Development and evaluation of Recombinase Polymerase Amplification assay for diagnosis of Canine Leptospirosis

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Abstract

Canine Leptospirosis pose a public health risk in addition to life threatening disease to dogs. The molecular methods enable faster and sensitive diagnosis of leptospirosis at early stage of infection in contrast to dark field microscopy and culture. The Recombinase Polymerase Amplification (RPA) assay is a versatile alternative to polymerase chain reaction by its simple, fast, inherent enzymatic amplification of nucleic acid at constant temperature. The RPA assay to detect Leptospira DNA was optimized with Leptospira reference strains and its performance characteristic such as analytical, diagnostics and reproducibility were assessed. The limit of detection of RPA assay was estimated as 10 2 copies of genomic DNA was similar to that of PCR assay and it is specific to amplify the pathogenic Leptospira strain rather than non-pathogenic Leptospira strains and other bacterial strains. Out of 150 dog samples screened Leptospira DNA was detected in 42.6% (64/150) by RPA assay and 44.6% (67/150) by PCR. The diagnostic sensitivity and specificity of RPA assay 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. The RPA assay has a good diagnostic agreement with a kappa value of 0.905 (0.837 to 0.974 at 95% CI). The performance of the assay on different occasion to amplify the DNA with mean concentration of 42.24 ng/µl ± 1.44 with a coefficient of variation of 5.9% showed its good repeatability. The reproducibility assessment with the third-party testing laboratory, revealed better agreement on the performance of the assay with a kappa value of 0.81(0.547 to 1.00 at 95% CI). Since this method is simple, rapid and less expensive enables to perform at resource limited laboratories or point of care testing at field level.

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 and more common in tropical or subtropical countries. The genus *Leptospira* comprises 66 different species that include more than 300 serovars (Caimi and Ruybal 2020). The disease incidence estimates among humans indicated that more than one million cases worldwide annually, with almost 59,000 deaths (Costa et al 2015). Apart from human being, the disease is also prevalent in the livestock population, despite the dogs are being vaccinated, leptospirosis results in acute and chronic disease with clinical manifestations of fever, icterus, haematuria, renal failure, and death (Greene 2012; Schuller et al 2015). In addition, the infected dogs have been shown to act as a carrier for this zoonotic pathogen and also pose a public health risk because of their close contact as companion animals (Bharti et al 2003; Martins et al 2012; Lelu et al 2015). The similarity of the clinical symptoms with other febrile illnesses, Leptospira infection may also complicate the clinical diagnosis (Schuller et al 2015; Miotto et al 2018). Early diagnosis of leptospirosis is very much essential for the application of the appropriate antimicrobial therapy and to reduce morbidity and mortality. There have been several approaches for diagnosis of leptospirosis such as Dark-Field Microscopy (DFM) (Faine et al 1999); culture and isolation (Faine, 1982); Polymerase Chain Reaction (PCR) (Harkin et al 2003; Miotto et al 2018); LAMP assay (Koizumi et al 2012) and Microscopic agglutination test (MAT) (Galton et al 1962; Fraune et al 2013; Senthilkumar et al 2021). The routine

diagnosis depends on the detection of specific antibodies to *Leptospira* by Enzyme-Linked Immunosorbent Assay (ELISA) or MAT. Most of the commercial ELISA kits to detect IgM / IgG antibodies to Leptospira employ the lipopolysaccharide (LPS) as capture agent and claim to have better sensitivity and specificity but their correlation with the OIE/WHO approved MAT assay is very poor (unpublished data). The MAT assay detects serogroup-specific antibodies in the late acute phase of the disease and needs the seroconversion to be assessed in paired sera samples. This imposes diagnostic difficulties during acute infection, the need to select representative strains of serogroups as antigens which is prevalent in that particular region, expertise to sustain these cultures and appropriate biosafety facilities (Levett 2001). Further, the application of serological assay will provide information on exposure to the disease but do not reveal the active shedders of leptospira, which is very much important because of their potential zoonotic.

The detection of antigen in the clinical samples will diagnose the disease at an early stage and detect the active shedders (Harkin et al 2003). The DFM has been routinely applied to detect the Leptospira in clinical samples but the sensitivity and specificity are low. The culture and isolation to confirm the disease are not beneficial for early diagnosis and initiation of treatment, since the organisms are fastidious in growth. Application of molecular diagnostic methods can enable faster and sensitive detection of Leptospira directly from the specimens in contrast to the routine approaches of DFM and culture. The polymerase chain reaction has been reported for clinical application in the diagnosis of Leptospira in livestock and humans because of their perceived sensitivity, capacity to provide an early diagnosis (Harkin et al 2003; Branger et al 2005) and its application to urine samples to detect active shedders (Harkin et al 2003; Rojas et al 2010). As a part of the treatment algorithm, PCR-based assays have been applied as a complementary tool to detect the residual leptospires in circulation (IDEXX, 2014). The reported PCR assays have been performed with hap1 (Branger et al 2005), lipL32 (Cheema et al 2007), ligB (Palaniappan et al 2005); flab (Gamage et al 2014) 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 alternate nucleicacid amplification method has been targeted. The Recombinase PolymeraseAmplification (RPA) is an isothermal amplification methodology developed in2006 (Twist Dx Ltd, UK) and had been applied for the detection of manypathogens (Piepenburg et al 2006;Ahmed et al 2014; Li et al 2018; Singpanomchai et al 2019). The RPA assay is a simple and fast isothermalamplification technique and employs recombinase enzyme, single-stranded DNA binding protein, homologousoligonucleotides, and strand-displacing polymerase, which aid in DNA synthesisfrom primer-paired target DNA. These enzymes amplify the target nucleic acid ina short time (20–30 minutes) at constant moderate temperatures (25°C to 42°C)and can be performed with simple and affordable types of equipment. The presenceof PCR inhibitors resulting in false-negative results could be greater due tothe contamination with faeces or autolysis in animal samples and most of thereported primer pairs for PCR have been evaluated for the diagnosis ofleptospirosis in human samples. A new method that has been claimed to beuseful for diagnosis has to be appropriately validated as per standard norms

