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Rapid detection of pathogenic leptospires by lyophilized reagent-based Polymerase Chain Reaction

Lee, S.V.*, Tai, E.S., Mutalib, A.R., Khairani-Bejo, S. and Bahaman, A.R.
Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Malaysia
*Corresponding author email: svlee7@gmail.com
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Abstract. A simple and reliable tool for the early diagnosis of leptospirosis is urgently needed. We report the development of a lyophilized reagent-based polymerase chain reaction (PCR) assay targeting *lipL32* gene, which is present only in pathogenic leptospires. To determine the effectiveness of the newly developed assay in the early diagnosis of leptospirosis, the sensitivity and specificity was evaluated. In simulated clinical samples, the assay was able to detect 10^2 and 10^3 leptospires/ml in spiked urine and blood samples, respectively. In experimentally infected animals, leptospiral DNA could be detected in blood and lung samples as early as Day 1 post infection. This assay was also shown to be stable and remained sensitive for up to five months at ambient temperature. Hence, this lyophilized reagent-based PCR assay with high specificity, sensitivity and stability would provide a simple, rapid and reliable method in diagnosing acute leptospirosis, especially in the field of veterinary medicine.

INTRODUCTION

Leptospirosis is a re-emerging zoonotic disease caused by the pathogenic species of *Leptospira*. Leptospiral infection occurs in humans and in animals, where it causes loss of productivity in livestock (Adler & Faine, 2006). Severe leptospirosis leads to systemic infections in both animals and humans characterized by jaundice, renal failure, and pulmonary haemorrhages (Sekhar *et al.*, 2000; Yang, 2006). The morbidity and mortality rate of leptospirosis is high but inestimable as it is highly underreported and misdiagnosed due to its protean manifestations, the lack of public awareness and insufficiency of rapid diagnostic capabilities (Bharti *et al.*, 2003; McBride *et al.*, 2005).

The conventional diagnosis of leptospirosis is based on culture isolation in specific media or demonstration of anti-*Leptospira* antibodies by serological techniques (Vinetz, 2001; Palaniappan *et al.*, 2005). The Microscopic Agglutination Test (MAT) is regarded as the gold standard test for leptospirosis despite several disadvantages (Levett, 2001; Bharti *et al.*, 2003, Palaniappan *et al.*, 2007). MAT can only be performed in certain reference laboratories by trained personnel as it requires cultures of life leptospires that are difficult to maintain (Levett, 2001). Besides, early sero-diagnosis, especially in acute cases is often hampered as antibodies will only be developed 5 to 7 days post infection (Ribotta *et al.*, 2000; Saengjaruk *et al.*, 2002; Levett, 2004). Confusing results will also be produced due to the persistence of anti-*Leptospira* antibodies in the body for a long period of time even after recovery. Drawbacks associated with serodiagnosis had prompted great interest in the development of rapid and simple molecular diagnostic methods.

The Polymerase Chain Reaction (PCR) has been utilized in the detection of pathogenic leptospires from clinical samples such as blood, urine, and infected organs (Levett, 2001; Palaniappan *et al.*, 2005). It

has been shown to be a promising assay in the diagnosis of leptospirosis as it is rapid, sensitive and highly specific. However, the possibilities of errors occur during multi-step preparation of PCR mixtures and contaminations may perhaps lead to false positive results. Thus, trained personnel are required to perform PCR and strict measures have to be taken in order to avoid contamination (Siegmund *et al.*, 2005). Besides, conventional PCR which comprises heat-sensitive reagents must be transported and stored under cold condition in order to prevent degradation.

To overcome these limitations, this study aims to develop a lyophilized reagent-based PCR for the detection of pathogenic leptospires, which is simple, rapid and reliable. It can be performed by the addition of only sterile water and DNA template before subjecting to amplification. This is especially useful when the number of samples is large. Besides, it is also user friendly and time-saving. Klatser *et al.* (1998) had reported that freeze-dried PCR mixes are stable at ambient temperature for up to a year. Therefore, storage and transportation of the PCR reagents in cold condition is not a requirement. In this study, a set of primers, V1/V2 previously described by Lee *et al.* (2009) was included in the developed lyophilized reagent-based PCR for the diagnosis of leptospirosis. This developed PCR assay was then evaluated on simulated clinical samples and samples obtained from experimentally infected hamsters.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

Leptospiral reference strains involved in this study (Table 1) were from the WHO/OIE Collaborating Centre for Reference and Research on Leptospirosis, Centre for Public Health Sciences, Queensland Health Scientific Services, Australia. A highly virulent strain, *Leprospira interrogans* serovar Lai strain Langkawi, was kindly provided by WHO/FAO/OIE *Collaborating*

