Full Length Article

VISUAL DETECTION OF RECOMBINASE POLYMERASE AMPLIFIED DNA FOR RAPID DIAGNOSIS OF LEPTOSPIROSIS IN CANINE

K. Senthilkumar*[1], K. Nirmala[2] and K.G. Tirumurugaan[3]

Zoonoses Research Laboratory
Centre for Animal Health Studies
Tamil Nadu Veterinary and Animal Sciences University
Chennai, Tamil Nadu, India

ABSTRACT

Rapid, sensitive and accurate diagnosis of Leptospirosis in canines is very much helpful in areas of increased prevalence. This study aimed to visualise Recombinase Polymerase amplified DNA of pathogenic Leptospira for rapid diagnosis of Leptospirosis in canine. The Recombinase Polymerase Amplification (RPA) assay for amplifying the lipL32 gene of pathogenic Leptospira was optimised. The visual detection of the amplified DNA by incorporating an optimised concentration of SYBR Green I dye in an indigenously built low-cost detection device was also optimised. Light green fluorescence was observed in the positive samples, while the negative samples appeared orange. The visual detection format of the RPA can be integrated as a point of a diagnostic test in any veterinary clinic or in remote areas where resource settings are limited.

Keywords: Canine, leptospirosis, recombinase polymerase amplification assay

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INTRODUCTION

Leptospirosis is a zoonotic bacterial disease affecting most mammalian species (Bharti *et al*., 2003). Canine Leptospirosis is considered as most serious, since leptospiral pulmonary haemorrhage syndrome (LPHS) has emerged as a life-threatening complication (Schuller *et al*., 2015). In addition, leptospires are zoonotic pathogens pose public health risk. Leptospires are motile, helically coiled bacteria, having a hook at both ends (Faine *et al*., 1999). It causes septicaemia and systemic infection and subsequently causes fatalities owing to delayed treatment in dogs. Rapid, sensitive and accurate diagnosis of leptospirosis in canines is very helpful for areas where leptospirosis prevalence and intensity are high. The available diagnostic methods based on dark field microscopy examination are rapid but less specific; which, require experienced personnel to distinguish leptospires from other artefacts

* Corresponding author, email: senthilkumar.k@tanuvas.ac.in

[1] Assistant Professor

[2] Project Associate, DBT Scheme

[3] Professor and Head

due to similarity in morphology; therefore, need a dark-field microscope. The serological tests such as the microscopic agglutination test (MAT), ELISA and others detect the antibodies to leptospires at the late acute phase (5-7 days of infection or later). The MAT is confirmative, referred diagnostic test by World Organisation for Animal Health and is serogroup specific. However, this test is laborious to perform, requires technical expertise for reading and interpretation of results and involves biosafety issues (OIE,2018).

Most of the studies use molecular methods, like PCR-based detection assays for the detection of residual *Leptospira* DNA in samples for the diagnosis of leptospirosis. However, it requires expensive thermal cycler and reagents that are not affordable in resource-limited areas and diagnostic laboratories with insufficient funding. The real-time PCR-based method with a total turnaround time of approximately two hours has been endorsed by many authors (Stoddard *et al*., 2009; Rojas *et al*., 2010) for detection without post PCR procedures. However, the cost of equipment, reagents and stable electric power supply limit its use in poor resource areas.

The isothermal amplification methods are considered as an alternative to PCR assays which amplify products at a constant temperature and can be supported with a simple heat block avoiding the need for thermal cycler and other specific instruments. This method is a major advantage for low-resource settings and can be applied as a point-of-care test. The Recombinase Polymerase Amplification assay (RPA) is one of the isothermal methods and has been widely used to detect viruses, bacteria and parasite-specific nucleic acids since the advent of this technology in 2006 (Piepenburg *et al*., 2006; Singpanomchai *et al*., 2019; Lalremruata *et al*., 2020). The three core enzymes, namely, recombinase, single-stranded binding protein and DNA polymerase are used in RPA amplification (Piepenburg *et al*., 2006). The amplification process occurs under an optimum temperature that ranges from 37 to 42°C and takes <10-40 minutes (Crannell *et al.*, 2014). Previous studies have reported the detection of *Leptospira* by the RPA assay employing a real-time method (Ahmed *et al*., 2014) but required specific devices to detect the amplified DNA. Hence in this study, we have optimised the Recombinase Polymerase Amplification Assay using SYBR Green I (RPA-S) and have developed a low-cost indigenous device for visual detection of the amplified DNA targeting the application in low-resource settings or field laboratories.

