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REVIEW ARTICLE

MULTIPLEX PCR-BASED DETECTION OF *LEPTOSPIRA CANICOLA* IN ENVIRONMENTAL WATER SAMPLES OBTAINED FROM NAGAPATTINAM AREA

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ABSTRACT

The aim of this study was to apply a molecular protocol to detect *Leptospiral canicola* DNA in environmental water samples. The study was carried out in a pullulated area in nagapattinam town.. A multiplex PCR method employing the primers LipL32 and 16SrRNA was used. Three out of 100 analysed samples were positive in the multiplex PCR, two were considered to have saprophytic leptospires and one had pathogenic leptospires. The results obtained supported the idea that multiplex PCR can be used to detect *Leptospira* spp in water samples. This method was also able to differentiate between saprophytic and pathogenic leptospires and was able to do so much more easily than conventional methodologies.

INTRODUCTION

Leptospirosis is a worldwide zoonotic disease of cattle associated with the *Leptospira interrogans* infection. It causes significant economic losses in the cattle industry worldwide due to abortion, reduced milk production and infertility. There have been some disputes over the classification of *Leptospira* species in recent years.

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Although *L. borgpetersenii, L. noguchii, L. santarosai, L. weilii* and *L. kirschneri* have been proposed as pathogen species in addition to *L. interrogans* in light of DNA hybridisation-based studies (Yasuda *et al.,* 1987), the term *L. interrogans* is still widely used in reference to pathogenic leptospires. *L. interrogans* serovar hardjo (L. hardjo) is the primary causative agent of bovine leptospirosis throughout the world and responsible for most of the losses attributable to the

disease (Ellis *et al.,* 1982). Lptospirosis is a zoonosis caused by *Leptospira* spp, including saprophytic and pathogenic species and it is recognized as an important public health problem worldwide, especially in tropical countries (Mathur *et al.,* 2009). Humans can become infected directly through contact with the urine of infected animals such as rodents and domestic (dogs, cattle and swine) or may be indirectly infected through contaminated water (Tansuphasiri *et al.,* 2006a; Vijayachari *et al.,* 2008; Adler and de la Peña Moctezuma 2009). In Brazil, leptospirosis is endemic to both rural and urban areas. In urban areas, the incidence of this disease may be increased in poor slum communities where the lack of basic sanitation contributes to the ecological conditions for rodent-borne transmission of pathogenic *Leptospira* (Sarkar *et al.,* 2002; Dias *et al.,* 2007). Outbreaks in these areas have high mortality rates and can result in death within only a few days of hospitalization. These outbreaks have a significant impact on public health and contribute to the increased number of reported cases (Ko *et al.,* 1999; McBride *et al.,* 2005).

The major problem in determining the environmental qualitative risk for leptospirosis exposure has been the difficulty of isolating pathogenic *Leptospira* from surface waters. This fact is attributable, at least in part, to the observation that the culture of these bacteria may be hindered by the predominance of saprophytic leptospires, which are morphologically similar and grow faster than the pathogenic ones and also by the fact that this methodology is time-consuming (Ganoza *et al.,* 2006; Tansuphasiri *et al.,* 2006a). Molecular tools based on PCR have been proven to be valuable in overcoming these limitations. This methodology can be used to specifically detect the pathogen since it can target specific genes, especially those related to surface macromolecules that are not present in the saprophytic group (Adler and de la Peña Moctezuma 2009). The aim of this study was to apply a molecular protocol to detect *Leptospiral canicola* DNA in environmental water samples.

MATERIALS AND METHODS

The aim of this study was to adapt and evaluate a protocol based on multiplex PCR, which is currently used to detect leptospiral DNA in clinical samples, for environmental water samples using two sets of primers that allow the differentiation of pathogenic from non-pathogenic species. Water samples were collected from Nagapattinam town of may 2010. This area shows characteristics similar to slum areas in urban areas of Nagapattinam district and is home to around 5440 people. There is no basic sanitation and the water that comes from a small riverhead is stored in a reservoir that is used for the whole community. In the absence of a sewage system, the sewage is disposed directly into the environment without prior treatment. A total of 100 samples of water were collected from the water supply that is distributed to the whole community (50 different points) and from small lakes (25 different points) and surface water (25 different points) where humans and domestic and wild animals may share the risk of infection. A total of 125 mL of water was collected from each site in sterile polypropylene flasks and transported in an icebox to the laboratory. The samples were kept at 4ºC and processed within 24 h.