todetermine its fitness for the intended purpose. An assay that has undergone such a validation process is considered to be properly developed, optimized andstandardized. A diagnostic assay for use in livestock and other animal specieshas to complete at least the first three stages as per the OIE adopted formalvalidation standard (Chap. 1.1.6 as per the Manual of Diagnostic Tests and Vaccinesfor Terrestrial Animals, 2021). Such a formal and complete validation process hasbeen attempted in only a few of the studies for the PCR methods applied for thediagnosis of leptospirosis in animal samples. Hence, in this study, wedeveloped the RPA assay for the quick and efficient detection of Leptospira in dogs and validated thesame for its analytical, diagnostic and repeatability characteristics as perthe OIE pathway of validation.

Materials And Methods

Reference culture, collection of samples, and biological reagents

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 DNA (Table 1). The clinical samples included 150 serum samples from dogs with clinical signs of fever, jaundice, vomition, hematuria, renal failure submitted to ZRL for Leptospira diagnosis (from Madras Veterinary College Teaching Veterinary Clinical Complex and Veterinary University Peripheral Hospital of TANUVAS and some Private Veterinary Clinics from Chennai). The kidney tissues of wild rats (n=28) and water samples (n=15) collected from selected locations in and around Chennai during the monsoon rain (during December 2020) were also used in this study. The other materials used in the study included Prime TaqTM DNA polymerase (GeNet Bio, Korea), TwistAmp® (TwistDx, UK), QIAamp DNeasy Blood and Tissue Kit, QIAamp DNA Mini Kit (Qiagen, India), 100bp DNA ladder (New England Biolabs, MA), Oligonucleotide primers (synthesized from IDT, Singapore) and QIAexpert (Qiagen, MA). The volumes dispersed by the different micropipettes used in the study were also verified at different time points as part of the validation process.

DNA extraction

The genomic DNA of Leptospira reference strains and other related bacterial species were extracted with QIAamp DNA Mini kit. DNeasy Blood & Tissue Kit was used for the dog blood and serum samples. The extraction was performed as per the manufacturer's protocols and the QIAexpert® system was used to determine the concentration and quality of the DNA.

PCRassay for detection of pathogenic Leptospira in animals

The primers of the PCR assay were designed by Primer 3 software employingthe LipL32 gene sequence of Leptospira interrogans serovarCanicola strain RTCC 2805 (GenBank Accession No. JN831363.1) and the primercharacteristics were verified with the Oligo AnalyzerTM Tool (available at www.idtdna.com/pages/tools/oligoanalyzer). The primers used for the studyinclude the RPA-11F 5'-

CTGCCGTAATCGCTGAAATGGGAGTTCGTATG - 3' (position 260 to291) and RPA-11R 5'-GTGGCATTGATTTTTCTTCTGGGGTAGCCG- 3' (position 385 to 356). The PCR assay was performed in avolume of 20 µl with the 2x PCR reaction mixture (Taq DNA Polymerase Master MixRED from Amplicon, Denmark) and the PCR cycling conditions included an initialdenaturation at 95ºC for 5 minutes, 35 cycles of 92ºC for 30 sec, 56ºC for 30sec, and 72ºC for 30 sec and a final extension for 5 min at 72ºC. The amplifiedproducts were electrophoresed in 2 % agarose gel and the results were documented inthe gel document system (M/s Bio-Rad, India). The initial PCR amplificationexperiments were performed with the DNA extracted from the 23 different pathogenic reference strains of Leptospira(strains are based on the circulating antibodies determined by MAT assay in animalsera samples since 1997; data unpublished) and the non-pathogenic Patocserovar. The PCR assay was also performed with the DNA extracted from other Gram–vebacteria (*E. coli, Salmonella* and *Brucella*) to confirm the specificity as well as with different dilutions ofthe DNA to determine the limit of detection. This optimized PCR assay was used toscreen the clinical samples (serum and tissue) and used as the standard assay to validate the developed RPAassay.

