Comparison of two PCR methods for detection of *Leptospira interrogans* in formalin-fixed and paraffin-embedded tissues

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In this study we compared two polymerase chain reaction (PCR) methods using either 16S ribosomal RNA (rRNA) or 23S rRNA gene primers for the detection of different Leptospira interrogans *serovars. The performance of these two methods was assessed using DNA extracted from bovine tissues previously inoculated with several bacterial suspensions. PCR was performed on the same tissues before and after the formalin-fixed, paraffin-embedding procedure (FFPE tissues). The 23S rDNA PCR detected all fresh and FFPE positive tissues while the 16S rDNA-based protocol detected primarily the positive fresh tissues. Both methods are specific for pathogenic* L. interrogans*. The 23S-based PCR method successfully detected* Leptospira *in four dubious cases of human leptospirosis from archival tissue specimens and one leptospirosis-positive canine specimen. A sensitive method for leptospirosis identification in FFPE tissues would be a useful tool to screen histological specimen archives and gain a better assessment of human leptospirosis prevalence, especially in tropical countries, where large outbreaks can occur following the rainy season.*

Key words: diagnostics - PCR - *Leptospira* - FFPE tissue

Leptospirosis is a worldwide disease that affects both humans and animals. Leptospirosis is caused by pathogenic *Leptospira* species, which have the ability to survive in a wide range of environmental reservoirs, including several mammalian hosts. Disease presentation can vary from sub-clinical infection to a severe illness with multi-organ involvement (Adler & de la Pena Moctezuma 2010). In tropical, developing countries, large epidemic outbreaks of leptospirosis in humans often occur following the rainy season (Ko et al. 1999, Levett 2001). However, in developed countries, leptospirosis cases are often associated with outdoor leisure activity or sport events (Ciceroni et al. 2000, Morgan et al. 2002).

Due to the variety of clinical symptoms, leptospirosis is often misdiagnosed as influenza, hepatic disease or fever of unknown origin, leading to an underestimation of prevalence (Romero et al. 1998, Lucchesi et al. 2004). The development of a reliable and specific technique for *Leptospira* detection in post-mortem samples is required for the correct diagnosis and for a more accurate understanding of leptospirosis epidemiology. Polymerase chain reaction (PCR) methods for the detection of *Leptospira* in different fresh clinical specimens are sensitive, specific and rapid (Letocart et al. 1997) and numerous protocols have been developed (Savio et al. 1994, Zuerner et al. 1995, Woo et al. 1997, Faber et al. 2000, Levett et al. 2005, Majed et al. 2005, Vitale et al. 2005). On the contrary, the reported PCR protocol for post-mortem samples and formalin-fixed, paraffin-embedded (FFPE) tissues has low sensitivity (Brown et al. 2003). The fixation process for histological samples can variably degrade cellular components, such as nucleic acids (Ben-Ezra et al. 1991, Coombs et al. 1999, Gloghini et al. 2004). Consequently, no reliable diagnostic PCR methods for the detection of *Leptospira* in FFPE tissues have been reported to date.

SUBJECTS, MATERIALS AND METHODS

We compared two PCR protocols to detect diverse *Leptospira interrogans* serovars in lung and kidney samples fixed in 10% buffered formalin. Briefly, lung and kidney samples were cut into small pieces (approximately 5 g) and inoculated with 100 µL of a bacterial suspension (approximately 5 x 10^2 colony-forming unit/mL) using a 1 mL syringe. Organ samples were taken from the carcass of a slaughtered cow with no clinical signs of disease that was approved for meat consumption by the official veterinary inspector at the slaughterhouse. Different portions of the same organ were inoculated with *L. interrogans* serovars and other bacterial species (Table I). Some pieces of inoculated, fresh tissue (approximately 25 mg/piece) were used directly for DNA extraction. DNA extraction was carried out using the Fast DNA Kit (Q-BIOgene) following homogenisation of the tissue samples with the FastPrep instrument (BIO 101) according to the manufacturer's instructions. Other tissue pieces were immediately put in

