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Research Article

DEVELOPMENT OF A REAL-TIME PCR ASSAY FOR DETECTION OF PATHOGENIC *LEPTOSPIRA* IN GOATS

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ABSTRACT

Leptospirosis is considered as a neglected, underreported and underdiagnosed zoonotic disease. Confirmatory diagnosis of leptospirosis can be done by bacterial culture and/or serology but without the right knowledge and experience, it can be challenging and time-consuming too. So to overcome these, many PCR assays have been developed. However, with added benefit the real-time PCR targeting 227 bp fragment within the LipL32 gene was developed to detect pathogenic *Leptospira* spp. from goat urine samples. Twenty one urine samples of goats positive for leptospiral by using conventional PCR were used for this assay wherein DNA was extracted by boiling method. In real time PCR, the reactions were read by SYBR green fluorescence and melting curve analysis. This assay had high analytic sensitivity with 3-4 genome copies per PCR reaction volume using *L. interrogans* serovar *icterohaemorrhagie* strain RGA DNA. In this assay *Leptospira* organism's DNA was detected and quantified from goat urine samples and hence it may be used for rapid diagnosis or for screening of carrier animals in an endemic area.

Keywords: Goats, *Leptospira*, RT-PCR

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Leptospirosis, caused by pathogenic spirochetes belonging to the genus *Leptospira*, isglobally recognized reemerging zoonosis impacting human and animal health. Pathogenic *Leptospira interrogans* complex has over 24 serogroups and more than 300 serovars based on the expression of surface exposed lipopolysaccharides. The disease represents major threat to health of animals like dogs, cattle, buffaloes, swine, horses, sheep and goats etc. and infection runs in different forms like acute, subacute or chronic which can have major impact on health resulting in significant economic losses (Perez *et al.*, 2020). Leptospirosis is reported to be endemic in several parts of India such as Kerala, Gujarat, Maharastra, Tamil Nadu, Karnataka, Pondicherry and Andamans (Shekatkar *et al.*, 2010). The disease has been one of the underreported diseases in India.

Direct contact with infected urine, tissue from infected animals, or coming into contact with water or soil that has previously been contaminated by urine can all result in leptospirosis in humans or animals (Adler and de la Peña Moctezuma, 2010). Among goats leptospirosis is exhibited as the subclinical and silent chronic form which can lead to reproductive abnormalities like reduced fertility, abortion and stillbirths in affected animals. It also affects milk production of animal and all these factors lead to significant economic losses (Lilenbaum *et al.*, 2008). Infected animals shed leptospires and this contaminates the environment which acts as a continuous source of infection to others (Vihol *et al.*, 2017). Because of lack of specific clinical signs, laboratory confirmation of disease is necessary for diagnosis. Laboratory diagnosis includes use of indirect methods like microscopic agglutination test, ELISA etc. and/or direct methods like bacterial culture, immunoûuorescence assays (IFAs). The MAT or other serological approaches are little challenging and complicated by means of its time-consuming nature, live culture maintenance and contamination issues. Isolation or culture of bacteria for clinical diagnosis may not be suitable as it is difficult to isolate organism because of very slow growth (13 weeks) and high rate of contamination (Perez *et al.*, 2020). So now days molecular methods like PCR, isothermal amplification and quantitative real time PCR have caught the attention of many laboratories or researchers being simple, effective and inexpensive diagnostic methods. These assays may be used for detection of leptospiral DNA from samples like urine, blood, serum, semen, and aborted fetus (Fornazari *et al.*, 2012; Miotto *et al.*, 2018; Perez *et al.*, 2020) and reported to be useful as the most important methods for the diagnosis of leptospirosis in animals (Di Azevedo and Lilenbaum, 2020).The real time PCR assay can detect the desired nucleic acid as well as it also can quantify the load of organism.