MATERIALS AND METHODS

Reference culture, collection of samples and biological reagents

A reference pathogenic (*L. interrogans* serovar Canicola) and non-pathogenic *(L. biflexa* serovar Patoc*) Leptospira* strain maintained at Zoonoses Research Laboratory, Tamil Nadu Veterinary and Animal Sciences University, Chennai were used as the source of positive and negative DNA in this study. The clinical samples included 117 serum samples from dogs with clinical signs of fever, jaundice, vomiting, hematuria, renal failure submitted to Zoonoses Research Laboratory for *Leptospira* diagnosis from the Madras Veterinary College, Teaching Veterinary Clinical Complex and Veterinary University Peripheral Hospital of

Tamil Nadu Veterinary and Animal Sciences University (TANUVAS) and some private veterinary clinics in Chennai. The materials used in the study included TwistAmp® (TwistDx, U.K), QIAamp DNeasy blood and Tissue kit (Qiagen, India), 100bp DNA ladder (New England Biolabs, MA), Oligonucleotide primers (synthesised from IDT, Singapore) and QIAexpert (Qiagen, MA) to assess the DNA quality and quantity. The primers used in the RPA assay are listed in Table 1.

DNA extraction

Genomic DNA of *Leptospira* reference strains was extracted with QIAamp DNA Mini kit (M/s Qiagen, India). The DNA from dog blood and serum samples were extracted with DNeasy Blood and Tissue Kit. The extraction was performed as per the manufacturer's protocol. The concentration of DNA was determined in the QIAexpert® system. A total of 117 genomic DNA samples extracted from blood samples collected from dogs were used in this study.

Recombinase polymerase amplification assay and agarose gel electrophoresis (RPA-AGE)

The concentration of primer, DNA, magnesium acetate used in the RPA assay, and the temperature and time required for the isothermal reaction are the critical parameters to be optimised (Piepenburg *et al.,* 2006). In this study, the assay was performed in a 50 µl reaction with slight modifications to optimise the above parameters. The reaction mixture consisting of 29.5 µl of buffer, 2.4 pmol each of forward and reverse primer, 20 ng of template DNA in a volume of two µl and 11.2 µl of nuclease-free water was added to the reaction pellet tube, mixed by vortexing. As magnesium acetate initiates the polymerase activity, 2.5 µl was added to the inner side of the reaction tube lid, lid closed and spun to mix with the reagents. The reactions were initially incubated at 39º C for 5 minutes in the water bath, taken out, vortexed and again incubated at 39º C for 20 minutes The proteins were separated from the amplified DNA by denaturing at 65º C for 10 minutes and the addition of SDS to a final concentration of 1% w/v. The amplicons were detected by electrophoresis in 1.5% agarose gel in 1X TAE, and results were documented. All the DNA extracted from 117 blood samples were subjected to RPA assay to amplify the *LipL32* gene. The DNA extracted from pathogenic and non-pathogenic leptospires were used as control.

Visual detection of RPA amplified products

For visual detection of RPA assay

Table 1. Primers used in RPA assay

Primer pair	Sequence 5′-3	Region targeted	Amplicon length (bp)
RPA 11F	CTGCCGTAATCGCTGAAATGGGAGTTCGTATG	260-291	126 bp
RPA 11R	GTGGCATTGATTTTTCTTCTGGGGTAGCCG	385-356	

amplified *lipL32* gene, two µl of different concentrations of SYBR Green I (Invitrogen, USA) (62.5x, 125x, 250x, 375x, 500x, 625x, 750x and 1000x) was added to 10 µl of RPA product in each tube (RPA-S), and then the change of colour was viewed by the naked eye (light green in positive samples vs orange in negative samples). The same tube was also visualised in an indigenously built portable prototype electrical device with blue LED (460 nm wavelength) using 3D printing technology to observe the light green fluorescence in the positive samples. This device works on a stand alone battery that can be charged through an USB port and withstand for a period of 8-9 hours, once charged. The RPA assay with different concentrations of DNA (100ng, 10ng, 1ng) was performed to determine the detection limit of amplified DNA for visual detection. Data on detection of RPA amplification in respect of RPA-AGE and RPA-S were statistically analysed by ANOVA test using STATA software (TANUVAS, Chennai) and $P<0.05$ values were considered statistically significant.

