.5ml nuclease free water (Qiagen) was added and centrifuged at 14000 rpm for 10 minutes at 4⁰C and this step was again repeated. After discarding supernatant 0.1ml nuclease free water was added to the pellet and vortexed. The tube was placed in boiling water bath for 15 minutes and immediately snap chilled for 20 minutes. After vortexing, samples were stored at -20⁰C until further analysis. Quality and purity of DNA was checked by submarine agarose gel electrophoresis using 0.8 per cent agarose in 1X TAE (PH 8.0) buffer (Sambrook and Russel, 2001). Ethidium bromide was added @ 0.5µg/ml. The wells were charged with 4µl of DNA preparations mixed with 1µl of 6X gel loading buffer dye. Electrophoresis was carried out at 5V/cm for 20 min at room temperature and then the DNA was visualized under UV transilluminator.

Genomic DNA isolation from blood samples

DNA was extracted from blood samples collected in K3 EDTA vacutainers using DNeasy® Blood and Tissue kit (Cat. No. 69504, QIAGEN, Germany).DNA extraction was performed as per the manufacturer's protocol: (I). In sterile 1.5 ml microcentrifuge tube 20 µl proteinase K was pipetted in. (II) Anticoagulated blood (100 µl) was added and vortexed for 15 seconds. The volume was

adjusted to 220 µl with PBS. (III) 200 µl buffer AL was added and vortexed for 15 seconds. (IV) The tube was incubated at 56°C for 10 minutes. (V) 200 µl ethanol (96-100 %) was added to the sample and mixed by vortexing for 15 seconds. (VI) The mixture was carefully pipetted out from the previous step (No. V) to the DNeasy Mini spin column with a 2 ml collection tube. The cap of minispin column was closed and centrifuged at 8000 rpm for 1 min. The DNeasy Mini spin column was taken out and placed in another clean 2ml collection tube and the tube containing the filtrate was discarded. (VII) The DNeasy Mini spin column was placed in a new 2 ml collection tube provided in kit and 500 µl buffer AW1 (prepared by adding 25ml absolute ethanol) was added. The cap was closed and centrifuged at 8000 rpm for 1 min and the collection tube with filtrate was discarded as it was done at step VI. (VIII) The DNeasy Mini spin column was placed in a new 2 ml collection tube provided with the kit and 500 µl buffer AW2 (prepared by adding 30 ml absolute ethanol) was added. The cap was closed and centrifuged for 3 min at 14,000 rpm to dry the DNeasy membrane. Filtrate was discarded with collection tube (IX) The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube having built in cover cap. (X) The DNeasy Mini spin column was opened carefully and 100 µl buffer AE was added directly onto the DNeasy membrane. (XI) The tube was incubated at room temperature for 1 minute and centrifuged at 8000 rpm for 1 min. (XII) The filtrate in the collection tube at this stage contained the eluted DNA from the DNeasy minispin column and it was stored at -20°C for future use.

Oligonucleotides primers for PCR

PCR was performed using two sets of primers of these one being conventional i.e. G1/G2 (Gravekamp *et al.*, 1983) and the other was

gene based i.e. LipL32 (Cheema *et al.*, 2007). Primers G1 and G2 had sequence 5'CTGAATCGCTGTATAAAAGT3' and 5'GGAAAACAAATGGTCGGAAG3', respectively. The sequence of primers LipL32 gene was 5'GTTCGACATGAAAAAAGTTTCGATTTT G3' and 5'CTGCAGTTACTTAGTCGCGTCAGAAGC3', respectively, for forward and reverse primer.

PCR assay

The PCR assay was performed in a final volume of 25 µl mixture containing DNase - RNase-free water: 7.5 µl, Taq PCR master Mix (M/S: Cat. No. 201443, QIAGEN, Germany): 12.5 µl (having 2.5 units Taq DNA Polymerase, 1X PCR Buffer containing 1.5 mM MgCl₂ and 200 µM each dNTPs), forward Primer: 1.0 µl (20 pmoles/ µl), reverse Primer: 1.0 µl (20 pmoles/ µl) and template DNA: 3.0 µl. The PCR assay was performed as described previously with minor modification in thermal cycler protocol (M/S: BIORAD) (Cheema *et al.*, 2007).

Briefly, initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR protocol designed for both G1/G2 and LipL32 primers were same. The product was checked for amplification and absence of spurious products by electrophoresis using 0.8 % agarose gel. The gel was visualized by gel documentation (M/S: Syn Gene, Gene Genius BioImaging System, UK) system and photographed.

Standardization of PCR

During standardization of the method the G1/G2 and LipL32 primers showed positivity in conventional PCR with amplification using DNA from reference serovars.