Table 1. *Leptospira* strains used in this study

Species	Serovar	Strain	Status
L. interrogans	Australis	Ballico	Pathogenic
L. interrogans	Autumnalis	Akiyami A	Pathogenic
L. interrogans	Bataviae	Swart	Pathogenic
L. interrogans	Canicola	Hond Utrecht IV	Pathogenic
L. interrogans	Grippotyphosa	Moskva V	Pathogenic
L. interrogans	Hardjo-prajitno	Hardjoprajitno	Pathogenic
L. interrogans	Hebdomadis	Hebdomadis	Pathogenic
L. interrogans	Pomona	Pomona	Pathogenic
L. interrogans	Pyrogenes	Salinem	Pathogenic
L. interrogans	Djasiman	Djasiman	Pathogenic
L. interrogans	Icterohaemorrhagiae	RGA	Pathogenic
L. interrogans	Lai-langkawi	Langkawi	Pathogenic
L. borgpetersenii	Javanica	Veldrat Batavia 46	Pathogenic
L. borgpetersenii	Sejroe	M84	Pathogenic
L. borgpetersenii	Tarassovi	Perepelitsin	Pathogenic
L. borgpetersenii	Hardjo-bovis	Sponselee	Pathogenic
L. borgpetersenii	Ballum	Mus 127	Pathogenic
L. kirschneri	Cynopteri	3522C	Pathogenic
L. weilii	Celledoni	Celledoni	Pathogenic
L. meyeri	Semaranga	Veldrat Semarang 173	Non-pathogenic
L. biflexa	Patoc	Patoc I	Non-pathogenic

Centre for Reference and Research on *Leptospirosis* of the Royal Tropical Institute, *Amsterdam*. Leptospires were propagated in EMJH liquid medium (Ellinghausen & McCullough, 1965; Johnson & Harris, 1967) at 30°C.

Eight other pathogenic bacteria (*Staphylococcus aureus*, *Streptococcus pyrogenes*, *Klebsiella pneumonia*, *Pasturella multocida*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, and *Salmonella typhi*) were obtained from the Bacteriology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia.

Genomic DNA from all bacteria was extracted, purified and eluted using the Promega Wizard Genomic DNA purification kit (Promega, USA), according to the manufacturer's instruction.

Lyophilisation of PCR reagents

Five percent (w/v) of Trehalose (Merck, USA) was included in each tube of PCR mixture containing 200 µM of dNTP, 2 mM of $MgCl_2$, 0.5 µM of V1/V2 primers (NHK biosciences, Korea) and 1 U of GoTaq® Flexi DNA polymerase (Promega, USA), to stabilize the DNA polymerase (Klatser *et al*., 1998). PCR mixtures were lyophilized using freeze-dryer (FreeZone, Labconco, USA). The lyophilized PCR cocktails were incubated at ambient temperature (25-30°C) over a period of 6 months for stability observation.

Conventional PCR

Reactions were performed in a total volume of 25 µl by adding sterile water and 1 µl of DNA template extract from culture, or 5 µl of DNA extract from spiked or clinical specimens into the lyophilized PCR assay. PCR was conducted in My Cycler (Bio-Rad, USA) using the following profile: an initial denaturation at 94°C for 5 minutes, followed by 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min. The cycles were followed by a 7 min extension at 72°C. Ten µl aliquots from each amplicon were analyzed via agarose gel electrophoresis at 5V/cm and visualized using Gel Doc® System (Bio-Rad, USA) after ethidium bromide staining.

Analytical sensitivity of the PCR assay

The lower limit of detection (LOD) of the PCR assay was determined using purified genomic DNA from *L. interrogans* serovar Icterohaemorrhagiae strain RGA. The quantity of genomic DNA was estimated by measuring the absorbance of DNA using a spectrophotometer (Cecil Instruments, UK) according to the method described by Sambrook *et al.* (1989). Serial dilutions of genomic DNA were made from 0.1 µg/µl to 10 fg/µl. A genome size of 4.659 Mb was used to determine the genomic equivalent (GE) of the purified DNA (Nascimento *et al.*, 2004).

Spiking experiments and DNA extraction

Samples of canine blood and urine were collected from the Small Animal Ward, University Veterinary Hospital (UVH), Universiti Putra Malaysia. Blood and urine samples were inoculated into separate semi-solid EMJH media for culture and maintained for up to 3 months before discarding as cultures with negative growth. Serum samples were collected for screening of antibodies against leptospires via MAT.