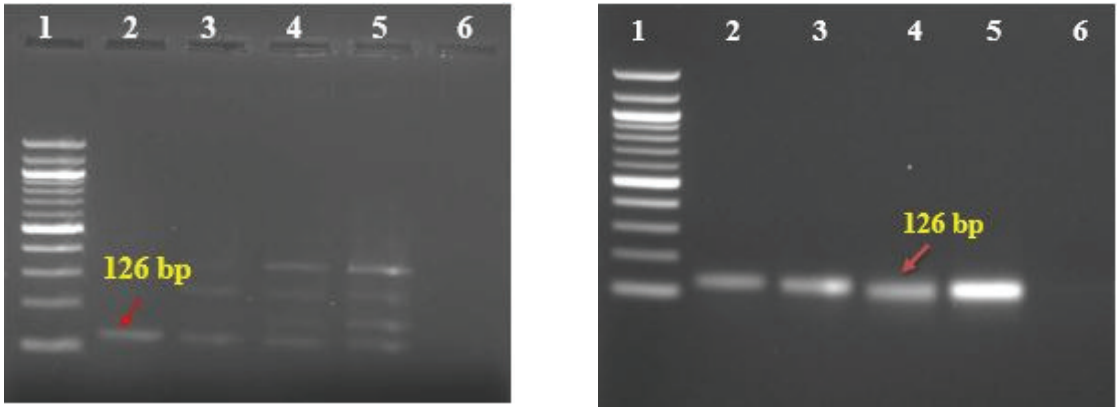
RESULTS AND DISCUSSION

Recombinase polymerase amplification assay and agarose gel electrophoresis

The RPA assay was optimised with the RPA primer concentration of 2.4 pmol with 2.5 µl of Magnesium acetate (280 mM) and 20 ng of DNA at an incubation temperature of 39ºC for 25 minutes. The presence of 126 bp amplicon in the RPA assay with pathogenic reference strain was visible upon agarose gel electrophoresis. This indicated the efficiency of the RPA assay to amplify the *LipL32* gene of *Leptospira*. The simple and low turnaround time for amplification of DNA by RPA assay overcome the limitations such as cost and complexity in the utility of conventional and commercial nucleic acid amplification methods, even though they are sensitive and rapid (Ahmed *et al*., 2009; Hartskeerl *et al*., 2011). The RPA assay has been applied to diagnose several diseases (Singpanomchai *et al*., 2019; Piepenburg *et al*., 2006; Li *et al*., 2018). This method does not require a thermalcycler, and the whole amplification processes occur under isothermal conditions (Piepenburg *et al*., 2006). Out of the 117 samples screened by RPA assay, leptospiral DNA was detected in thirty-nine samples (Fig. 1). RPA assay with a variety of detection formats, including real-time detection method (Ahmed *et al*., 2014; Li *et al*., 2018), Lateral flow assay-based detection (Wang *et al*., 2018) and agar gel-based detection (Singpanomchai *et al*., 2019) were reported. However, these detection methods require specific instruments that become costly and cumbersome, necessitating an alternate detection method.

Visual detection of RPA amplified products

The RPA amplified products were viewed by addition 2 µl of 375x SYBR green dye I was found to be optimum for detection by the naked eye (Fig. 2), and this concentration of SYBR Green I is in agreement with the previous report (Lai and Lau, 2020). However, the LED device enables better visualisation of the amplified DNA due to the light green fluorescence (Fig. 3). The amplified products were detected up to 10 ng of DNA in the sample by RPA-S by the naked eye. The detection level depends on the efficiency of



A B

Fig. 1. Visualisation of RPA amplified DNA in agarose gel electrophoresis

A- Optimisation of RPA assay (lane 1- an optimised condition of RPA assay; lane 2-5 different concentrations of magnesium acetate, DNA, incubation time)

B. RPA assay amplified DNA of clinical samples (lane 2-4 - dog blood samples; lane 5 -*L. interrogans* serovar Canicola DNA; lane 6- no template control)

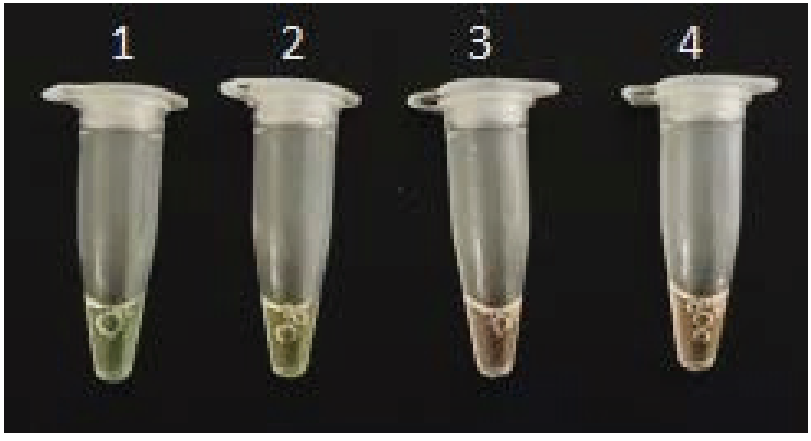


Fig. 2. Visual detection of RPA amplification of *LipL32* gene

1-*L.interrogans* serovar Canicola; 2&3 - dog blood samples; 4 - no template control

