Indian Journal of Animal Sciences **82** (10): 1133–1136, October 2012

Comparison of DNA extraction methods and gene targets from *Leptospira interrogans* serovar icterohaemorrhagiae for application in polymerase chain reaction

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Received: 10 May 2011; Accepted: 5 May 2012

ABSTRACT

The objective of this study was to identify the appropriate DNA extraction method and gene target from *Leptospira interrogans* serovar icterohaemorrhagiae for subsequent application in polymerase chain reaction (PCR) for diagnosis. DNA was extracted from pure cultures of *Leptospira interrogans* serovar icterohaemorrhagiae, spiked serum and urine samples using 4 different protocols,viz. STET buffer method, boiling method, nucleospin kit and the QIAamp DNA extraction kit using LipL41, LigA/B and LipL32 genes as targets. The lowest concentration of DNA that showed a visible band in PCR was taken as its analytical sensitivity in terms of concentration of DNA for each type of sample, target gene and DNA extraction method respectively. It was observed that DNA extraction from suspected clinical human sera samples using the QIAamp kit with either LipL41 or LipL32 as targets were most suitable for diagnosis by PCR since it had the greatest sensitivity of detection in PCR and the lowest amount of contaminating protein in the extracted DNA.

Abbreviations: STET: Sucrose- Tris – EDTA and Triton X 100; PCR: polymerase chain reaction; MAT: microscopic agglutination test; DFM – Dark field microscopy.

Key words: Analytical sensitivity, DNA extraction methods, Gene targets, *Leptospira*, Polymerase chain reaction

Leptospirosis, a worldwide zoonosis caused by spirochetes of the genus *Leptospira* (Office International des Epizooties 2005, Hartskeerl 2006), is an under-recognised problem with major health impact. It affects both humans and animals (Vinetz 2001) mainly due to exposure to water, food or soil contaminated with urine from infected animals (Levett 2001). Its potential carriers include rats, cattle, dogs, horses and pigs (Goldstein and Charon 1990). The species *Leptospira interrogans* comprises at least 250 serovars and 24 serogroups (Dutta and Christopher 2005). Among humans major serovars were Australis followed by Pyrogens, Canicola and Hebdomadis, and in domestic animals were Australis followed by Hebdomadis Sejroe, Pomona, Pyrogens, Autumnalis, Canicola, Ballum and Icterohaemorrhagiae (Koteeswaran 2006). The diagnosis of leptospirosis is conventionally based on detecting leptospires in body fluids by dark field microscopy (DFM). The other approved assay for diagnosis is microscopic agglutination test (MAT). Commercial kits based on immuno chromatographic methods or latex agglutination tests are available. The polymerase chain reaction (PCR) was developed for *Leptospira* detection using various target genes (Vinodh *et al*. 2008, Bomfim *et al*. 2007, Palaniappan *et al.* 2005) in pure culture, serum, semen and urine. However the assays have used different protocols for DNA extraction and gene targets with variable sensitivities. The main objective of this study was to apply PCR assay in relation to the method of DNA extraction by targeting portions of different genes of pathogenic leptospira in culture, spiked serum and spiked urine samples.

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MATERIALS AND METHODS

Source and propagation of leptospires: Leptospira interrogans serovar icterohaemorrhagiae maintained at Leptospirosis Research Laboratory, Madhavaram Milk Colony, Chennai in semisolid Ellinghausen McCullough Johnson and Harris (EMJH) media was used as inoculum. Eight- day-old liquid cultures of leptospires were enumerated using Petroff Hauser counting chamber and 4×10^8 organisms

/ ml was used for DNA extraction by different methods and for spiking serum and urine.

DNA extraction methods

Extraction of DNA from culture, spiked urine and spiked serum samples was done by following methods.

STET buffer method (11Lemarchand et al. 2005): One ml of 8-days-old liquid culture was taken in a microfuge tube and pelleted by centrifugation at 15000×g for 15 min. The pellet was resuspended in 300 µl of STET buffer (8% sucrose, 50 mM Tris HCl, pH 8.0; 50 mM EDTA and 5% Triron×) and 30 µl of lysozyme (10 mg/ml) and properly mixed. The above mixture was incubated at boiling temperature for 75 sec and then centrifuged at 15000 ×g for 15 min. The supernatant was collected by centrifugation at 10 000 rpm for 1 min and 1/10 volume of 4M lithium chloride was added and kept on ice for 5–10 min. Then, the supernatant is precipitated by isopropanol, washed with 80% ethanol and resuspended in 50–100 µl of nuclease free water.

Boiling method: The pellet, as obtained above, was resuspended in 50 µl of nuclease free water and boiled for 10 min and used as template for PCR.

