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Detection of *Leptospira* Species in Environmental Samples by Amplification of 16S *rRNA* and *rpoβ* Genes

(Pengesanan Spesies *Leptospira* dalam Sampel Alam Sekitar melalui 16S *rRNA* dan Gen *rpoβ* Diperkuat)

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ABSTRACT

This study attempted to identify and determine distribution of Leptospira spp. in environmental samples using 16S rRNA and rpoβ genes amplification. The samples were collected from high risk areas in Selangor, Malaysia. A total of 105 environmental samples consisting of soil and water were subjected to direct DNA extraction and PCR reaction. PCR products were analysed using gel electrophoresis and subjected to sequence analysis. Thirteen out of 105 (12.38%) samples were amplified for 16S rRNA with an expected amplicon size of 330 bp, while 50 out of 105 (47.62%) samples showed amplification using rpoβ primers, but were not of expected size. Of the 13 16S rRNA amplified samples, only 5 were identified as Leptospira in the gene sequence analysis and clustered under uncultured group via phylogenetic tree. This study showed the DNA-based approach using PCR and sequence analysis is able to detect the presence of Leptospira, although environmental samples may contain diverse microbial populations that may complicate the detection. Overall, the study suggested the importance of surveillance for Leptospira from environmental samples.

Keywords: Environment; *Leptospira*; *rpoβ*; Selangor; 16S rRNA

ABSTRAK

Kajian ini dijalankan untuk mengenal pasti dan menentukan penyebaran Leptospira spp. dalam sampel alam sekitar menggunakan 16S rRNA dan gen rpoβ diperkuat. Sampel diperolehi daripada kawasan yang mempunyai risiko tinggi di Selangor, Malaysia. Sampel keseluruhan adalah 105 yang terdiri daripada tanah dan air yang tertakluk secara langsung kepada pengekstrakan DNA dan reaksi PCR. Produk PCR dianalisis menggunakan elektroforesis gel dan tertakluk kepada analisis urutan. Tiga belas sampel daripada 105 (12.38%) menunjukkan amplifikasi untuk 16S rRNA dengan saiz amplicon terjangka 330 bp, manakala 50 sampel daripada 105 (47.62%) menunjukkan sampel amplifikasi menggunakan pencetus rpoβ, tetapi tidak mengikut saiz yang dijangka. Daripada 13 16S rRNA sampel diperkuat, hanya 5 dikenal pasti sebagai Leptospira dalam urutan gen analisis dan berkelompok di bawah kumpulan tidak dibiak melalui pohon filogenetik. Kajian ini menunjukkan pendekatan berasaskan DNA menggunakan PCR dan analisis urutan dapat mengesan kehadiran Leptospira, walaupun sampel alam sekitar berkemungkinan mengandungi pelbagai populasi mikrob yang boleh merumitkan pengesanan. Secara keseluruhan, kajian ini mencadangkan kepentingan pengawasan Leptospira daripada sampel alam sekitar.

Kata kunci: Alam sekitar; *Leptospira*; *rpoβ*; Selangor; 16S rRNA

INTRODUCTION

Leptospirosis is a fatal zoonosis that is widely spread in numerous tropical regions especially in frequently flooding districts, causing large epidemics (Haake & Levett 2015). Leptospirosis cases in Malaysia recently shows an increasing pattern from 263 cases in year 2004 to 5370 cases in 2015 (Garba et al. 2017). *Leptospira* spp. are an aerobic spirochete, motile and a Gram-negative bacterium which can be divided into 2 groups; non-pathogenic (saprophytic) and pathogenic strains. Saprophytic leptospires do not cause infections to humans and are more abundant in the environment due to their rapid growth (Ismail et al. 2014). Meanwhile, pathogenic leptospires need a host for reproduction and surviving. Generally, rodents and wild animals such as rats, cattle, dogs and

pigs are the main sources of pathogenic leptospires, as they may excrete leptospires that can survive in urine for months or even years (Lim et al. 2011). Unfortunately, data on identification and distribution of individual *Leptospira* spp. (local strains) from various areas in Malaysia are still lacking. Rapid and simple assays for the identification of individual *Leptospira* spp. are currently not well established especially in Malaysia, due to the fastidious and sensitive nature of the microorganism that hinders a wide surveillance study to be feasibly conducted on the distribution of the microorganism. 16S *rRNA* and *rpoβ* genes have been earlier demonstrated as suitable targets for identifying and discriminating bacterial spp. (Case et al. 2007). Therefore, this study attempted to detect the presence and investigate the distribution of

