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Development and Evaluation of Recombinase Polymerase Amplification Assay for Diagnosis of Canine Leptospirosis

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ABSTRACT

Background: Canine Leptospirosis is a life-threatening disease and zoonosis. Usually, PCR assay is carried out for early diagnosis but requires a thermal cycler and post-PCR procedures. This limits its use in resource-limited areas. Hence, the isothermal amplification of nucleic acid by recombinase polymerase amplification assay was developed as a versatile alternative for the diagnosis of canine leptospirosis in this study.

Methods: The RPA assay to detect Leptospira DNA was optimized with Leptospira reference strains and its performance characteristics such as analytical, diagnostics and reproducibility were assessed.

Result: The limit of detection of RPA assay was estimated as 10^2 copies of genomic DNA and specific to amplify the pathogenic Leptospira. Out of 150 dog samples screened, Leptospira DNA was detected in 64 (42.6%) by RPA assay and 67 (44.6%) by PCR. The diagnostic sensitivity and specificity of the RPA assay were 92.5% and 97.59% respectively. The RPA assay has a good diagnostic agreement with a kappa value of 0.905. The reproducibility assessment with the third-party testing laboratory revealed a better agreement with a kappa value of 0.81. The simplicity, rapid and less expensive enable this assay to perform at resource-limited laboratories or point-of-care testing.

Key words: Canine, Leptospirosis, PCR, Recombinase polymerase amplification.

INTRODUCTION

Leptospirosis is a bacterial zoonotic disease caused by the pathogenic species of the genus *Leptospira* affecting all mammals including aquatic animals, with a worldwide distribution. Canine leptospirosis results in acute and chronic disease with clinical manifestations of fever, icterus, haematuria, renal failure and death (Schuller *et al*., 2015). In addition, the infected dogs have been shown to act as a carrier and pose a public health risk (Bharti *et al*., 2003). The similarity of the clinical symptoms with other febrile illnesses, leptospirosis complicates the clinical diagnosis (Miotto *et al*., 2018). Early diagnosis of leptospirosis is essential for the application of antibiotic therapy and to reduce mortality. Several approaches have been used for the diagnosis of leptospirosis such as Dark-Field Microscopy (DFM); culture and isolation (Faine *et al*., 1999); Polymerase Chain Reaction (PCR) (Miotto *et al*., 2018) and Microscopic agglutination test (MAT) (OIE, 2021a). The referred serodiagnostic test, MAT detects serogroup-specific antibodies but needs multiple strains of serogroups as antigens, expertise to sustain cultures and biosafety facilities, making the assay too difficult (Levett, 2001). Further, the assay does not reveal the active shedders of leptospira.

The polymerase chain reaction has been reported for clinical application in the diagnosis of Leptospira in livestock and humans (Harkin *et al*., 2003) and its application to urine samples to detect active shedders (Rojas *et al*., 2010). The PCR assays have been performed with *ligB* (Palaniappan *et al*., 2005); *flab* (Gamage *et al*., 2014); *lipL32* (Senthilkumar *et al*., 2021), as gene targets that are restricted to pathogenic Leptospira. However, the PCR assay requires a thermal cycler, molecular reagents and post-PCR procedures to detect the amplicons. This limits its use in resource-limited areas and the presence of amplification inhibitors in clinical samples can result in false-negative results (Ahamed *et al*., 2009). To overcome these, an

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isothermal nucleic acid amplification method, recombinase polymerase Amplification (RPA) was developed in 2006 (Twist Dx Ltd, UK) and had been applied for the detection of many pathogens (Piepenburg *et al*., 2006; Singpanomchai *et al*., 2019). The RPA assay employs recombinase enzyme, single-stranded DNA binding protein, homologous oligonucleotides and strand-displacing polymerase, which aid in DNA synthesis from primer-paired target DNA. These enzymes amplify the target nucleic acid in a short time (20-30 minutes) at constant moderate temperatures (25°C to 42°C) and can be performed with affordable equipment.

A new assay that has been claimed to be useful for diagnosis has to be appropriately validated as per standard norms to determine its fitness for the intended purpose. A diagnostic assay for use in livestock and other animal species has to complete at least the first three stages as per the OIE adopted formal validation standard (OIE, 2021b). In this study, the RPA assay was developed for the quick and efficient detection of Leptospirosis in dogs and validated the same for its analytical, diagnostic and repeatability characteristics as per the OIE pathway of validation.