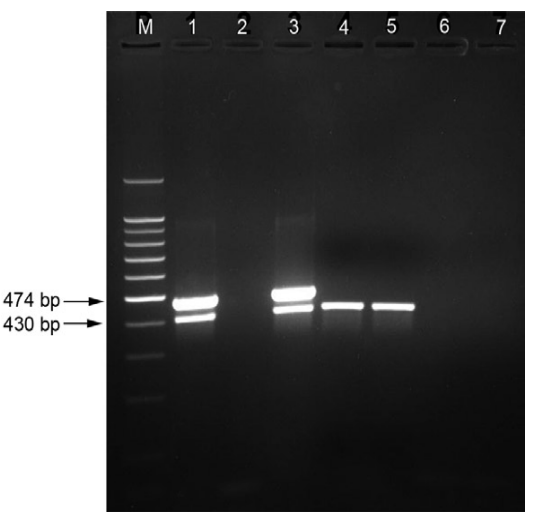
All of the procedures were done aseptically, as previously described by Tansuphasiri *et al.,* (2006b) with some modifications. Briefly, in order to concentrate the water samples, they were filtered twice through nitrocellulose membranes (Millipore, Ireland) of different pore sizes: a 0.45 μm filter followed by a 0.22 μm. Each of the filters used for each sample were separately cut into small pieces and transferred to a 15 mL polypropylene tube and stored at -20ºC prior to DNA extraction. As positive controls, two different samples of 125 mL of sterile water were artificially inoculated with 10^6 cells obtained from cultures of reference strains *Leptospira interrogans* serovar Copenhageni strain M20 (pathogenic) and *Leptospira biflexa* serovar Patoc serovar Patoc 1 (saprophytic) and processed as described above. Also, negative controls were generated using 125 mL of sterile unseeded water artificially contaminated with *Escherichia coli, Listeria monocytogenes* and *Salmonella enterica*.

First, 1 mL of lysis solution (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% Triton X-114) containing 1 mg/mL proteinase K (Invitrogen, USA) was added to the tubes containing the pieces of the filters and they were then incubated at 65ºC for 15 min. Next, 1.2 mL of chloroform was added and mixed by vortexing. The samples were centrifuged at 15,000 *g* for 10 min (4°C) and the aqueous phase was transferred to a microcentrifuge tube containing 0.8 mL 80% isopropyl alcohol and mixed again by vortexing. After centrifugation at 15,000 *g* for 20 min (4°C), the supernatant was discarded and the pellet was dissolved in a solution containing 170 µL of 1.2 M NaCl and 4 mg/mL RNase (Invitrogen, USA) and then incubated at 37ºC for 15 min. As a final step, 425 µL of cold absolute ethanol was added to precipitate the DNA and each sample was then incubated at -20ºC overnight. Subsequently, the samples were centrifuged at 14,000 *g* for 15 min. The supernatant was then discarded and the pellet was washed three times with 100 µL of cold 75% ethanol. The DNA was dried, dissolved in 100 µL of sterile TE buffer (1 M Tris-HCl pH 8.0, 0.5 M EDTA) and stored at -20ºC.

The amplification of the *LipL*32, which encodes the outer membrane lipoprotein LipL32 and 16S ribosomal RNA genes, was performed using the primers previously described by Ahmed *et al.,* (2006) and Tansuphasiri *et al.,* (2006a), respectively. The forward primer of *LipL*32 was 5'ATCTCCGTTGCACTCTTTGC3' and the reverse was 5'ACCATCATCATCATCGTCCA3'. The forward primer of 16S rRNA was 5'GAACTGAGACACGGTCCAT3' and the reverse was 5'GCCTCAGCGTCAGTTTTAGG3'. These two sets of primers allowed us to distinguish between pathogenic and saprophytic *Leptospira* species. Both genes are amplified in pathogenic species, but only 16S rRNA is amplified in the saprophytic species (Tansuphasiri *et al.,* 2006a). Multiplex PCR amplification was performed in a final reaction volume of 25 µL. All reactions contained 1X magnesium free PCR buffer, 1 mM $MgCl_2$, 200 µM dNTPs, 10 µM of each primer, 1 U Taq polymerase (QIAGEN). PCR amplification was performed using the following conditions: one denaturation cycle at 95ºC for 5 min, 35 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 30 sec and extension at 72ºC for 1 min, and a final extension at 72ºC for 7 min. The amplified products were electrophoresed on ethidium bromide-stained 2% agarose gels and observed using UV light. The *LipL*32 and 16S rRNA primers produced 474 bp and 430 bp fragments, respectively, as estimated using a 100-bp ladder (Invitrogen, USA).