For the diagnosis of *Leptospira* infection, several real-time PCR techniques that amplify various target sequences have been established (Smythe *et al.*, 2002; Levett *et al.*, 2005; Stoddard *et al.*, 2009; Villumsen *et al.*, 2012). The most often used target for *Leptospira* detection

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is the lipL32 gene of pathogenic *Leptospira* spp., which encodes an outer membrane lipoprotein absent in nonpathogenic species and possibly involved in virulence (Naddaf *et al.*, 2020). Real-time PCR tests for the detection of pathogenic *Leptospira* species are widely in use presently as it is faster than conventional PCR, however there aren't many methods that have been successfully documented for the analysis of urine samples. This study depict the real-time PCR assay using LipL32 gene target for the rapid detection of pathogenic *Leptospira* species in goat urine samples.

MATERIALS AND METHODS

Real time assay design

The standard curve method was used for development of the quantitative real-time PCR (qPCR) assay to detect and quantify the pathogenic leptospires from urine samples.

Standards for *Leptospira organism*

The standard curve method was used to determine the absolute target quantity of pathogenic leptospires. In this study quantitative standards of *Leptospira* spp. genomic DNA were prepared. For preparation of standards, the genomic DNA was extracted using commercial kit (DNeasy® Blood and Tissue kit, Cat. No. 69504, QIAGEN, Germany) as per the manufacturer's instructions from culture of *L. interrogans* serogroup *Icterohaemorrhagiae* serovar *Icterohaemorrhagiae* strain RGA. This strain was received from Regional Medical Research Centre (Indian Council of Medical Research), WHO Collaborating Centre for Diagnosis, Reference, Research & Training in Leptospirosis, Port Blair 744 101, Andaman & Nicobar Islands. Concentration of extracted DNA was measured by Nanodrop UV spectrophotometer (Thermo Fisher Scientific Inc.). Concentration of extracted DNA was 5.9 ng/ l. Numbers of genomes were calculated assuming a *Leptospira* spp. genome size of 4.63 Mb using the web tool http://www.uri.edu/research/gsc/resources/cndna.html. It was converted to number of copies or Genome equivalent. Five serial dilutions i.e. 1:10 of the gDNA (Genome equivalent 1.17×10^6/ l) were made in nuclease free water and were used for standard curve analysis. The same gDNA was used as positive control.

Primers

The gene LipL32 sequence of *Leptospira interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was used to commercially synthesize the primers (eurofinsmwg/operon, Bangalore, India). For primer design Primer3 software (http://frodo.wi.mit.edu/) was used and the specificity was checked by NCBI blast programme

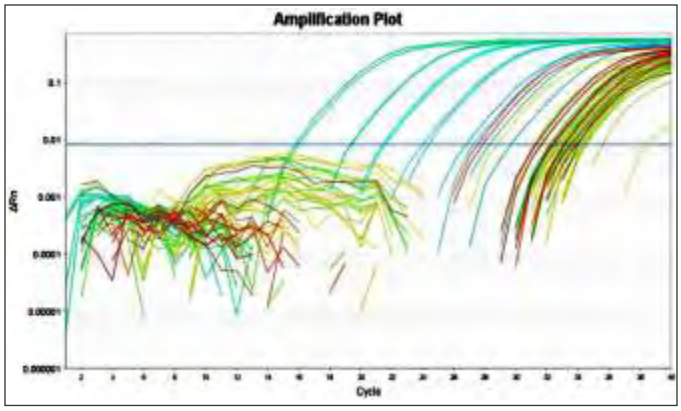

Fig. 1. Real time PCR amplification graph for *Leptospira organism* in SYBR green chemistry