DNA was extracted using tissue kit or DNA mini kit from 1 ml of 8- day-old culture following the procedures recommended by the respective manufacturers.

Spiking of serum and urine

Urine and serum collected from apparently healthy individuals and animals that were found negative for leptospira under DFM was spiked with the pellet of 4×10^8 *Leptospira* cells and used for DNA extraction by the above methods.

DNA quantification and estimation of contaminating protein in the extracted DNA preparations

DNA extracted from *Leptospira* cultures, spiked urine or serum was quantified with the DNA assay kit using a fluorimeter. Protein contamination in the extracted DNA was also assessed using the protein estimation kit.

PCR for Leptospira

PCR was performed using thermal cycler with different sets of primers (Table 1) and thermal cycling conditions of 94°C for 5 min, 94°C for 1 min, 55°C for 1 min (for LipL32), 72°C for 1 min (32 cycles) and a final extension of 72°C for 7 min. The annealing temperature for LipL41 and Lig A/B was 48°C. The final concentration of the reagents in 25 µl reaction mixtures was as follows: primers 10 picomoles each, template 5 µl, RedDye master mix 12.5µl and DNAase free water to make up to 25 µl. The PCR products were run on 2% agarose gel prepared in 1X TAE buffer.

Analytical sensitivity of PCR for Leptospira *in terms of DNA extraction method and gene target:* DNA was extracted from 1 ml of pure culture of *L.interrogans* serovar icterohaemorrhagiae using 4 different methods. The concentration of the extracted DNA by different methods was adjusted to 10 ng / µl and then 10–fold serial dilutions were made and used as template for assessing the analytical sensitivity of the PCR with different gene targets namely LipL32, LipL41 and Lig A/B genes. The lowest concentration of DNA that showed a visible band in PCR was taken as the analytical sensitivity of PCR in terms of concentration of DNA for each type of sample, target gene and DNA extraction methods respectively.

RESULTS AND DISCUSSION

The minimum amount of DNA needed to amplify specific targets in PCR with culture, spiked serum and urine samples and LipL41, Lig and Lip L32 gene targets are shown in Table 2. The highest analytical sensitivity with pure cultures was obtained with DNA mini kit (0.01ng of DNA) with all 3 gene targets. With spiked urine samples, highest analytical sensitivity was seen with DNA kit for LipL32 and LipL41 genes and STET and kit methods for LigA/B target. A representative gel picture is given in Fig. 1. With spiked serum, highest sensitivity was seen with STET buffer and DNA mini kit for all 3 gene targets. The boiling method and the other kit showed variable sensitivity with culture and spiked samples. Since serum is the sample of choice for

Table 1. Primers used in PCR for leptospiral DNA detection

	Primers	Sequence 5'—3'	Position of genome	Size of amplified product	Reference
LipL32 EU871723	Forward	TTCTGAGCGAGGACACAATCCC	101–122	183 bp	Fernanda *et al*. (2003)
	Reverse	CTCCCATTTCAGCGATTACG	264–283		
LipL41 AY642287	Forward	AAGCGGTAGTTTCCAGTCC	605–623	459 bp	Designed in this study
	Reverse	TTGCGTTGCTTTCGTCA	1047–1063		
Lig A/B AF368236 AF534640	Forward	TCAATCAAAACAAGAGGCT	632–650	468 bp	Palaniappan *et al*. (2005)
	Reverse	ACTTGCATTCGAAATTGAGAG	1080–1100		

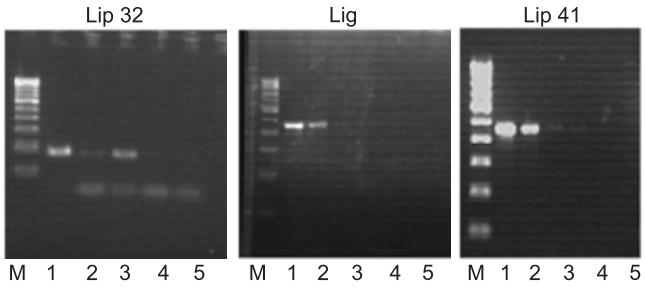

Fig. 1. Agarose gel electrophoresis showing the result of analytical sensitivity of PCR using pure culture of leptospira with DNA extracted using STET buffer method and different gene targets. Lane M-100bp- ladder, Lane 1-5: PCR amplification performed of 10 ng, 1ng, 0.1 ng, 0.01, 0.001 ng DNA of leptospira. Note no amplification 0.01 ng of LipL 32, 0.1 ng of Lig and 0.1 ng of LipL41.

Leptospira diagnosis LipL32 or Lip L41 gene as targets for detection and either STET or DNA kit methods were potential candidates for application in diagnostic PCR.