A known quantity of *L. interrogans* serovar Lai strain Langkawi was spiked into Phosphate-buffered saline (PBS), pH 7.4, whole blood in EDTA and urine to reach the final concentration of 1×10^6 leptospires/mL. The number of bacteria was determined by counting in a Petroff-Hausser counting chamber (Hausser Scientific, USA) according to the standard protocol. Subsequently, serial 10-fold dilutions of 1×10^5 down to 1×10^0 leptospires/mL were made. Unspiked whole blood and urine samples were used as negative controls.

DNA extraction of both blood and urine samples for PCR was performed using Promega Wizard® SV Genomic DNA Purification kit (Promega, USA), in accordance to the manufacturer's instruction. *However, samples from each of the spiked urine dilution series were centrifuged at 10,000 rpm for 30 minutes followed by a*

washing step with PBS, prior to DNA extraction.

Animal experimental infection and DNA extraction

Four weeks old Golden Syrian hamsters were injected intraperitoneally with a sub-lethal dose (10^6) of *L. interrogans* serovar Lai strain Langkawi in 500 µl liquid EMJH medium. Three hamsters were euthanized by exsanguinations on Day 1, 3, 5 and 7 post infections. Three hamsters injected with un-inoculated liquid EMJH medium were used as negative controls, and were sacrificed on the last day of experiment. These animals were managed as in the proposal approved by the Institutional Animal Care and Use Committee (IACUC). Blood was collected from the hamsters via cardiac puncture and cultured by direct inoculation of a few drops of blood into EMJH medium. The liver, kidneys and lungs were removed aseptically from each animal. Briefly, these tissue samples (liver, kidneys and lungs) were homogenized in 10 ml sterilized PBS and 0.5 ml of the lysates were inoculated into 5 ml semi-solid EMJH medium for culture. Recovery of leptospires from these samples was monitored under dark field microscope and negative cultures were discarded after three months. DNA extraction of organs was performed using Promega Wizard® SV Genomic DNA Purification kit (Promega, USA) according to the manufacturer's instructions.

RESULTS

Analytical sensitivity and specificity of V1/V2 primers

The assay was found to be highly specific (100%) towards the identification of pathogenic *Leptospira* species. None of the saprophytic strains and other bacterial species was detected by the assay. Our results showed that the lyophilized reagent-based PCR could detect as low as 0.5 pg of leptospiral DNA, or 1 x 10^2 leptospiral genome copies (assuming one genome equivalent per leptospire).

Stability of lyophilized reagent-based PCR

PCR was performed at monthly intervals on the lyophilized PCR cocktails stored at ambient temperature (25-30°C). The lyophilized reagent-based PCR were stable at ambient temperature for up to five months. A decrease in sensitivity was shown after storage for more than five months.

Simulated clinical samples

The developed assay was able to detect 10^3 leptospires/ml in the spiked blood samples and 10^2 leptospires/ml in the spiked urine samples, respectively (Figure 1).

Experimentally infected hamsters

Lyophilized reagent-based PCR and isolation of leptospires from blood, kidney, liver and lungs of experimentally infected hamsters

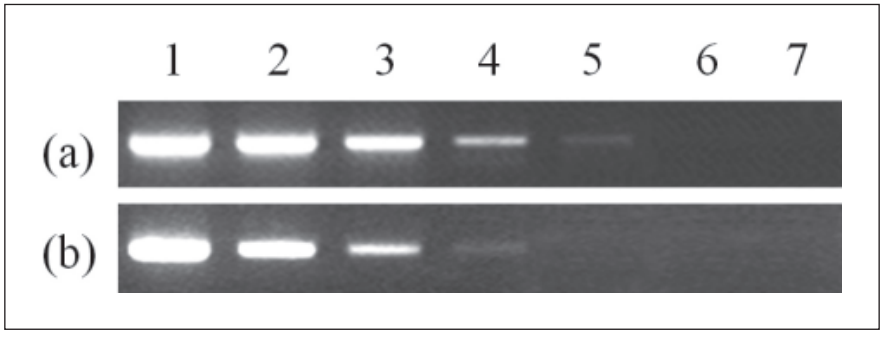

Figure 1. 819 bp PCR products amplified by lyophilized reagent-based PCR on samples spiked with *L.interrogans* serovar Lai strain Langkawi. Serial dilutions were performed from 10^6 to 10^0 leptospires/ ml in a) canine blood sample, b) canine urine sample; Lane 1-7: 1x 10^6 to 10^0 leptospires/ ml