Leptospira spp. by amplifying 16S *rRNA* and *rpoB* genes from environmental samples (soil and water) collected in high risk areas in Selangor, Malaysia. High risk areas are categorised based on the population intensity, number of food stores or activities that might attract rodents which in turn can contaminate the environment particularly areas with many food stores and rubbish assembly sites in the housing or recreational vicinities.

MATERIALS AND METHODS

SAMPLE COLLECTION

Water and soil samples from 4 different regions in Selangor, mainly in high risk areas, were collected in early morning. One hundred and five samples were collected aseptically from the areas with a potential source of *Leptospira* transmissions such as wet markets, housings or food courts around Serdang, Seri Kembangan, Bangi and Kajang which were among the highly populated areas in Klang valley (Figure 1). Water samples were collected from lakes or drain waters, while soil samples were collected at a distance of 1 to 3 m from the edge of the water sources area (Figure 2). Approximately, 30 mL of water and 5 g of soil from each sampling site were collected in falcon tubes and placed in a sterile container. All samples were transported to the laboratory and proceeded with DNA extraction directly from the samples within 12 h.

DNA extraction was performed on all of the fresh samples using the soil DNA isolation mini kit (Favorgen) following the instruction by the manufacturer which involves bead beating technique (Gabor et al. 2003). Briefly, the environmental samples were vortexed together in a mixture of 200 mg glass beads and 600 μ L SDE1 buffer

at maximum speed to enhance cell disruption followed by 10 min incubation at 70°C and vortexing twice during the incubation. After incubation, 200 μ L of SDE2 buffer was added, vortexed and incubated for 5 min on ice. The supernatants were collected by centrifugation and the pellet was extracted by combining the supernatant with equal volume of isopropanol, vortexed and centrifuged. The dried down pallet was dissolved again in 200 μ L of pre-heated elution buffer and 100 μ L of SDE3 buffer. The supernatant was collected by centrifugation for 2 min and mixed again with equal volume of each SDE4 buffer and 96% ethanol. The sample mixture was bound to the SDE column and washed twice by using 750 μ L of wash buffer. The DNA was eluted by adding the pre-heated elution buffer to the membrane centre of the SDE column, stand column at room temperature and centrifuged at 18000 \times g for 1 min to elute the DNA.

MOLECULAR CHARACTERIZATION OF DNA SAMPLES

The extracted DNA samples were amplified by using standard PCR method. A set of primers against *Leptospira* were used to target and amplified 16S *rRNA* (*rrs*) and *rpoB* (Merien et al. 1992; Scola et al. 2006). The forward primer of 16S *rRNA* was 5'-GGCGGCGCGTCTTAAACATG-3' and the reverse was 5'-TTCCCCCATTTGAGCAAGATT-3'. The forward primer of *rpoB* was 5'-CCTCATGGGTTCCAACATGCA-3' and the reverse was 5'-CGCATCCTCRAAGTTGTAWCCTT-3'. The cycling conditons for amplifying 16S *rRNA* consisted of initial denaturation for 3 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 57°C, extension for 2 min at 72°C and final extension for 10 min at 72°C. The cycling conditions for amplifying *rpoB* consisted of initial denaturation at 95°C for 2 min, 35