MATERIALS AND METHODS

Reference culture and collection of samples

A panel of Leptospira reference strains to represent twenty-four serogroups (23 pathogenic and one non-pathogenic strain) maintained at the Zoonoses Research Laboratory (ZRL), Tamil Nadu Veterinary and Animal Sciences University, Chennai was used as the source of positive control (Table 1) and cultured in EMJH medium (Senthilkumar *et al*., 2022). The clinical samples included 150 serum samples from dogs with clinical signs of fever, jaundice, vomition, hematuria and renal failure submitted to ZRL for diagnosis of Leptospirosis during the period from 2019 to 2021. The kidney tissues of wild rats (n=28) and water samples (n=15) collected from in and around Chennai during the monsoon rain (December 2020) were also used and research work was carried out in the Zoonoses Research Laboratory.

DNA extraction

The genomic DNA of Leptospira reference strains and other related bacterial species were extracted with QIAamp DNA Mini kit (Qiagen, India. DNeasy Blood and Tissue Kit (Qiagen, India) was used for the extraction of DNA from the dog blood and serum samples and the QIAexpert® system was used to determine the concentration and quality of the DNA.

PCR assay for detection of pathogenic Leptospira

The primers of the PCR assay were designed by Primer 3 software employing the *LipL32* gene sequence of *Leptospira interrogans* serovar Canicola strain RTCC 2805. The primers include the RPA-11F 5′-CTGCCGTAATC GCTGAAATGGGA GTTCGTATG-3′ and RPA-11R 5′-GTGGCATTGATTT TTCTTCTGGGGTAGCCG-3′. The PCR assay was performed in a volume of 20 µl with the 2x PCR reaction mixture (M/s Amplicon, Denmark) and the PCR cycling conditions included an initial denaturation at 95°C for 5 minutes, 35 cycles of 92°C for 30 sec, 56°C for 30 sec and 72°C for 30 sec and a final extension for 5 min at 72°C. The amplified products were electrophoresed and documented in the gel document system (M/s Bio-Rad, India). The initial PCR amplification experiments were performed with the DNA extracted from the 23 different pathogenic reference strains of Leptospira and the non-pathogenic Patoc serovar. The PCR assay was also performed with the DNA extracted from other Gram-ve bacteria (*E. coli, Salmonella* and *Brucella*) to confirm the specificity as well as with different dilutions of the DNA to determine the limit of detection. This optimized PCR assay was used to screen the clinical samples and used as the standard assay to validate the developed RPA assay.

Optimization of the RPA assay for the detection of pathogenic Leptospira

The RPA assay was carried out using the reagents in TwistAmp ® Basic kit (TwistDx Limited, UK). The reaction mixture consisted of buffer (29.5 µl), forward and reverse primer (24 pmol each), template (2 µl) and nuclease-free water (13.2 µl) that was added to the pellet in the reaction tube. The contents were mixed by vortexing and 0.5 µl of 2.8 mM magnesium acetate was added to the lid of the PCR

Table 1: Pathogenic and Non-pathogenic *Leptospira* reference strains used in PCR and RPA assay.

Serogroup	Serovar	Strain
Australis	Australis	Ballico
Autumnalis	Rachmati	Rachmati
Ballum	Ballum	Mus 127
Canicola	Canicola	Hond Utrecht IV
Grippotyphosa	Grippotyphosa	Moskva V
Hebdomadis	Hebdomadis	Hebdomadis
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
Javanica	Poi	Poi
Pomona	Pomona	Pomona
Pyrogenes	Pyrogenes	Salinem
Sejore	Hardjo	Hardjoprajitno
Tarassovi	Tarassovi	Perepelitsin
Bataviae	Bataviae	Swart
Cynopteri	Cynopteri	3522 C
Djasiman	Dasiman	Djasiman
Hurstbridge	Hurstbridge	BUT 6
Louisiana	Louisiana	LSU 1945
Manhao	Manhao	L 60
Mini	Mini	Sari
Panama	Panama	CZ 214 K
Ranarum	Ranarum	ICF
Sarmin	Weaveri	CZ 390
Shermani	shermani	1342 K
Semeranga	Patoc	Patoc 1

tube and spun to mix with the reagents. The reactions were initiated by incubating in a water bath at 39°C for 5 min then taken out for gentle mix, spun and again incubating at 39°C for 25 min and stopped by holding at 12°C. The RPA amplified DNA was purified using either Gel and PCR clean-up (M/s Macherey-Nagel) kit or the proteins in the reaction mix were separated from the DNA by denaturing at 65°C for 10 minutes. The purified /denatured amplicons were visualized upon electrophoresis in agarose gel and the results were documented in the gel documentation system (M/s Bio-Rad, USA). The positive control provided in the kit, the DNA from known positive and negative samples were used as controls to determine the validity of the assay performance. In addition, the DNA from the Leptospira reference strains (cultures spiked in the negative serum samples) was used as an in-house reference standard to assess the ability of the RPA assay to detect pathogenic serovars of Leptospira. The optimized assay was validated for the detection of Leptospira as per the OIE pathway (OIE, 2021b).