Optimization of the RPA assay for detection of pathogenic Leptospira in animals

The RPA assay was first carried out using the reagents in TwistAmp® Basic kit (TwistDx Limited, UK). The optimization included the critical parameters for the assay namely concentration of RPA-11F/ RPA-11R primers, the concentration of the target DNA, magnesium acetate levels, the assay temperature, and time (Piepenburg et al 2006). The RPA assay 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 2.8mM magnesium acetate was added to the lid of the PCR tube and spun to mix with the reagents. The reactions were initiated by incubating in water bath at 39º C for 5 min then taken out for gentle mix, spun and again incubating at 39º C for 25 min and stopped by holding at 12º C. The RPA amplified DNA was purified using either NucleoSpin® Gel and PCR clean-up (M/s Macherey – Nagel) kit or the proteins in the reaction mix was separated from the DNA by denaturing at 65º C for 10 minutes. The purified /denatured amplicons were visualized upon electrophoresis in 2 % 1X TAE agarose gel and the results were documented in the gel documentation system (M/s Bio-Rad, USA).

As a part of the standardization and optimization process, samples from known infected and uninfected animals from the population were included in the screening. 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 for a particular day. 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 most of the circulating pathogenic serovars of Leptospira. The optimized assay was validated for the detection of Leptospira as per the OIE pathway (Chapter 1.1.6 Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2021) as in **Figure 1**.

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

The first three stages of the OIE pathway of validation estimate the analytical, diagnostic and repeatability characteristics and including the performance characteristics can designate the assay as "validated for the original intended purpose (s)". As a diagnostic testing unit, the laboratory has a typical three-room setup with designated purposes for performing the PCR and RPA assay. In addition to the laboratory set-up, the precision of the volumes dispensed could be a major contributing factor to the false positive/negative results in a molecular assay and hence the accuracy and precision of the pipettes used in the study were verified at appropriate intervals as a part of the validation process (triplicates at each time point). The details on the precision checks performed at different time points are listed in **Supplementary Table S1**. The following activities were performed as a part of the approved validation process and the PCR assay described above was as the reference standard to generate data on the performance of the RPA assay.

Stage 1: Analytical performance of the developed RPA assay

Two major characteristics of the assay, which included analytical sensitivity and analytical specificity were first evaluated.

(a) Analytical sensitivity*:* The pooled cultures of the available reference pathogenic Leptospira serovars in the laboratory were used in this step to determine the analytical sensitivity. The size of the Leptospira genome was taken as 4.77 Mb to calculate the copy number of the Leptospira genome in the DNA samples used for this step (calculated using the web link - 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).

(b) Analytical specificity*:* For this step, 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.

Stage 2: Diagnostic characteristics of the developed RPA assay

The ability of the PCR and RPA to specifically amplify the DNA from pathogenic Leptospira strain was primarily confirmed in this step also. In addition, the DNA extracted from different clinical samples (blood, urine, tissues and body secretions) from animals was tested by PCR and RPA. 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.

Stage 3: Reproducibility characteristics of the developed RPA assay

a) Common reproducibility characteristics: As a primary step, the results of the PCR and RPA were valid for the particular run only when they met the validity criteria (amplification with the kit control and positive control and no amplification with the negative control). 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.

b) Reproducibility potential of the developed RPA: For this purpose, a set of known positive and negative samples (25 Nos) were blind coded by an independent and unrelated person to the laboratory. The blind coded samples, reaction components for the assay and the SOP were shared with the testing laboratory. The test was performed as per the SOP by the independent laboratory and results were shared with the person involved in the blind coding of the samples. The results of inter-laboratory reproducibility were determined.

Result

Optimized PCR and RPA assay

For the PCR assay, the annealing temperature of 56º C was found to be optimum for amplification of the lipL32 gene and resulted in an amplicon size of 126bp (Figure 2). The primer pair RPA11F/RPA11R was found to be efficient in amplifying the 126bp LipL32 gene from twenty-three pathogenic reference strains and not from the non-pathogenic Leptospira serovar Patoc. For the RPA assay, the optimized conditions included 24 pmol of each of the RPA 11F and RPA 11R primers, 2.8mM Magnesium acetate (0.5 µl), 20 ng of template DNA, incubation temperature of 39º C and reaction time of 30 minutes. The optimized RPA resulted in an amplicon of 126 bp with the DNA from the pathogenic Leptospira reference strains (**Figure 3**). The RPA assay was valid only when the kit control DNA and the positive control Leptospira DNA resulted in an amplicon size of 140 bp and 126 bp respectively.

Performance of the RPA assay in comparison with the PCR assay

Analytical performance of the developed RPA assay

The concentration of the stock DNA was 53 ng/µl which is equivalent to 1.014×10^7 copies. The different concentrations and copies of genomic DNA as detailed in **Supplementary Table S3**. The lower limit of detection of PCR assay was estimated as 10^2 copies of genomic DNA. The detection limit for the developed RPA assay was also similar (**Figure.4**). The RPA assay amplified the *LipL32* gene from twenty-three pathogenic reference strains with an amplicon size of 126 bp but not from non-pathogenic Leptospira serovar Patoc and other Gram-negative bacterial species such as *Brucella, Salmonella*, and *E.coli* DNA, confirming the specificity of the RPA assay to amplify only leptospiral DNA (**Figure 5**).