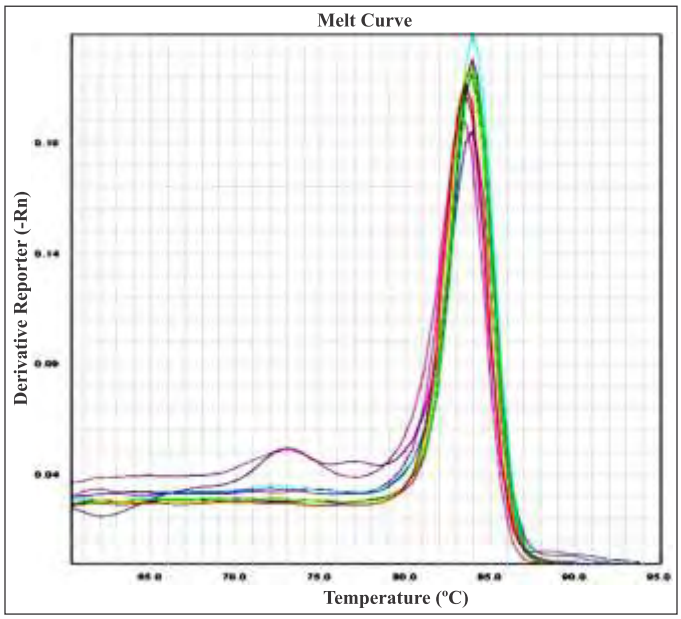

Fig. 2. Real time PCR melt curve graph of pathogenic *Leptospira* after real-time amplification with LipL 32 gene specific primers

(http://www.ncbi.nlm.nih.gov/BLAST/). The forward primer LipL32-F (5'-GGACGGTTTAGTCGATGGAA-3') and reverse primer LipL32-R (5'-GCATAATCGC CGACATTCTT-3') were used. The LipL32 gene specific fragment of size of 227bp was amplified.

Real time assay:

Real-time PCR assay was performed in final reaction volume of 20 l using optical 96 well plates (Applied Biosystems) in 7500 Real Time PCR instrument (Applied Biosystem, USA). This assay was based on SYBR Green I chemistry. In this assay each reaction volume was made up of the 1.0 l each forward and reverse primer solutions (10 pmol/ l), 10.0 l 2X QuantiFast® SYBR® Green PCR master mix solution (Cat. No. 204054, Qiagen, containing Hot Start Taq DNA polymerase, SYBR green PCR buffer, dNTPs, SYBR green I, ROX-passive reference dye), 5.0 l RNase Free

water and 3.0 l template DNA. The amplification protocol consisted of 2 minutes at 50º C, 10 minutes at 95º C followed by 40 cycles of amplification (95º C for 0.15 minutes, 60º C for 1 minute), after which the reaction was melted for one cycle at 95º C for 0.15 minutes, 60º C for 1 minute, 95º C for 0.15 minutes and 60º C for 1 minute. For each run, serially diluted five standards, positive control and no template control were used along with the clinical samples. All the samples were run in duplicate. Five serial dilutions i.e. 1:10 of the gDNA (Genome equivalent 1.17×10^6/ l) made in nuclease free water were used for standard curve analysis and as positive control. The plate was sealed using adhesive. At the end of each extension step, fluorescence was measured once every cycle using SYBR Green filters (excitation at 492 nm and emission at 530 nm). In real time machine itself, the fluorescence data were converted into a log scale and the threshold was obtained for the calculation of the threshold cycle value (Ct; the cycle at which the threshold line crosses the amplification curve). For every cycle, the threshold was set above the background (0.01) normalized fluorescence value. Upon completion of real-time PCR run, data were automatically analyzed for melt curve and quantification by 7500 system Sequence Detection Software (SDS).

Evaluation of assay using clinical samples

The archived goat urine samples positive for leptospiral DNA by conventional PCR were used to check the developed real time assay. These samples have been described in detail previously (Vihol *et al.*, 2017). Briefly, Goat urine samples were collected and DNA was extracted using a boiling method. The extracted DNA was stored at -80º C until further use. Later on these samples were used for PCR to detect nucleic acid of leptospira organism using primers G1/G2. In realtime PCR, randomly selected twenty one PCR positive samples were used to check sensitivity for detection of pathogenic leptospires and to quantify the bacterial load.

Procedure followed for sample collection, DNA extraction and PCR assay for urine samples was as described below:

Collection of urine samples

Midstream urine samples were collected in plastic containers transported to laboratory as soon as possible in ice container. In laboratory, these samples were filtered in sterile 50 ml conical centrifuge tubes using 0.45 micrometer pore size filters (Pall Life Science). The filtrate was centrifuged at 7800 rpm for 20 minutes at 4º C. The supernatant was discarded and pellet was vortex and transferred to sterile 1.5 ml microcentrifuge tube. These pellet were resuspended in phosphate-buffered saline (pH 7.2).