Results and Discussion

In the present study a total of 120 blood and 304 urine samples (cattle and buffaloes) were subjected to PCR, out of these 9 (7.50 %) and 33 (10.85 %) samples, respectively were found to be positive with G1/G2 primer. In cattle a total 86 blood and 232 urine samples were screened using PCR and out of which 7 (8.14 %) and 24 (10.34 %) samples, respectively were found to be positive for leptospiral DNA. Whereas in buffaloes out of 34 blood and 72 urine samples screened, 2 (5.88 %) and 9 (12.50 %) samples, respectively were found to be positive for the presence of leptospiral DNA (Table 1).

All the positive samples were retested using another set of primers i.e. LipL32. These samples equally detected leptospiral DNA in blood and urine samples indicating the comparative usefulness of LipL32 (Fig. 1). In the present study attempt were made to

determine whether conventional PCR with primers derived from the LipL32 sequence and G1/G2 could be used to directly detect pathogenic leptospire in biological samples as an alternative to traditional diagnostic methods like leptospiral isolation, ELISA and serology using the MAT. The production of antibodies against *Leptospira* in the body occurs several days after the occurrence of leptospirosis and rapidly starts clearing bacteria from blood and tissue by immune system. Some of the leptospire usually escape the reach of the immune system and may persist in kidney tubules, liver, uterus, eye and meninge. Animals that recovered from acute leptospirosis may be carrying the infection and leptospire may continue in their kidney tubules from a few days to several years. In such cases the agent may not found in blood but may excrete through urine. So in both these acute and chronic condition PCR assay may be used to detect leptospiral DNA.

Table.1 Detection of pathogenic leptospire in clinical samples by PCR

Sr. No.	Type of clinical sample	Animal Species	No. of samples tested	No. of positive samples using G1/G2 Primer pair
1.	Blood	Cattle	86	7 (8.14 %)
		Buffaloes	34	2 (5.88%)
		Total	120	9 (7.50%)
2.	Urine	Cattle	232	24 (10.34 %)
		Buffaloes	72	9 (12.50%)
		Total	304	33(10.85%)

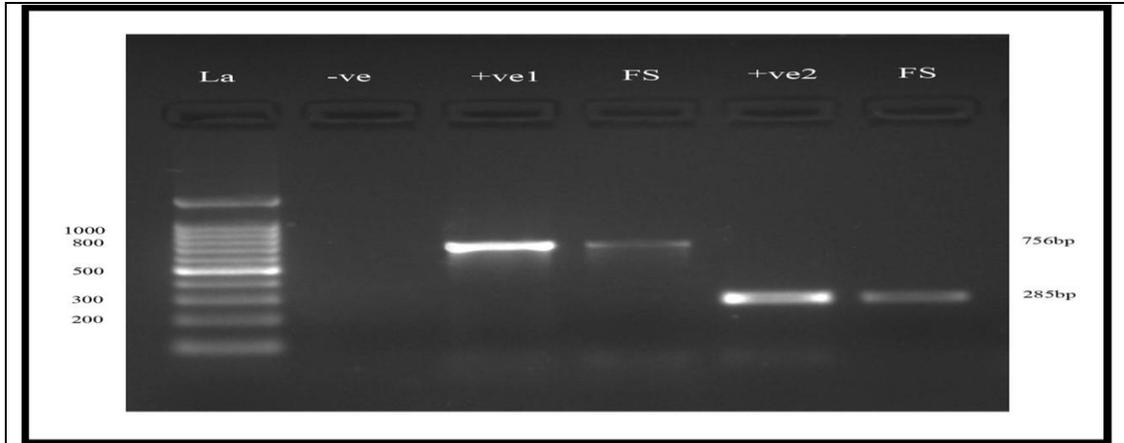


Figure 1:

- (A) Agarose gel electrophoresis pattern of PCR product of template DNA amplified with primer pair G1/G2 (approximately 285bp)
 (B) Agarose gel electrophoresis pattern of LipL32 gene (approximately 756bp) specific PCR product of template DNA amplified with primer pair LipL32
 La: DNA molecular weight ladder 100bp+
 -ve: Negative Control
 +ve 1: Positive Control (*Leptospira interrogans*) using primer pairs LipL32
 +ve 2: Positive Control (*Leptospira interrogans*) using primer pairs G1/G2
 FS: Field sample

Earlier Van Eys *et al.*, (1989) and Gerritsen *et al.*, (1991) used PCR with primer derived from *L. interrogans* serovars Hardjobovis to detect *Leptospira* in bovine urine samples. However, all of these experiments were performed on urine collected from animals experimentally infected with *Leptospira* under controlled laboratory conditions. In the present field study it was revealed that a total 304 urine and 120 blood samples successfully screened by PCR using two sets of primer and supported the findings of Cheema *et al.*, (2007) and Jafari *et al.*, (2011) who too carried out the investigation on field samples. The PCR positive samples also showed positivity in microscopic agglutination test (MAT) with *L. interrogans* serovar Hardjo, Canicola, Hebdomadis, Icterohaemorrhagiae, Pomona, Autumnalis, Australis, Pyrogenes; *L. kirschneri* serovar Grippotyphosa and *L. borgpetersenii* serovar Javanica (Patel *et al.*, 2014). Further the amplicons size of G1/G2 and LipL32 primers was 285bp and 756bp, respectively and supported the earlier findings (Meenambigai *et al.*, 2011; Gravekamp *et al.*, 1993; Bomfim *et al.*, 2007; Baquero *et al.*, 2010).