Diagnostic characteristics of the developed RPA assay

The diagnostic characteristics were determined by comparing the results of the PCR assay with clinical serum samples from dogs. The PCR assay detected 67 samples as positive, while 64 samples were found to be positive by RPA assay. The results of the testing by both the assay are provided in **Supplementary Table S4**. 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 which tested negative by the PCR assay. 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 respectively by amplifying the *lipL32* gene with an amplicon size of 126 bp. To rule out the false positivity and negativity with these assays, the samples were tested by Taqman real-time PCR using primer set of *LipL32*-45F 5'-AAGCATTACCGCTTGTGGTG-3'), LipL32-286R 5'-GAACTCCCATTTCAGCGATT-3') and probe, LipL32-189P (FAM-5'-AAAGCCAGGACAAGCGCCG-3'-BHQ1) as described (Stoddard et al 2009) and the results are provided in **Supplementary Table S4** *(the discordant samples are highlighted)*. The *Leptospira* DNA was detected in 74 samples by the real-time PCR with a positive rate of 49.3% (the results of the testing with the tissue and water samples were similar). 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 and slightly lower sensitivity with both assays. Both the assays were also suitable for application to screen tissue and environmental samples.

Repeatability and Reproducibility characteristics of the developed RPA assay:

The mean DNA concentration of amplified products at different occasions was 42.24 ng/ul ± 1.44 with a coefficient of variation of 5.9% indicating good repeatability of the assay **(Supplementary Table S2)**. Out of 25 blind coded samples that were used for the reproducibility assessment (including two of the discordant samples) the Leptospira DNA was detected in 19 samples in the third-party testing laboratory, while 17 samples tested positive in our laboratory (results are shown in the **Supplementary Table S5**). 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).

Discussion

Leptospirosis in dogs is considered the most serious disease since it causes life-threatening complications such as leptospiral pulmonary hemorrhage syndrome (LPHS), and hepatorenal failure (Schuller et al 2015). In addition, leptospires are zoonotic pathogens that pose a public health risk, its early diagnosis in clinical cases is beneficial for the initiation of appropriate antibiotic therapy not only to save the life of the animal but also to prevent excretion of the leptospires into the environment. The detection of leptospiral genetic material nucleic acid amplification methods is considered to be a confirmative diagnosis (OIE 2021). The PCR assay can diagnose leptospirosis at an early acute stage when serology does not allow early rationalization of antibodies and also in fulminating cases, in which death occurs before the seroconversion (Brown et al 1995; Hall and Lambourne 2014). Isothermal amplification methods have been described as a potential point of care alternate for the amplification of nucleic acids and among them, the Recombinase Polymerase Amplification Assay (RPA) requires only a water bath and a shorter duration for amplification (to a maximum of 30 minutes) when compared with

PCR. This study reports the optimization and a standard validation pathway to assess the appropriateness of the RPA to detect Leptospiral DNA from clinical samples.

PCR-based assays to detect leptospira have targeted the genes restricted to pathogenic Leptospira like the *lipL21, lipL32, lipL41, ligA* and *ligB* and except for a few, many of the reported primers sets have been evaluated for use in human samples. In this study, we used the *lipL32* as the gene target for both the PCR and the RPA assay. Even though PCR has been applied for the diagnosis of leptospirosis in animals, only three real-time PCR assays have been evaluated following the standard validation pathway (Ahmed et al 2009; Ahmed et al 2012; Slack et al 2007; Thaipadungpanit et al 2011). Among the isothermalamplification methods, the RPA assay had been applied to detect the pathogenmethicillin-resistant *Staphylococcus aureus* (Piepenburg et al 2006), Foot and Mouth disease virus (Abul El Wahed et al 2013), BovineCoronavirus (Amer et al 2013); *Orientia tsutsugamushi* or *Rickettsia typhi* (Chao et al 2015); *Mycobacteriu*m sp (Singpanomchai et al 2019) and Duplex real-time RPA assay was applied for detection of *Babesia* and *Theileria* infection at field level (Lei et al 2020). The *LipL32* gene was targeted for thedetection in the RPA as it is a potential target (abundantly expressed duringinfection and restricted to pathogenic strains) that has been applied in othernucleic acid-based amplification methods (Haake et al 2000). The optimizedRPA assay targeting the lipL32 gene wasable to amplify the 126 bp amplicon from all the reference serogroups of Leptospira (23 that are available at our laboratory) demonstrating the selectivity to thepathogenic Leptospira as alreadyobserved with the PCR.

Following the confirmation of the selectivity of the RPA to detect Leptospira DNA, we determined to validate this optimized assay for its performance characteristics as per the OIE pathway of validation (as per Chap. 1.1.6 Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 2021) as it ascertains the assay to be intended for the purpose developed. Performing the first three stages of validation (Analytical characteristics, Diagnostic characteristics and Repeatability characteristics) is sufficient to determine the suitability of the assay for the purpose intended. To meet the demands of an appropriate validation methodology, the study initially ascertained minimum variability in pipetting of the reaction components. The coefficient of variation ranged from 0.3 to 1.1% indicating negligible contribution for dispensing the respected volumes with the micropipettes used in this study.