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a solution of 10% buffered formalin for seven days and then paraffin-embedded. After one week, two 3 µm sections were deparaffinised in 1.2 mL of xylene for 20 min at 40ºC. After centrifugation at 8,000 *g* for 15 min (Beckman Coulter Microfuge 18), the supernatant was carefully aspirated and the pellets were dried at room temperature. The desiccated samples were resuspended in 200 µL of lysis buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0) and boiled for 15 min. A total of 2 µL of DNA from each sample was used as template for PCR reactions. The 16S-based PCR protocol amplified a 571 bp fragment from 16S ribosomal RNA (rRNA) genes using previously published (Faber et al. 2000) *L. interrogans*-specific primers (16S-For, 5'-AGGGAAAATAAGCAGCGATGTG-3' and 16S-Rev, 5'-ATTCCACTCCATGTCAAGCC-3'). The 23S-based PCR method generated a 115 bp amplicon from 23S rRNA genes using previously published (Woo et al. 1997) *L. interrogans*-specific primers (F1, 5'-GAACTGAAACATCTAAGTA-3' and Ri, 5'-CAGCGAATTAGATCTG-3'). Both PCR reactions (50 µL in total volume) contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, 100 µM (each) dATP, dTTP, dCTP and dGTP, 20 pmol of primers and 3 U of Taq DNA polymerase.

The reactions were performed using an Applied Biosystems 9600 thermal cycler for 40 cycles. A total of 10 µL of the PCR products was analysed on a 2% agarose gel and visualised with ethidium bromide staining under ultraviolet light. The amplified products were gel-purified with the DNA Gel Extraction Kit (Genomics Millipore) according to the manufacturer's instructions. Sequencing of the gel-purified amplicons was performed using the BigDye Terminator v1.1 Cycle Sequencing Kit followed by capillary electrophoresis on the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

The five FFPE tissue samples analysed were obtained from the histology archive of the Institute of Tropical Medicine Pedro Kourì, in Cuba.

RESULTS

The specificity of the PCR method for pathogenic *L. interrogans* was confirmed with DNA extracted from eight *L. interrogans* serovars, eight *Leptospira biflexa* saprophytes and eight other bacterial reference strains (Table I). PCR amplification from all *L. interrogans* serovars was positive, while the *L. biflex*a saprophytes and other bacterial reference strains resulted in no amplification (data not shown).

The 16S-based PCR method detected *Leptospira* DNA in all of the fresh animal tissue samples tested; however, the 16S-based PCR method only detected *Leptospira* DNA in two of eight FFPE samples after fixation and embedding processing (Table II). However, PCR with 23S primers detected *Leptospira* DNA in all the samples regardless of fixation processing. The relative results of the two PCR methods for the detection of different *Leptospira* serovars in FFPE tissues are shown in Table II.

The success of the 23S rDNA PCR method for the detection of *Leptospira* DNA in FFPE specimens is likely due to the short length of the amplified fragment (115 bp) compared with the 16S rDNA fragment (571 bp). The extensive DNA cross-linking that occurs during formalin fixation can inhibit the activity of the Taq DNA polymerase (Ben-Ezra et al. 1991); therefore, the amplification of a shorter fragment may be more efficient for formalin-processed tissues.

To assess the validity of the method, the 23S rDNA PCR was also used to screen five specimens from dubious cases of human fatal leptospirosis and one specimen of confirmed canine leptospirosis. The human archival specimens were from the kidneys (n = 2), livers (n = 2) or lung (n = 1) of suspected leptospirosis cases, while the canine specimen came from the kidney of a dog with confirmed leptospirosis. In total, four out of the five uncertain human cases were positive for *Leptospira* (Figure, Lanes 2-5). The negative result for the other sample (Lane 1) may be caused by either the inability to amplify *Leptospira* DNA or the presence of a different pathogen, such as dengue virus, in patients with clinical signs similar to leptospirosis.

The products that were able to be amplified from the DNA of paraffin-embedded tissues were purified and sequenced. All sequenced fragments showed > 98% sequence homology to the *Leptospira* 23S rRNA gene in GenBank (accession X14249), confirming the amplification specificity of the 23S rDNA primers.

TABLE I

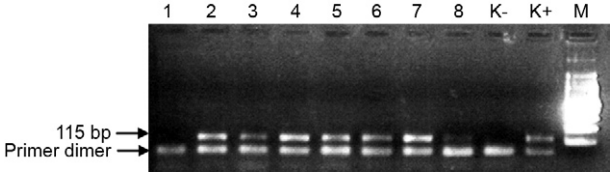
Leptospira serovars and bacterial strains used polymerase chain reaction analysis

Leptospira interrogans Serogroup/serovar/strain	*Leptospira biflexa* strains	Other bacteria
Australis/Bratislava	Basovizza	*Mycoplasma agalactiae*
Ballum/Ballum/MUS 127	Ondina	*Brucella melitensis*
Canicola/Canicola/Alarik	V-2	*Staphylococcus aureus*
Grippotyphosa/Grippotyphosa/Moskva V	Iran-1	*Salmonella typhi*
Icterohaemorrhagiae/Copenhageni/Wjinberg	Jequitaia	*Escherichia coli*
Pomona/Pomona/Pomona	Nazareni	*Mycobacterium bovis*
Sejroe/Hardjo/Hardjoprajitno	AR-18	*Pseudomonas aeruginosa*
Tarassovi/Tarassovi/Mitis Johnson	Patoc1	*Mycobacterium avium*