Table 2. Lyophilized reagent-based PCR and isolation data on samples harvested from experimentally infected hamsters. Three hamsters were euthanized on each day 1, 3, 5, and 7 post infection

Samples	Tests	Day 1			Day 3			Day 5			Day 7			Positive (%)
Blood	PCR	+	–	–	+	+	+	+	+	+	+	+	–	75.0
	isolation	+	–	–	+	+	+	+	+	+	+	+	+	83.3
Kidney	PCR	–	–	–	+	+	+	+	+	+	+	+	+	75.0
	isolation	–	–	–	–	–	–	–	+	+	–	+	+	33.3
Liver	PCR	–	–	–	–	–	+	+	+	+	+	+	+	58.3
	isolation	–	–	–	–	–	–	-	+	+	+	+	–	33.3
Lungs	PCR	–	+	+	–	+	–	+	+	+	+	+	+	75.0
	isolation	–	–	–	+	+	–	+	+	–	+	+	–	50.0

Table 3. Relative sensitivity and specificity of lyophilized reagent-based PCR on blood and organs samples as compared to isolation

Sample		Culture		Total
		Positive	Negative	
Blood	PCR (+)	9	0	9
	PCR (–)	1	2	3
	Total	10	2	12
Organs	PCR (+)	13	712	25
	PCR (–)	1	10	11
	Total	14	22	36

* Relative sensitivity for blood samples (%) = 90.0%; Relative specificity (%) = 100.0%
** Relative sensitivity for organs samples (%) = 92.86%; Relative specificity (%) = 45.45%

were performed. Leptospiral DNA can be detected in blood and lungs samples by lyophilized reagent-based PCR as early as Day 1 post infection. In kidneys and liver samples, positive PCR results were shown on Day 3, 5 and 7 post infections. However, the results for culture isolation from tissues samples corroborated to a lesser extent with lyophilized reagent-based PCR than found in the blood samples (Table 2). Leptospires were only recovered from kidney and liver samples starting from Day 5 post infection. As for lung samples, cultures were negative on Day 1 post infection despite the positive result shown by lyophilized reagent-based PCR. Both lyophilized reagent-based PCR and isolation of leptospires from control animals were always negative.

Relative specificity and sensitivity

By using culture as a standard, the relative sensitivity and specificity of the developed lyophilized reagent-based PCR on blood samples were 90.0% and 100.0%, respectively. For organ samples, the relative sensitivity was 92.9% while the relative specificity was shown to be as low as 45.5% (Table 3 and Table 4).

DISCUSSION

Rapid diagnosis of leptospirosis plays a significant role in the treatment of patients because early administration of appropriate antibiotic therapy was shown to be effective in shortening the duration of illness (Yang, 2006; Suputtamongkol *et al.*, 2010). Confirmation of diagnosis also facilitates the management of control and preventive measures by respective authorities. Hence, any delay resulted from the conventional diagnostic methods will directly affect an early and effective antibiotic treatment for leptospirosis. Rapid molecular diagnostic methods such as PCR with high sensitivity and specificity are therefore highly in demand. In this study, V1/V2 primers were incorporated into a lyophilized reagent-based PCR assay for the rapid diagnosis of leptospirosis. Earlier study has shown that

this set of primers, targeting the *lipL32* gene, were highly specific and sensitive towards identifying pathogenic leptospires (Lee *et al.*, 2009). Therefore, further evaluation of the assay, in lyophilised form, was carried out in this study using simulated clinical samples and experimentally infected hamsters.

Similarly, our results showed that the lyophilized reagent-based PCR assay was specific and sensitive towards pathogenic leptospires as there was no cross-reactivity with DNA from saprophytic leptospires and/or other bacterial species. Subsequent evaluation of the assay was performed on simulated clinical samples obtained through spiking *L. interrogans* serovar Lai strain Langkawi into blood and urine samples. Isolation or detection of leptospires from blood samples collected during leptospiremic phase and urine samples during convalescent phase are useful for diagnosis (Levett, 2004). The developed lyophilized reagent-based PCR assay was able to detect 1 x 10^2 leptospires/ml in the spiked urine and 1 x 10^3 leptospires/ml in the spiked blood sample. As noted, the sensitivity of the assay on blood samples may be lower as compared to urine samples due to the presence of PCR inhibitors such as haemoglobin, Immunoglobin G (IgG), and lactroferrin found in the blood which may not have been completely removed during DNA extraction (Al-Soud *et al.*, 2000; Al-Soud & Radstrom, 2001). Despite a perfectly optimized PCR assay, accurate results may not be produced when the extraction of DNA is performed improperly. Therefore, DNA extraction on clinical specimens has to be performed precisely in order to exclude all possible effects of PCR inhibitors that can significantly decrease the sensitivity of PCR.