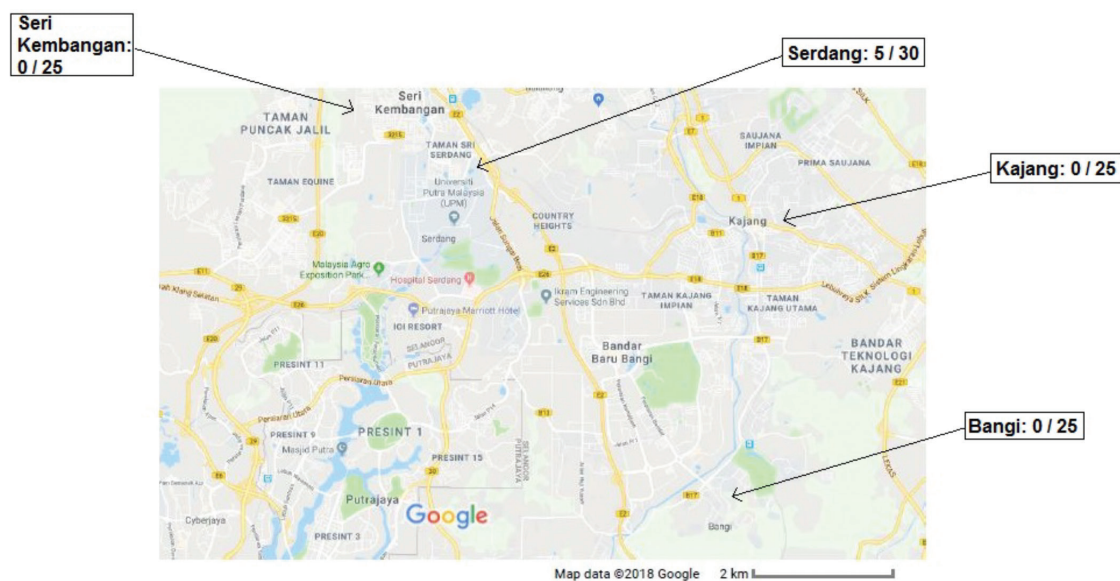


FIGURE 1. Sample collection sites in Selangor, Malaysia. Name of region, number of positive samples/number of samples, were stated in the figure. Five of 105 samples were uncultured *Leptospira*-positive

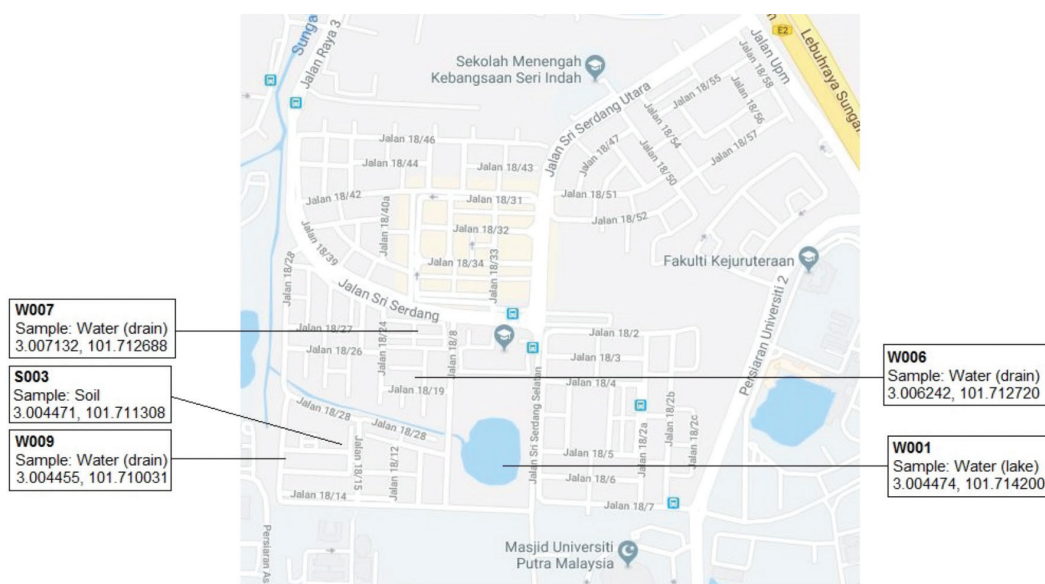


FIGURE 2. Sampling points for positive samples. Designated sample codes, type of samples, and GPS coordinates were stated in the figure

cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, extension at 72°C for 2 min and final extension at 72°C for 10 min. All PCR products were analysed by electrophoresis on a 1.5% agarose gel (Vivantis) and viewed under UV light.