RESULT AND DISCUSSION

The epidemiological history of this case showed a possible occupational association and risk exposure linked to prolonged activities in small lakes located in the above-mentioned settlement without the use of proper protective equipment. Three out of 100 water samples collected from different locations had positive reactions according to multiplex PCR analysis. The adapted protocol, based on the protocol previously used to assess clinical samples in our laboratory was effective in detecting leptospiral DNA in environmental water samples. There were no positive reactions with DNA from the other bacterial species used as controls (data not shown). This result shows that both primer sets, 16S rRNA and *LipL*32, specifically amplify DNA from *Leptospira*.



Agarose gel (2%) electrophoresis of multiplex-PCR products showing the detection of saprophytic and pathogenic *Leptospires* in environmental water

samples obtained from Nagapattiman town. M: 100 bp DNA ladder; Lane 1: positive reaction control (*Leptospira interrogans* serovar Copenhageni strain M20); 2: negative reaction control; 3: water sample collected near to the garbage deposit; 4: water sample from the water supply distributed in the whole extension of the community (point 23); 5: water sample from the small lake localized in front of a child care day nursery; 6: water sample from the main water reservoir; 7: water sample from the water supply distributed in the whole extension of the community. We showed that 97% of the analyzed samples were negative for the amplification of both 16S rRNA and *LipL*32 genes. In the period preceding the sample collection, there were no records of rainfall and the average temperature was 15ºC. The high incidence of negative samples observed in our work may be due to the fact that temperature is a limiting factor in the survival of *Leptospires* (Levett 2001; Bharti *et al.,* 2003). Also, it is well recognized that rainy weather plays a crucial role in the environmental dissemination of this pathogen from the urine of infected animals (Kupek *et al.,* 2000; Levett 2001; Vinetz 2001; Tassinari *et al.,* 2008).

The Figure (Lanes 4, 5) shows the positive amplification of only the 16S rRNA gene using a universal primer for *Leptospira* species (Postic *et al.,* 200; Tansuphasiri *et al.,* 2006b). The detection of this specific band by multiplex PCR occurred in only 2% of the overall samples, including samples from a small lake located in front of a child care day nursery and in the water supply which is distributed throughout the whole area. The amplification of a ribosomal RNA-related gene does not permit the differentiation between saprophytic and pathogenic *Leptospira* because the 430 bp fragment of 16S rRNA seems to be conserved between both groups of microorganisms (Mérien *et al.,* 1992; Tansuphasiri *et al.,* 2006a). It should be pointed out that saprophytic *Leptospires* are commonly found in environmental water samples (Adler and de la Peña Moctezuma 2009). In contrast, pathogenic *Leptospires* are usually maintained by animal carriers and are rarely isolated from surface water samples (Ganoza *et al.,* 2006). In addition, the Figure (Lane 3) shows PCR products resulting from both sets of primers, indicating the presence of pathogenic leptospires (Tansuphasiri *et al.,* 2006b). The PCR positive water sample was collected near a garbage deposit that was probably contaminated by the urine of rodents and wild or domestic animals whose presence was previously observed on the collection day. Our data agree with other authors in that it suggests that these animals are an important factor in determining the risk of leptospirosis transmission and they can be considered a persistent source of this pathogen in the community analyzed (Ganoza *et al.,* 2006). Multiplex PCR targeting of the two genes used here allows the detection of pathogenic leptospires, because LipL32 is the major outer membrane protein found on the surface of pathogenic *Leptospira* and has been highly conserved among these species (Stoddard *et al.,* 2009).

CONCLUSION

In conclusion, the results obtained reinforce previous observations that multiplex PCR, now using the specified two sets of primers, allowed the detection of *Leptospira* spp in water samples and was able to differentiate between saprophytic and pathogenic leptospires. The amplification of the *lipL*32 gene proved to be a valuable tool for identifying pathogenic leptospires in water samples. Future studies will be conducted in order to evaluate if the methodology employed here is able to predict environmental contamination and evaluate the risk related to occupational activities involving immersion in water over prolonged periods.

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