Standard OIE validation pathway to evaluate the performance of the RPA assay

The accuracy and precision of the pipettes were verified at the appropriate intervals as a part of the validation process. The following activities were performed as a part of the approved validation process and the PCR assay described above was the reference standard to generate data on the performance of the RPA assay.

Analytical sensitivity

The cultures of reference pathogenic Leptospira serovars were used to determine the analytical sensitivity. The size of the Leptospira genome was taken as 4.77 Mb and copy number of the Leptospira genome in the sample was calculated (https://cels.uri.edu/gsc/cndna.html). The PCR and the RPA assay were performed with different copy numbers to assess their limit of detection (LOD).

Analytical specificity

The DNA extracted from other Gram-ve bacteria (*E. coli, Salmonella* and *Brucella)* and cultures of reference pathogenic and non-pathogenic Leptospira strains were tested by both the PCR and RPA assay.

Diagnostic characteristics of the developed RPA assay

The DNA extracted from different clinical samples was tested by PCR and RPA assay. The test results of the RPA (150 sera samples, 28 tissue samples and 15 water samples) were compared with the PCR assay to determine the diagnostic sensitivity and specificity. To rule out the false positivity and negativity with PCR and RPA assays, the samples were tested by Taqman real-time PCR as described (Stoddard *et al*., 2009).

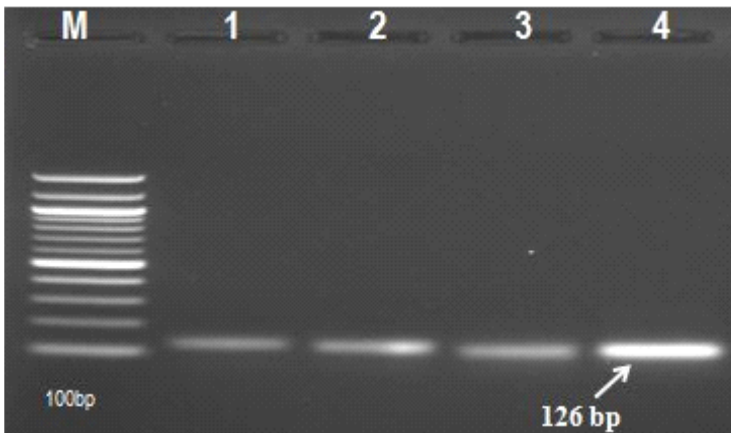
Reproducibility characteristics of the developed RPA assay

The inter-assay repeatability was performed with a set of twenty positive and negative samples and the intra-assay repeatability testing was performed in triplicates on selected days. The intra-assay repeatability was performed with a set of known positive and negative samples (25 Nos) that were blind-coded and the test was performed as per the SOP by the independent laboratory. The results of inter-laboratory reproducibility were determined.

RESULTS AND DISCUSSION

Optimized PCR and RPA assay

The primer pair RPA11F/RPA11R was found to be efficient in amplifying the 126 bp *LipL32* gene from twenty-three pathogenic reference strains and not from the non-pathogenic Leptospira by PCR. The PCR assay targeting the pathogenic gene is considered a confirmative diagnosis (OIE, 2021a). Hence *lipL32* gene was chosen



M- 100 bp DNA ladder; Lane 1- Kit control (140 bp); Lane 2-4- DNA from known serovars of Leptospira- 126 bp.

Fig 1: Optimization and specific detection *Leptospira* DNA by recombinase polymerase amplification assay.

Table 2: Performance of RPA assay in comparison with the PCR assay for detecting leptospira DNA in clinical samples.

		PCR assay result (Nos)			Performance characteristics (%)	
		Positive	Negative	Total	Sensitivity	Specificity
RPA assay result (Nos)	Positive	62	2	64	92.5%	
	Negative				(83.44% to 97.53%,	97.59% (91.57 to 99.71%,
	Total	5	81	86	95% CI)	95% CI)
		67	83	150		

as the target for both the PCR and the RPA assay, which is highly expressed during infection (Haake *et al*., 2000).