Genomic DNA extraction

For DNA extraction, resuspended pellet samples were centrifuged at 14000 rpm for 15 minutes at 4º C. Again the obtained pellet was resuspended with 1ml PBS (7.2 pH) and centrifuged at 14000 rpm for 10 minutes at 4º C. This step was repeated twice. Then supernatant was discarded and 0.5 ml nuclease free water (Qiagen) was added in pellet and centrifuged at 14000 rpm for 10 minutes at 4º C. This step was again repeated. Lastly, in pellet 0.1 ml nuclease free water was added and vortexed. These tubes were placed in boiling water bath for 15 minutes and immediately snap chilled for 20 minutes. Then the tubes were vortexed and samples were stored at -20º C until further analysis.

PCR assay

The final reaction mixture of 25 l for PCR comprised of 7.5 l DNase -RNase-free water, 12.5 l Taq PCR master Mix (having 1X PCR Buffer containing 1.5 mM MgCl2, 2.5 units Taq DNA Polymerase and 200 M each dNTPs, QIAGEN, Germany), 1.0 l (20 pmoles/ l) forward primer, 1.0 l (20 pmoles/ l) reverse primer (20 pmoles/ l) and 3.0 l template DNA. Primers G1 (forward) and G2 (reverse) had sequences 5'CTGAATCGCT GTATAAAAGT3' and 5'GGAAAACAAATGGTCGG AAG3', respectively. In PCR programme consisted of initial denaturation at 95º C for 4 min followed by 35 cycles of denaturation at 95º C for 1 min, annealing at 53º C for 1 min, extension at 72º C for 1 min and final extension at 72º C for 10 min. The visualization of the band at the predicted product size of 285bp in electrophoresis was considered PCR positive. For each PCR run, positive and negative control samples were also used. For positive control, the genomic DNA extracted from culture of *L. interrogans* Serogroup Icterohaemorrhagiae Serovar Icterohaemorrhagiae strain RGA was used while as negative control nuclease free water was added in place of gDNA in PCR reaction mixture (Vihol *et al.*, 2017).

RESULTS AND DISCUSSION

The Real time qPCR was developed for detection and quantification of leptospiral nucleic acid/load in the urine samples. The DNA concentration (5.9 ng/µl) was converted into genome equivalent (1.17×10^6) using GE calculator and these were serially diluted 10 fold up to five dilutions and used for generation of standard curve. The PCR assay was able to detect 3-4 genome copies per PCR reaction. The melt curve temperature of standards was

around 83º C.

The 21 PCR positive urine samples were used for detecting the load of leptospires and the results are depicted in Table 1. Out of a total of 21 urine samples examined, melt curve analysis were near to the melt curve temperature of standards i.e. 83.34 to 83.84º C in 4 samples only. While in rest of the samples the melt curve temperature was recorded between 71.98 to 80.84º C. The calculated threshold cycle (Ct) value is shown in amplification plot (Fig. 1) and melt curve obtained in assay is depicted in Fig. 2. The quantification was automatically done by SDS software with the help of standard curve. The quantity obtained was in genome equivalent number.

In the present study the load of leptospiral organisms ranged from 16.08 to 1047.74 GE/reaction in urine samples (Table 1). On the basis of melt curve analysis the detection limit of this Real Time PCR assay was found up to 3-4 leptospires (considering 5 GE is equal to one leptospiral organism). When PCR products of serially diluted DNA were visualized by agarose gel electrophoresis at 15 min of electrophoresis run, no band was observed in sample having concentration of 16.08 GE/reaction of leptospiral DNA. However, real-time PCR detected the amplification and melt curve of the same sample. These observations indicated the higher sensitivity of the real-time PCR especially when microbial load is low.