Primer set LipL32 and G1/G2 can be used in PCR for early and chronic stage diagnosis of leptospirosis from various clinical samples in bovines. Thus the PCR possesses advantages over more traditional methods like MAT, isolation and culture of leptospires.

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References

- Ellis, W.A. 1994. Leptospirosis as a cause of reproductive failure. *Vet. Clin. North Am. Food Anim. Pract.* 10: 463-478.
 Ellis, W.A. 1984. Bovine leptospirosis in the tropics: prevalence, pathogenesis and control. *Prev Vet Med.* 2:411-421.
 Turner, L.H. 1970. Leptospirosis. Maintenance,

- isolation and demonstration of leptospires. *Trans R Soc Trop Med Hyg.* 64: 623-646.
- Szeredi, L. and Haake, D.A. 2006. Immunohistochemical identification and Pathologic finding in natural case of equine abortion caused by *Leptospira* infection. *Vet Pathol.* 43: 755-761.
- Sreekumari, R., Berghaus, R.D., Overton, M.W., Pence, M.E. and Baldwin, C.A. 2010. Comparison of fluorescent antibody and microscopic agglutination testing for *Leptospira* in pregnant and nonpregnant cows. *J Vet Diagn Invest.* 22: 51-54.
- Meenambigai, T.V., Ravikumar, G., Srithar, A., Balakrishnan, G., Saranya, C. and Muralimanohar, B. 2011. Simultaneous detection of LipL32 and LipL21 genes of pathogenic leptospira from serum samples of bovines by multiplex PCR. *Veterinary Science Development.* 1:15.
- Ambily, R., Mini, M., Joseph, S. and Vamshi Krishna, S. 2012. LipL 41 gene specific PCR for the detection of pathogenic leptospires in cattle. *J. Ind. Vet. Assoc.* 10(2): 5-7.
- Sambrook, J. and Russel, D.W. 2001. *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, PP: 188-192.
- Gravekamp, C., Kemp, H.V.D., Franzen, M., Carrington, D., Schoone, G.J., Van Eys, G.J.J.M., Everard, C.O.R., Hartskeerl, R.A. and Terpstra, W.J. 1993. Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Journal of General Microbiology.* 139: 1691-1700.
- Cheema, P.S., Srivastava, S.K., Amutha, R., Singh, S., Singh, H. and Sandey, M. 2007. Detection of pathogenic leptospires in animals by PCR based on lipL21 and lipL 32 genes. *Indian J Exp Biol.* 45: 568 – 573.
- Van Eys, G.J., Gravekamp, C., Gerritsen, M.J., Quint, W., Cornelissen, M.T., Schegget, J.T. and Terpstra, W.J. 1989. Detection of leptospires in urine by polymerase chain reaction. *J. Clin. Microbiol.* 27(10): 2258-2262.
- Gerritsen, M.J., Olyhoek, T., Smits, M.A. and Bokhout, B.A. 1991. Sample Preparation Method for Polymerase Chain Reaction-Based Semiquantitative Detection of *Leptospira interrogans* Serovar Hardjo Subtype Hardjovovis in Bovine Urine. *J Clin Microbiol.* 29(12): 2805-2808.
- Jafari, D.A., Shahbazkia, H.R. and Ronagh, N. 2011. Evaluation of pathogenic serovars of *Leptospira interrogans* in dairy cattle herds of Shahrekord by PCR. *Indian J Microbiol.* 3(3): 135-139.
- Patel, J.M. 2014. Pathoepidemiological and molecular studies in on leptospirosis in bovines of South Gujarat. PhD thesis, Navsari Agricultural University, Gujarat, India.
- Bomfim, M.R.Q., Barbosa-Stancioli, E.F. and Koury, M.C. 2007. Detection of pathogenic leptospires in urine from naturally infected cattle by nested PCR. *Vet. J.* 178: 251–256.
- Baquero, M.I., Lopez, N., Mejía, M.E. and Trueb, G. 2010. Evaluation of a Polymerase Chain Reaction for the Diagnosis of Leptospirosis in Cattle. *Open Vet Sci J.* 4: 31-35.

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