It was observed that both PCR and RPA had an analytical sensitivity of up to 10^2 copies of the *Leptospira* genomic DNA. In addition, the PCR and RPA assay also were found to be specific in detecting Leptospira DNA and resulted in no specific amplification with other related Gram -ve bacteria confirming its analytical specificity. Both these observed features confirm the analytical characteristics of the optimized RPA assay with the *LipL32* gene as the target. The diagnostic sensitivity and specificity of the RPA assay revealed good agreement with the PCR assay (kappa value of 0.905). However, when applied to kidney tissues and water samples the RPA assay had similar positivity percentage as that of PCR. The reported RPA assay not only showed a very good intra and inter-assay repeatability, the reproducibility was also very good. The repeatability of assay performed at different occasions showed a co-efficient of variation of 5.9% is minimal and in agreement with the report of Reed et al (2002). In an earlier report, the limit of detection was claimed to be ≤ 10fg or ≤ 2 genome equivalents with the use of a real-time RPA assay

(Ahamed et al 2014) and similar to that of an earlier validated real-time PCR. A real-time RPA assay can have a lower detection due to the inherent ability of the fluorescent tags that are used as the reporter however, this lower detection limit was not supported with data. If the RPA assay reported in this study is modified to include a probe and coverted to a real-time RPA, the same detection limit could be achieved.

The manufacturer (M/s TwistDx) recommends the purification of products using PCR purifying columns for removal of DNA-binding proteins from RPA-generated amplicons before visualization by electrophoresis that increases the time and expense of the assay. The alternate method for denaturation of protein by heat was applied in this study resulted in fast and less expensive detection approach. The application of RPA assay to detect the leptospiral DNA in kidney and water samples, implies the use of the assay for surveillance of Leptospira in the environment which are considered to be source infection to dogs. RPA assay is a versatile molecular diagnostic tool that extends the capabilities of diagnostic facilities without access to a thermocycler. This technique is more rapid generating the result in 30 minutes when compared to that of PCR assay, but the reagent is costly when compared to PCR reagent. On factoring in the time and cost, RPA assay is less expensive than PCR assay. RPA is less sensitive to inhibitors than PCR (Piepenburg et al 2006) and is capable of amplifying DNA from a variety of samples from humans, animals, and the environment (Ahamed et al 2014). 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 setting.

Declarations

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Consent to Participate

Not required since the samples were collected for diagnostic purpose

Consent to Publish: All authors gave explicit consent to publish the research work

Ethical Approval : Ethical committee approval is not required for this study since the samples were used for diagnostic purpose

Availability of data and materials: All data generated or analyzed during this study are included in this article and also available in Zoonoses Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India.

Conflict of Interest: The authors declare no conflict of interest

Authors contribution

Senthilkumar had involved in conceptualization, sample collection, performance of the research work and original draft preparation of the manuscript.Nirmala performed the research work. Ravikumar and Tirumurugaan had involved in supervision of work, formal analysis of investigation and editing the manuscript. Aravindh babu had coordinated for the statistical analysis of investigation and editing the manuscript.Thirumurugaan reviewed the article

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Tables

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

S.No	Serogroup	Serovar	Strain
1.	Australis	Australis	Ballico
2.	Autumnalis	Rachmati	Rachmati
3.	Ballum	Ballum	Mus 127
4.	Canicola	Canicola	Hond Utrecht IV
5.	Grippotyphosa	Grippotyphosa	Moskva V
6.	Hebdomadis	Hebdomadis	Hebdomadis
7.	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA
8.	Javanica	Poi	Poi
9.	Pomona	Pomona	Pomona
10.	Pyrogenes	Pyrogenes	Salinem
11.	Sejore	Hardjo	Hardjoprajitno
12.	Tarassovi	Tarassovi	Perepelitsin
13	Bataviae	Bataviae	Swart
14	Cynopteri	Cynopteri	3522 C
15	Djasiman	Dasiman	Djasiman
16	Hurstbridge	Hurstbridge	BUT 6
17	Louisiana	Louisiana	LSU 1945
18	Manhao	Manhao	L 60
19	Mini	Mini	Sari
20	Panama	Panama	CZ 214 K
21	Ranarum	Ranarum	ICF
22	Sarmin	Weaveri	CZ 390
23	Shermani	shermani	1342 K
24	Semeranga	Patoc	Patoc 1

The above reference cultures are being routinely maintained in the laboratory to offer diagnostic testing for leptospirosis by Microscopic Agglutination Test (MAT)

Table 2 Performance of RPA assay

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

Table 2 Performance of RPA assay in comparison with the PCR assay for detecting leptospira

DNA in clinical samples (n = 150). Pos, positive; Neg, negative; CI, confidence interval.