High analytical sensitivity does not always correspond to its high diagnostic sensitivity. Presence of lysed leptospires and thus free DNA in the spiking media may perhaps contribute to an apparent similar, if not higher, sensitivity in the spiked samples as compared to the theoretical sensitivity which would be 10^2 leptospires/ml. Somehow, it remains to be clarified how long these free DNA from lysed leptospires can remain detectable in the clinical samples. Detection of the DNA sequences in spiked clinical samples does not indicate viability of the microorganism, therefore, may not facilitate the identification of true diagnostic sensitivity during leptospiraemia.

Also, in agarose gel electrophoresis, the quantities of DNA in the bands of interest (during analytical sensitivity) can be affected by the detection method post amplification as well as the amount of DNA loaded per well. Instead of ethidium bromide, the sensitivity for the detection of nucleic acids in agarose gels could be restored by using fluorescent stains such as SYBR Green Nucleic Acid Gel Stains (Schneeberger *et al.*, 1995). Conversely, the concept of more DNA loaded to produce more distinct band should be clarified. The minimal amount of an individual DNA fragment (5 ng) can be detectable by ethidium bromide staining when loaded into a 0.5-cm-wide x 0.2-cm deep sample well (Voytas, 2001). Therefore, in light of these ongoing problems, it is essential to emphasize that the development of this lyophilized reagent-based PCR assay targeting *lipL32* gene is not just one technique, but a method encompassing several techniques in the detection of pathogenic strains during leptospirosis.

The stability of our lyophilized reaction mixture was five months at ambient temperature (25° to 30°) which is consistent with the findings from Klatser *et al.* (1998) who had demonstrated the stability of their assay for a year at 20°C and for up to 3 months at 37°C. The use of different DNA polymerase may affect the stability of the final lyophilized PCR mixes (Klatser *et al.*, 1998). Therefore, glycerol-free *Taq* polymerases should be recommended for the increased stability and prolonged shelf-life of lyophilized PCR mixes.

Further evaluation of the developed PCR assay was carried out in Golden Syrian hamsters experimentally infected with 10^6 *L.interrogans* serovar Lai strain Langkawi. Blood, kidney, liver and lungs samples were subjected to PCR using assay developed and isolation in semi-solid EMJH medium. The recovery of leptospires from clinical specimens is considered the most definitive diagnostic test for leptospirosis (Adler & Pena

Moctezuma, 2010), thus culture was used as the reference test in determining the relative sensitivity and specificity of the developed lyophilized reagent-based PCR assay. The relative sensitivity and specificity of the developed assay on blood samples were 90.0% and 100.0%, respectively. As for the tissue samples, the relative sensitivity of the test was 92.86% while the specificity was 45.45%. The low specificity observed here probably reflects the occurrence of several false negative in cultures growth. Despite molecular evidence of the presence of pathogenic leptospires in the blood and organ samples, the causative agent was not isolated from all of the PCR positive clinical specimens. Isolation of leptopsires from tissues is often slow and notoriously difficult in artificial media (Lilenbaum *et al.*, 2008). Culture of *Leptospira* is therefore not practiced for rapid diagnostic of leptospirosis.

Also, in this study, the recovery of leptospires from blood was shown to be more successful compared to tissue samples. This could be attributed to the presence of blood components which promotes the proliferation of leptospires (Turner, 1970; Stoddard *et al.*, 2009). Besides, blood was harvested from hamsters by cardiac puncture, thus excluding chances of exposure to contaminants in the air. In contrast, organs of the hamsters were exposed to open air during sample collection leading to contamination. Our findings showed that one of the blood and lung samples was culture positive but PCR negative. This might be well explained by assuming that the number of viable leptospires in the samples might have been below the lower limit of detection (LOD) of the PCR but were sufficient for a positive culture, which in principle needs only one viable cell. Overall, culture is not practical as a routine diagnostic test for leptospirosis.

Based on the results achieved, the lyophilized reagent-based PCR is a rapid, reliable and simple tool for leptospiral detection as only the addition of nuclease free deionised water and template DNA is required. It is user friendly and time-saving, plus the transportation and storage of PCR reagents in cold conditions are no longer necessary. The premixed and pre-dispensed lyophilized PCR cocktails also reduce contaminations during the multi-step preparations. However, performance of the assay should be further evaluated on human clinical samples and stability of the assay should be improved for a longer shelf life.

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