Purified PCR products were subjected for DNA sequencing (First BASE, Malaysia). Online similarity searches were performed with the BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignment gaps were removed from pairwise distance estimations. The corresponding sequences of representative samples were deposited into Genbank (<https://submit.ncbi.nlm.nih.gov/subs>) and used for phylogenetic analysis using MEGA6 software with 1000 bootstrapping.

RESULTS

Agarose gel electrophoresis analysis showed 13 out of 105 (9.52%) samples were amplified for the 16S *rRNA* gene with an amplicon size of 330 bp (Figure 3), while 50 out of 105 (47.62%) samples were amplified using *rpoB* gene primers, showing a single amplicon band with around 400 bp instead of the expected size of 600 bp (Figure 4).

The amplified 16S *rRNA* of the 13 samples were further analysed through gene sequencing analysis. The results showed that only 5 samples were positive for *Leptospira*, while the others turned out as uncultured bacteria. Two of those *Leptospira* positive samples were from the lake and housing areas around Sri Serdang, while another 2 positive samples were drain water collected from drains food courts and housing areas in Sri Serdang. One of the sample was soil sample collected from housing area in Sri Serdang. Using BLAST analysis, the five *Leptospira*-positive samples showed relatively high matches, ranging from 88% to 99% homology, with

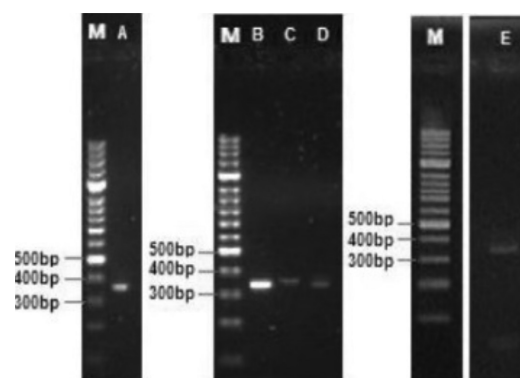


FIGURE 3. Representative agarose gel electrophoresis of 16S *rRNA* amplification from the collected samples. Lane M; 100bp DNA ladder marker GENESTA (GeneAll), Lanes A-E; 5 out of 13 positive samples were positive for leptospiral 16s *rRNA* with an expected amplicon size of 330 bp

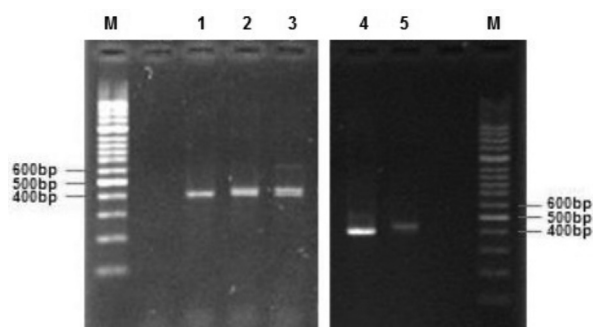


FIGURE 4. Representative agarose gel electrophoresis of *rpoB* amplification from the collected samples. Lanes M; 100bp DNA ladder marker GENESTA (GeneAll), Lanes 1-5; representative *rpoB* amplified samples with amplicon size smaller than the expected 600 bp

uncultured *Leptospira* spp. from freshwater samples (Thaipadungpanit et al. 2013) and sediment samples (Liu et al. 2011). The sequences were deposited into GenBank database and assigned with respective accession numbers as shown in Table 1. Due to only 88% similarity, S003 was not attained to get an accession number. The query coverage of deposited sequences has to be at least 90% in order to be accepted. On the other hand, based on sequencing analysis of the samples through BLAST analysis, all *rpoB* gene-amplicons of the unexpected sizes turned out as other bacteria such as *Streptococcus* spp. and *Paenibacillus* spp., while some have no significant similarity to any available genes at all.

Phylogenetic analysis was performed and a phylogenetic tree was constructed to identify the close relatives of the 5 *Leptospira* positive samples according to their 16S *rRNA* gene (Figure 5). Based on the phylogenetic tree, the results showed that all 5 positive samples of 16S *rRNA* gene resided in the cluster of uncultured *Leptospira*.