DISCUSSION

The molecular diagnosis of an infectious disease must consider that the pathogen load can be very low, particularly when the analysis is performed on thin paraffin sections. Reliable and reproducible methods for pathogen detection in FFPE tissues can be used to screen anatomic-pathological archives for dubious cases of fatal leptospirosis, particularly in tropical countries where *Leptospira* is a great public health concern. Due to the favourable climate and poor hygienic conditions, large outbreaks of leptospirosis with a high mortality rate can occur following the rainy season. Most of the histological archives store FFPE tissues samples; thus, it is important to develop a specific technique to assess the presence of *L. interrogans* in these types of specimens. In this paper, we compared two PCR methods to detect *L. interrogans* in fresh tissues that had been inoculated with bacterial suspensions, immediately fixed in formalin, embedded in paraffin and, after one week, processed for DNA. The PCR primers used target 16S and 23S rRNA giving amplicons of 571 and 115 bp, respectively (Woo et al. 1997, Faber et al. 2000). The results showed that histological procedures interfered with the activity of Taq polymerase, leading to the detection of the shorter 23S amplicon from all specimens both before and after FFPE processing (Table II). Although the method was effective for the detection of pathogenic *L. interrogans*, the most common *Leptospira* species associated with higher mortality, we were unable to rule out infection with other pathogenic *Leptospira* species. Moreover, four out of five human cases of possible fatal leptospirosis resulted positive using the 23S PCR-based assay. The negative result, however, does not completely exclude the possibility of *Leptospira* as the causative infectious agent. As far as we know, no standard procedures are internationally available for the processing of histological samples and many variables can affect the integrity of nucleic acids, such as the time lapse from organ removal to fixation. For instance, the size of the specimen can directly affect the diffusion of the fixative into the interior of the sample (von Ahlfen et al. 2007). These data suggest that the amplification of a short fragment (115 bp) reliably detects pathogenic *L. interrogans* DNA in FFPE tissues. Conversely, amplification of larger DNA fragments from the same samples is strongly impaired even when fixing and embedding procedures are optimised.

DNA detection from paraffin-embedded specimens by PCR is important for the retrospective study of different infectious diseases. The retrospective analysis of anatomic-pathological specimen archives is important for the study of infectious disease epidemiology and risk assessment, especially with infectious diseases, such as leptospirosis, that present with a variety of clinical symptoms, potentially leading to misdiagnosis and underestimation of the pathogen infection prevalence.



23S gene polymerase chain reaction analysis of DNA extracted from section of paraffin embedded tissues. Lanes 1-5: human archival specimens of dubious leptospirosis (1, 5: kidneys; 2: lung; 3, 4: livers); 6: kidney from positive dog: 7, 8: bovine kidney inoculated with *Leptospira interrogans* serovar Pomona; K-: bovine kidney; K+: positive control with reference strain DNA: M: DNA marker (100 bp ladder Biolabs). The presence of the smaller fragment is probably due to the primer dimers accumulation.

TABLE II

Results obtained by the amplification of 16S (571 bp) and 23S (115 bp) ribosomal RNA genes after formalin-fixed, paraffin-embedded (FFPE) procedures

	PCR	
Leptospira interrogans	571 bp/16S	115 bp/23S
Serogroup/serovar/strain	Kidney/lung	Kidney/lung
Australis/Bratislava	Negative/positive[a]	Positive/positive[a]
Ballum/Ballum/MUS 127	Negative/negative	Positive/positive[a]
Canicola/Canicola/Alarik	Negative/negative	Positive/positive[a]
Grippotyphosa/Grippotyphosa/Moskva V	Negative/negative	Positive/positive[a]
Icterohaemorrhagiae/Copenhageni/Wjinberg	Positive/negative[a]	Positive/positive[a]
Pomona/Pomona/Pomona	Negative/negative	Positive/positive[a]
Sejroe/Hardjo/Hardjoprajitno	Negative/negative	Positive/positive[a]
Tarassovi/Tarassovi/Mitis Johnson	Negative/negative	Positive/positive[a]

a: the 16S-based polymerase chain reaction (PCR) method only detected *Leptospira* DNA in two of eight FFPE samples after fixation and embedding processing.

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