To account for the differential performance of different DNA extraction procedures in PCR, contaminating protein in the extracted DNA preparations and the DNA – protein ratio was estimated. Differing amounts of contaminating proteins were seen in the DNA preparations made by different procedures (Table 3). DNA extracted from culture by boiling method had the highest levels of contaminating proteins, that was 3.91 to 31.00 times higher than the DNA kit method that was used as a calibrator. Although the DNA - protein ratio for the DNA extracted by the STET method was lower than the DNA kit method in pure cultures (0.49 times), with spiked serum and urine it was 3.91 and 1.77 times higher. The kit method also had 1.02 to 3.94 times higher DNA - protein ratio compared to another commercial DNA kit.

The study involved comparison of 4 different DNA extraction methods (STET buffer, Boiling method, Tissue kit and DNA mini kit) and 3 different gene targets (LipL41, LigA/B, LipL32) on pure culture, spiked serum and urine for subsequent application of PCR in clinical diagnostic mode. The combination of DNA extraction and gene targets that had maximum sensitivity of detection was to be used in development and optimization of PCR or other genome based detection methods such as loop mediated isothermal application (LAMP). The 4 methods of DNA extraction resulted in variable sensitivities with each gene target. This probably reflected the principle of each of the method of DNA preparation, purity of the DNA extracted and ability of the primer in initiating amplification.

STET buffer method had comparable sensitivity with DNA kit method and would have been a cheaper alternative to it. However protein contamination in the extracted DNA (with respect to DNA concentrations) was higher in spiked serum compared to DNA kit method, although it was lower in pure culture. In preliminary experiments, DNA extracted from known positive clinical serum samples with the STET method, did not show any amplification in PCR (data not shown).

Boiling method breaks the cell and releases the DNA which is used as template for PCR (Manterola *et al.* 2003). This method had slightly lower sensitivity of detection, when compared to other method of DNA extraction. This is probably due to the protein denatured and release by boiling, not being removed from DNA preparation. This was confirmed as the boiling method had DNA / protein ratios of 3.91 to 31-fold higher than DNA kit method. Our earlier study also showed that DNA extraction by boiling method had 10-fold lower sensitivity in detecting *Leptospira* and *Brucella*

Table 2. Analytical sensitivity of PCR using different DNA extraction methods and genes target

Samples used	DNA extraction methods	Gene target in PCR		
		LipL32	Lig	LipL41
Culture	STET method	0.10*	1.00	0.10
	Boiling method	1.00	10.0	0.10
	Tissue kit	1.00	1.00	0.10
	DNA kit	0.01	0.01	0.01
Spiked urine	STET method	0. 10	0.10	0.10
	Boiling method	0.10	10	1.00
	Tissue kit	1.00	0.10	1.00
	DNA kit	0.01	0.10	0.01
Spiked serum	STET method	0.01	0.10	0.01
	Boiling method	0.10	1.00	1.00
	Tissue kit	10.0	1.00	0.10
	DNA kit	0.01	0.10	0.01

*Minimum concentration of DNA in nanogram / ml that showed a visible band in PCR.

Table 3. DNA / protein ratio of leptospiral DNA extracted using different DNA extraction methods

Sample used	DNA extraction methods	DNA (µg)	Protein (µg)	DNA/ protein ratio	Fold difference in DNA/ protein ratio*
Culture	STET method	4.50	2.13	2.11	0.49
	Boiling method	1.10	19.60	0.06	17.12
	Tissue kit	1.01	1.00	1.01	1.02
	DNA kit	0.60	0.58	1.03	1.00
Spiked urine	STET method	1.74	1.10	1.58	1.77
	Boiling method	1.12	12.60	0.09	31.00
	Tissue kit	0.85	1.20	0.71	3.94
	DNA kit	2.24	0.80	2.80	1.00
Spiked serum	STET method	0.23	1.00	0.23	3.91
	Boiling method	1.49	6.39	0.23	3.91
	Tissue kit	0.86	1.00	0.86	1.00
	DNA kit	0.88	0.97	0.90	1.00

*DNA / protein ratio was calculated using DNA kit method as a calibrator

genomes in PCR (Vinoth *et al.* 2008).

Thus, it was found that DNA kit method of DNA extraction from suspected serum samples would be the method of choice for PCR, LAMP and other genome based detection methods with LipL32 or LipL41 as gene targets. This was further confirmed by using MAT positive sera from clinical cases that had not yielded an amplified product using STET buffer method of DNA extraction, however resulted in positive amplification when DNA extractions were performed using the DNA kit.

ACKNOWLEDGEMENTS

The authors thank the Tamil Nadu Veterinary and Animal Sciences University (TANUVAS) for providing all the facilities to carry out this research work. Our gratitude is also due to the Leptospirosis Research Laboratory, TANUVAS for providing the leptospira pure culture.

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