For the RPA assay, 24 pmol of each of the primers, 2.8 mM Magnesium acetate (0.5 µl), 20 ng of template DNA, incubation temperature of 39°C and a reaction time of 30 minutes were found to be the optimized conditions for amplification of 126 bp amplicon targeting *LipL32* gene (Fig 1) of pathogenic leptospira. The RPA assay efficiently amplified the *lipL32* gene (126 bp) from the reference strain of pathogenic serogroups. It is in concordance with the application of RPA assay to detect the methicillin-resistant *Staphylococcus aureus* (Piepenburg *et al*., 2006), *Leptospira* sp (Ahamed *et al*., 2014), *Mycobacteriu*m sp (Singpan omchai *et al*., 2019).

Analytical performance of the developed RPA assay

During ascertaining the variability in pipetting of the reaction components, the coefficient of variation ranged from 0.3 to 1.1% was noticed, indicating negligible contribution for dispensing the respected volumes with the micropipettes. The concentration of the stock DNA was 53 ng/µl which is equivalent to 1.014×10^7 copies. On serial dilution and detection of genomic copies, the lower limit of detection for developed RPA assay and PCR assay was estimated as 10^2 copies. The RPA assay amplified the *LipL32* gene from pathogenic reference strains but not from non-pathogenic Leptospira and other Gram-negative bacterial species such as *Brucella, Salmonella* and *E. coli* DNA, confirming the specificity of the RPA assay. This sensitivity and specificity in detecting Leptospira DNA confirm the analytical characteristics of the optimized RPA assay.

Diagnostic characteristics of the developed RPA assay

The PCR assay detected 67 samples as positive, while 64 samples were found to be positive by RPA assay. The test positivity to *Leptospira* DNA in the samples was 42.6% (64/150) and 44.6% (67/150) by the RPA assay and PCR respectively. The test results indicated a good diagnostic agreement between RPA assay and PCR with a kappa value of 0.905 (0.837 to 0.974 at 95% CI). The diagnostic sensitivity and specificity of RPA assay to detect *Leptospira* DNA when applied to clinical samples was 92.5% (83.44% to 97.53%, 95% CI) and 97.59% (91.57% to 99.71%, 95% CI) respectively, in comparison with PCR (Table 2). However, the RPA assay detected leptospiral DNA from two samples that tested negative by the PCR assay, the failure in the PCR assay could be due to the presence of inhibitory factors in the sample (Ahamed *et al*., 2009). When applied to the kidney tissues and water samples, both RPA assay and PCR detected Leptospir*a* DNA in twelve tissue and ten water samples showed a similar positivity percentage. It implies the use of the assay for surveillance of Leptospira in the environment which is considered to be a source of infection.

The real-time PCR detected Leptospira DNA in 74 samples with a positive rate of 49.3% (74/150). None of the samples that tested negative on either PCR or RPA assay was found to be positive on real-time PCR assay confirming the diagnostic specificity but showed slightly higher sensitivity than both the assays. The high sensitivity in real-time PCR assay is due to the inherent ability of the fluorescent tags that are used as the reporter (Thaipadunpanit *et al*., 2011).

Repeatability and reproducibility characteristics of the developed RPA assay

The mean DNA concentration of amplified products on different occasions was 42.24 ng/ul ± 1.44 with a coefficient of variation of 5.9% indicating good repeatability of the assay. Out of 25 blind-coded samples that were used for the reproducibility assessment, the Leptospira DNA was detected in 19 samples in the third-party testing laboratory, while 17 samples tested positive in this laboratory. Analysis of the results revealed better agreement on the performance of the assay across laboratories with a kappa value of 0.81 (0.547 to 1.00 at 95% CI). The reported RPA assay showed very good intra and inter-assay repeatability and reproducibility. The repeatability of the assay performed on different occasions showed a co-efficient of variation of 5.9% is minimal and in agreement with the report of Reed *et al*. (2002). The removal of DNA binding protein by heat as an alternative method in the study, before visualization by gel electrophoresis, reduces the time and expenses, compared to the manufacturer's recommended method.

RPA assay extends the capabilities of diagnostic facilities without access to a thermocycler and generates the result in 30 minutes, but the reagent is costly. On factoring, in the time and cost of equipment, RPA assay is less expensive than PCR assay. Taken all together, the RPA assay is a promising tool for canine leptospirosis detection, which is simple, rapid and reliable in resource-limited diagnostic laboratories and on-site facilities. Further, simplification is possible by using the endpoint detection through the lateral flow platform as a point care test, during an outbreak for early diagnosis of canine leptospirosis.

CONCLUSION

RPA assay developed for the early diagnosis of canine leptospirosis was evaluated. The RPA is an isothermal reaction, performed at a moderate constant temperature with affordable equipment. The analytical and diagnostic sensitivity and specificity of the assay were satisfactory. The method is rapid, less expensive and enables to perform at resource-limited laboratories or point of care and field settings.

Conflict of interest

All authors declare that they have no conflict of interest.

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