DISCUSSION

DNA-based approach using PCR has been commonly used to detect *Leptospira* spp. and identify specific microbial genes in environmental samples (Ganoza et al. 2006). In a similar approach, our results showed that 16S *rRNA* gene was amplified only in 13 samples using primers that were specifically designed to detect *Leptospira* in a previous study targeting 16S *rRNA* gene (Merien et al. 1992). Nevertheless, subsequent sequencing analysis confirmed only 5 samples to be positive for *Leptospira* of unknown spp. One the other hand, none of *rpoB* amplified samples yielded the expected amplicon size and without any match to *Leptospira* gene. The published primers, although had a high *in silico* specificity against *Leptospira* (data not shown), were optimized in clinical samples and thus their applicability in environmental sources such as soil and water was never shown. The usage of DNA extracted directly from uncultured samples of soil and water is expected to be problematic as there is a diverse pool of DNAs encompassing various known and

TABLE 1. 16s *rRNA* sequences of positive samples submitted to GenBank

Group	Sample / Isolate code	Species	Accession Number (GenBank ID)
Uncultured <i>Leptospira</i>	W001	Unknown	MF498487
	W006	Unknown	MF498488
	W007	Unknown	MF498489
	W009	Unknown	MF498490
	S003	Unknown	-

W= Water; S= Soil



FIGURE 5. Phylogenetic tree showing clustering of *Leptospira* species was constructed using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. The analysis involved 32 nucleotide sequences derived from GenBank database and the five DNA sequences from this study showing a segregation into the group of uncultured *Leptospira*. Evolutionary analysis was conducted using MEGA-6 program based on the nucleotide sequence of 16S *rRNA* gene. CP012584.1 (*P. aeruginosa*) serves as a sequence control for out-group

potential unknown microbial spp. including those from plants and other cellular contaminants. This may affect the accuracy and sensitivity of the PCR reaction, as well as competitiveness of the primers, against a diverse DNA background.

On the other hand, the 16S *rRNA* primers were able to identify 5 *Leptospira* of unknown origin, assigned as unculturable based on Genbank database. Interestingly, based on sequencing results and phylogenetic analysis, there was a clear distinction between the pathogenic, intermediate, saprophytic *leptospira* spp. and the unculturable group of *Leptospira* spp. in their own respective clusters. Thaipadungpanit et al. (2013), investigated the *Leptospira* spp. in floodwater in Bangkok region and also found in their study that the amplified 16S *rRNA* gene from some water samples were resided in unculturable groups based on the phylogenetic analysis. Adler and Moctezuma (2010) and Ganoza et al. (2006) reported that saprophytic *Leptospira* is commonly found in environmental samples whereas pathogenic *Leptospira* is often carried by animal hosts such as rats. Whether the unculturable *Leptospira* detected in this study and others belong to saprophytic or pathogenic group are not known as those have yet to be characterized for their spp and virulence potential. This indicates the diversity of *Leptospira* population which requires further investigation particularly the approach for direct detection and species characterization in unculturable samples. These uncultured leptospira could be environmentally originated but there is also a possibility that they could also originate from animal. As environment may be contaminated by animals through their urine, environment may temporarily harbour various *Leptospira* strains which may have potential to pose a health threat to humans, especially in areas frequently visited by humans such as food court, agricultural and recreational area (Mwachui et al. 2015).

CONCLUSION

In general, some environmental samples in this study were possibly contaminated by *Leptospira* although successful identification was low due to potential complexity of DNAs extracted from soil and water. The environmental samples may contain diverse microbial population and other contaminants that could have hindered efficient DNA extraction and PCR reaction. To a certain extent, this study indicates the importance of molecular screening on the identification of *Leptospira* spp. in the environment. A wide surveillance study may explore *Leptospira* population which in turn could facilitate understanding and managing *Leptospira* transmission and infection in Malaysia. However, further assessments should be conducted for an improved DNA extraction, primer selection and PCR system to allow a direct identification from environmental samples.

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