Figures

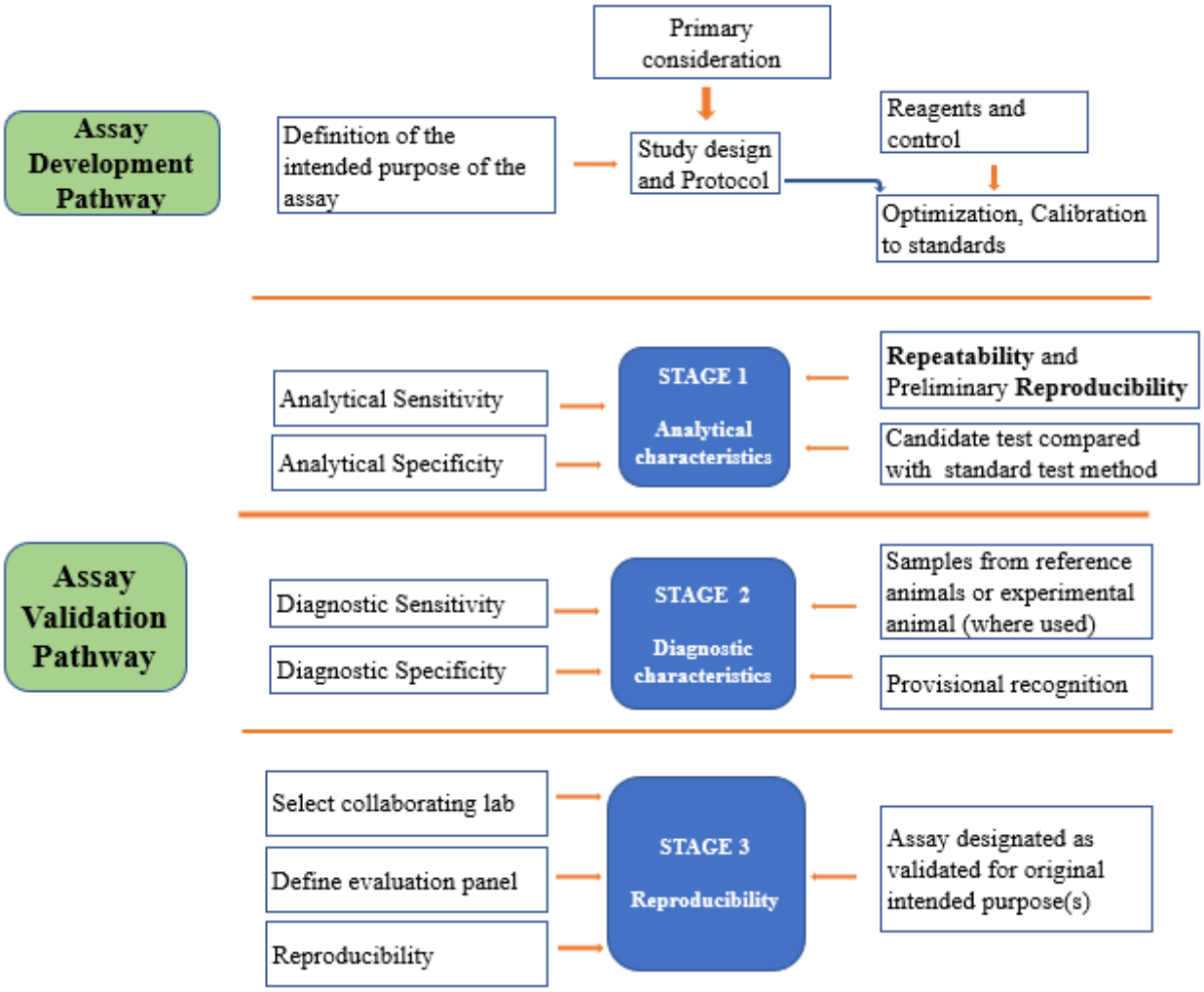

Figure 1

Flow diagram for validation of Assay

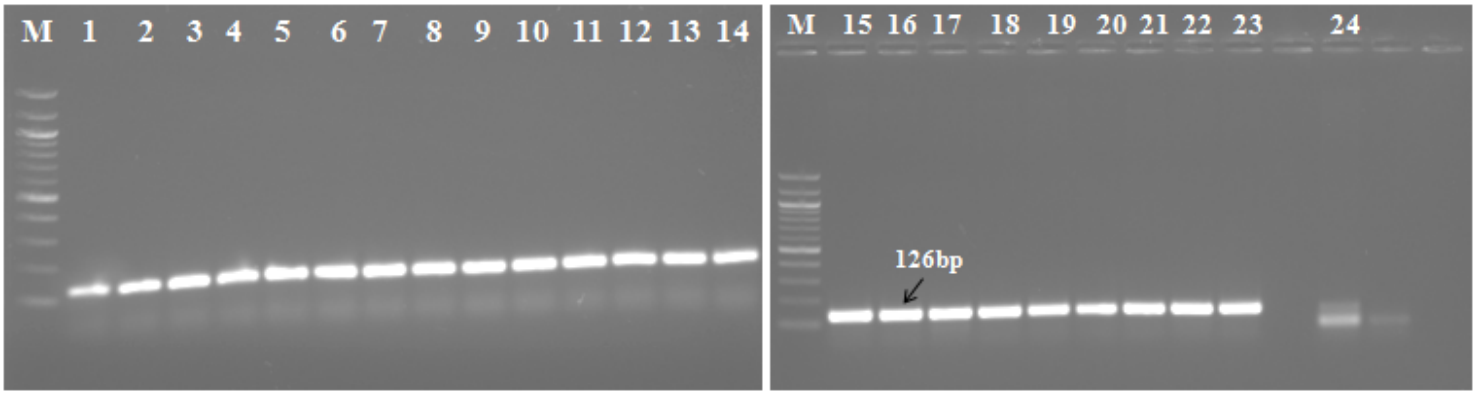

Figure 2

PCR Amplification of *LipL32* gene of *Leptospira* from different serogroups (*Details listed in Table 1*)

Note: The specific amplification of 126 bp amplicon in the optimized PCR assay from the 23 pathogenic Leptospira serogroup (Lanes 1 to 23) and no amplification in the non-pathogenic Leptospira biflexa serogroup Semaranga serovar Patoc(Lane 24)