The positive amplification (1&2) in the RPA assay resulted in mild yellowish green colour and negative amplification (3&4) as orange colour to the reaction mix.

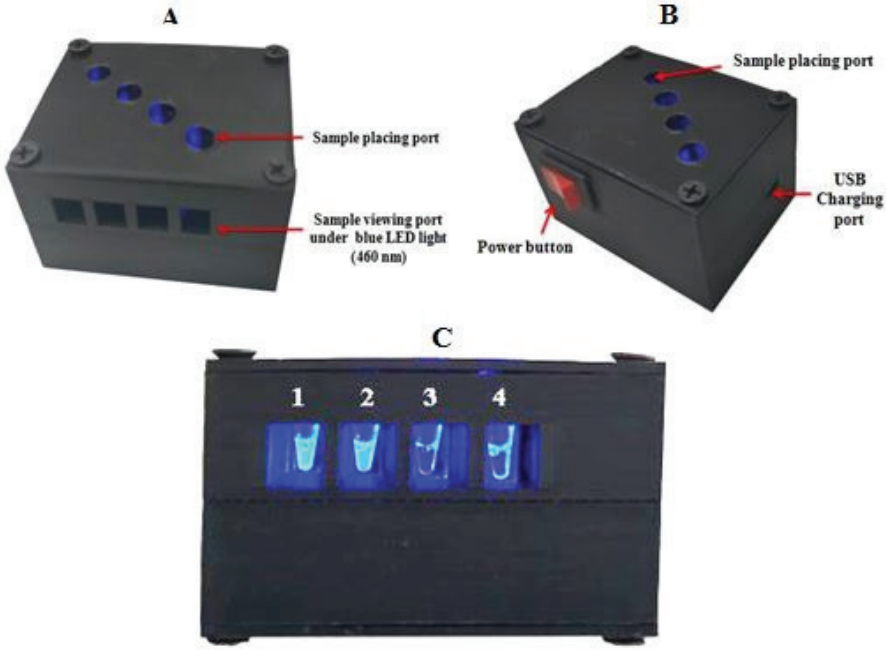


Fig. 3. Low-cost visualisation device developed by 3D printing technology

A-Front view

B - Lateral view (the availability of the USB port enables easy charging)

C - Viewing theresult of samples. 1- *L. interrogans* serovar Canicola; 2&3 - dog blood samples; 4- no template control.

The positive amplification (1&2) in the RPA assay was visible as a bright green fluorescence.

SYBR Green I to bind with double standard DNA. Out of 117 dog blood samples screened by RPA-S, leptospiral DNA was also detected in thirty-five samples. On statistical analysis, there is no significant difference between these two methods ((λ^2 – 0.32^{NS}, $P < 0.05$). The similar naked eye detection method was applied for *Mycobacterium tuberculosis* (Singpanomchai *et al*., 2019). The SYBR Green I bind to double-stranded DNA and results in a DNA-dye complex that emits green light and can be observed by the naked eye without the need for sophisticated equipment. The development of RPA assay in combination with SYBR Green I in this study becomes instrument-free nucleic acid amplification, and visual detection by naked eye provides the option to be used as a point-of-care test. A similar RPA assay combined with SYBR Green I was used as a POC test to detect *Plasmodium knowlesi* (Lai and Lau, 2020). The RPA-AGE requires electrophoresis to detect the amplicons of expected size but opening the tube with amplified products increases the chance of cross-contamination in a routine laboratory setting, thus results in

false positivity. The addition of SYBR Green I to the reaction tube reduces the contamination as the amplicons emit green fluorescence. The LED visualisation device prepared by 3D printing technology allows better visualisation of the amplicons due to the bright green fluorescence, and owing to simplicity, it can be applied as a point of the care detection device.

In conclusion, RPA, in combination with SYBR Green I have the potential to be developed for POC application with such detection devices. This approach is rapid, has no specific equipment to inspect the results, and does not require expertise. This method is also potentially valuable for poor resource settings.

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