Table 1. Load of leptospires in tested urine samples of goats

Sr. No.	Sample No.	Leptospires load in urine (GE/reaction)
1.	01/G	58.71
2.	02/G	1047.74
3.	03/G	16.08
4.	04/G	44.57

In this study, the primer that amplified the LipL32 gene fragment of pathogenic *Leptospira* spp. was used. In literature, for PCR or real time PCR based diagnosis either universal gene present in bacteria like *gryB*, *rrs* (16S rRNA gene) and *sec*Y or surface proteins restricted to *Leptospira*, such as *lipl21*, *lipl32*, *lipl41* and *ligB* (Guernier *et al.*, 2018) are reported to be a prime focus for amplification or primer design. Several real-time PCR assays that amplify different target sequences have been described for the diagnosis of *Leptospira* infection (Smythe *et al.*, 2002; Levett *et al.*, 2005; Stoddard *et al.*, 2009; Villumsen *et al.*, 2012; Fink *et al.*, 2015; Naddaf *et al.*, 2020). In many of these studies, the LipL32 gene, which encodes the *Leptospira* subsurface lipoprotein Lipl32 was commonly used (Levett *et al.*, 2005; Stoddard *et al.*, 2009; Fink *et al.*, 2015; Naddaf *et al.*, 2020). Because Lipl32 is thought to be a virulence factor that is only present in pathogenic species and hence it provides selective detection of the pathogenic *Leptospira* and also aid in increasing the specificity of these methods (Podgorsek *et al.*, 2020).

In present study, the PCR positive samples showed positivity for the LipL-32 gene which substantiate the appropriateness of DNA extraction and amplification procedures used. The qPCR assay described in this study presented high analytical sensitivity as it was able to detect low concentrations of leptospiral DNA extracted from goat urine samples (at least 3-4 copies per reaction). These results were compatible with the sensitivity levels of previous studies that used the LipL-32 gene as a target for molecular detection of leptospires in clinical samples like urine from dogs or human (Stoddard *et al.*, 2009; Rojas *et al.*, 2010; Fink *et al.*, 2015; Miotto *et al.*, 2018).

Previously, counting chambers were used for counting of leptospires under dark field microscope (Levett *et al.*, 2005) however with the recent technologies this is possible without risk of handling of organism directly and comparatively latest technique is more precise with higher sensitivity. Although the conventional PCR positive samples were tested in qPCR, only 4 samples showed amplification similar to standards in this assay. It is reported that the LipL-32 primers can amplify the DNA from the pathogenic *Leptospira* spp. strains like *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. kirschneri* and but not the intermediate and saprophytic strains *viz.*, *L. inadai*, *L. meyeri* and *L. biflexa* (Miotto *et al.*, 2018). So either infection with intermediate strain or possible loss of DNA integrity during sample processing may be the possible reason for less amplification in tested samples. Our findings supported the results of Smythe *et al.* (2002) who suggested that certain component(s) in urine samples could interfere with the qPCR in urine resulting in low percentage of positivity against *Leptospira*. Thus in their opinion urine in comparison to serum was not efficient sample in performing qPCR test. On the same line, it was noted that the presence of inhibitors in clinical samples may decrease efficiency of assay or entirely inhibit the reaction (Levett *et al.*, 2005).

In present study, assay was developed using SYBR green technology. Earlier, Levett *et al.* (2005) reported higher specificity derived from melting point analysis with the use of SYBR green technology for real time PCR. Commonly the real time PCR assays have been developed or are used with either SYBR technology (Levett *et al.*, 2005) or TaqMan probe (Smythe *et al.*, 2002) or, more recently, Light Upon eXtension (LUX) technology (Stoddard *et al.*, 2009). The omission of a probe from currently developed assay helped to limit the costs of

assay. A qPCR assay is a rapid method that can be used to detect *Leptospira* in clinical samples like whole blood, sera and urine (Stoddard *et al.*, 2009) and also facilitates the real time detection of amplified fragments and therefore it does not need end-point detection, which is comparatively less precise and time consuming too. However this assay is less specific in comparison with TaqMan probe assays as all double-stranded DNAs formed during the PCR reaction such as nonspecific PCR products and primer-dimers are detected with the SYBR green dye, whereas only sequence-specific amplification is measured with a TaqMan probe (Valasekand Repa, 2005).

CONCLUSION

The present study described the development of a reliable and highly sensitive real time PCR assay for detection and quantification of pathogenic leptospires using LipL 32 gene specific primers from urine samples and these may be applicable for diagnostic or screening approach.

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