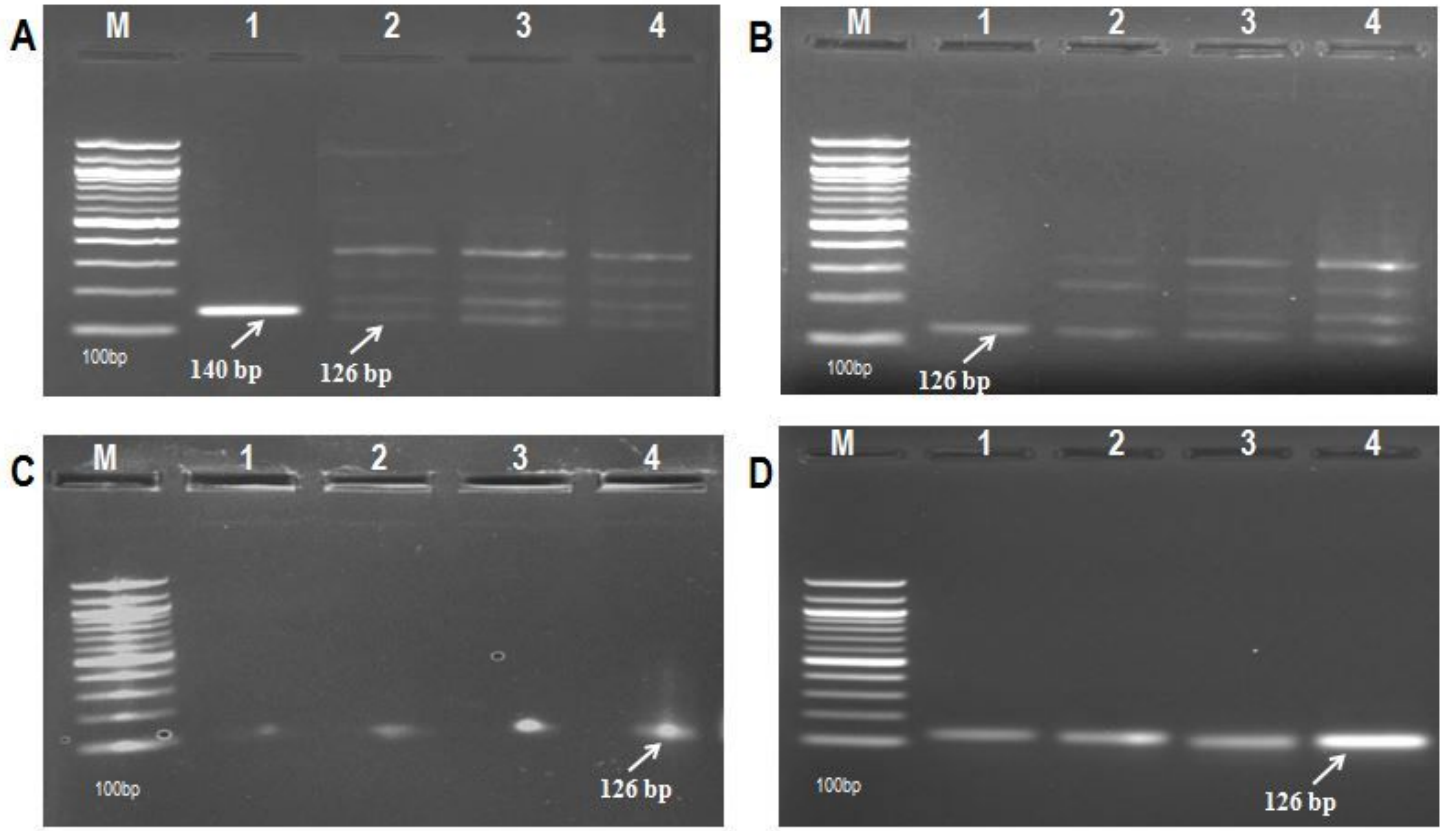

Figure 3

Optimization of the different reaction conditions for specific detection *Leptospira* DNA by Recombinase Polymerase Amplification Assay. A) Optimization of the DNA concentration (Lane1- Kit control, Lane 2 – 20ng, Lane 3 – 30ng, and Lane 4- 40ng); **B)** Optimization of Magnesium acetate concentration in the buffer (Lane1- 2.8mM, Lane 2- 5.6mM, Lane 3-8.4mM, and Lane 4-11.2mM); **C)** Optimization of the reaction time for the RPA assay **(**Lane 1-5 min., Lane 2- 10 min., Lane 3- 20 min., and Lane 4-25 min.); **D)** Representative gel image of the optimized RPA assay *(DNA concentration.- 20ng, Mag. acetate - 2.8mM and reaction time for 25 min.)* with DNA from known serovars of *Leptospira (Lanes 2 to 4 DNA of different serovars of Leptospira*). M – 100 bp DNA ladder. *Note: The specific amplification of 126 bp amplicon in the optimized RPA assay*

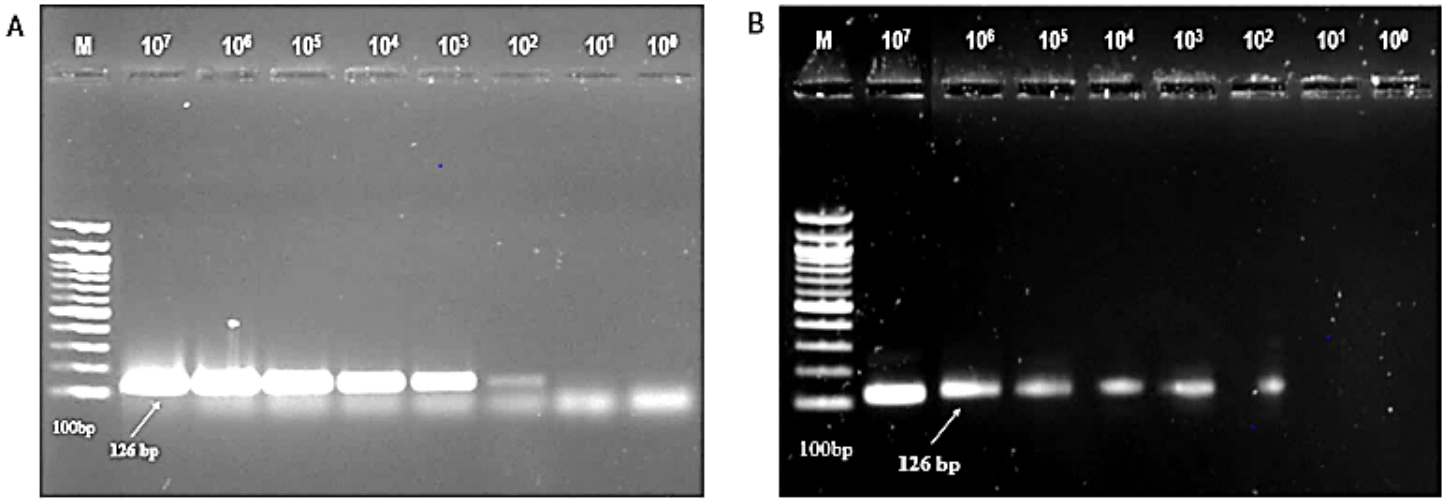

Figure 4

Analytical sensitivity of the RPA assay: A) Lower detection limit up to 10^2 copies by PCR assay **B)** Lower detection limit up to 10^2 copies by RPA assay

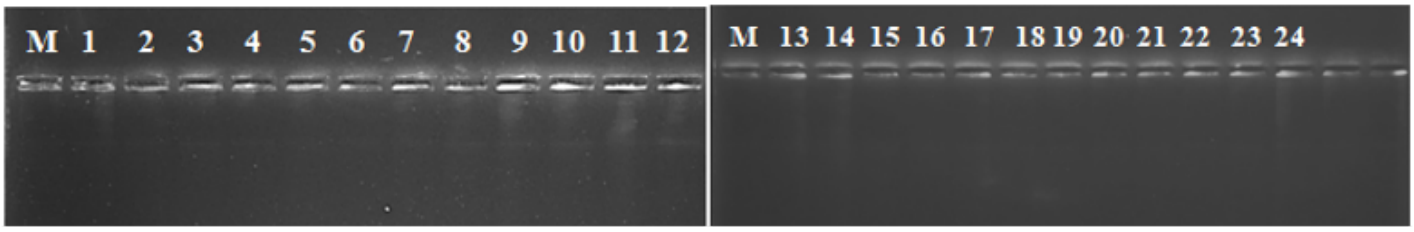

Figure 5

Specificity of RPA assay to detect the Leptospira DNA. A & B – Specific amplification of 126 bp amplicons from leptospiral DNA from different serogroups (1-23) and no amplification with Patoc (Lane 24) (*Details of the serogroup listed in Table-1*); C- No amplification with DNA from other related gram -ve bacterial species.