SHORT COMMUNICATION

Binary (*lipL32* and *gyrB)* gene marker analysis detects pathogenic *Leptospira interrogans* in a captured *Rattus norvegicus* in Marikina City, Philippines

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Leptospirosis is a major zoonotic disease endemic in the Philippines with an emerging pattern of post-typhoon increase of epidemiological cases. In 2009 after the typhoons Ondoy and Pepeng, a large number of cases were reported in Marikina City, Metro Manila, one of the major flood sites. The incidence pattern was in contrast with the absence of cases in prior years, suggesting the emergence of a vulnerable site with climatic change. The lack of information on infectious agent reservoirs like feral rats thriving in the site presents an opportunity for testing a biosurveillance protocol where gene-based detection methods could be explored. Thus, this study was aimed to test the utility of binary gene markers, *lipL32* and *gyrB* in the detection and identification of leptospires in feral rats in Marikina City. Traps were installed in two selected sites: Barangay Malanday and the Marikina Public Market where twenty-seven rats were recovered. The rats were dissected on site for their kidneys; genomic DNAs were extracted and assayed for leptospires by PCR amplification of *lipL32* and *gyrB*. Results showed one rat sample from Barangay Malanday to be positive for pathogenic *Leptospira* spp. and subsequent sequence analysis of the amplicons indicated that the DNA sequence belongs to *Leptospira interrogans*. The partial *gyrB* sequence obtained differed from the *L. interrogans* strain sequences deposited in the GenBank, suggesting a possible new strain. This study reports the utility of binary (*lipL32* and *gyrB*) gene marker analysis in the detection of pathogenic *Leptospira* in a sample biosurveillance of emerging infection of feral rats in Marikina City.

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Leptospirosis, rats, *gyrB, lipL32*, Marikina City, binary gene marker analysis.

INTRODUCTION

Leptospirosis is a zoonotic disease caused by a group of Gram-negative spirochetes belonging to the genus *Leptospira*

(Levett 2001). It is considered to be epidemiologically endemic in the Philippines with an incidence rising during the rainy and typhoon seasons (Yanigahara et al. 2006). During the typhoons Ondoy and Pepeng in 2009, a large number of cases of leptospirosis have been reported, mostly coming from Metro Manila particularly from Marikina City where no cases had been reported in previous years (National Epidemiology Center Department of Health, 2006; 2007; 2008; 2009). *Leptospira* infection in reservoir hosts like feral rats in Metro Manila has recently been studied by Villanueva et al. (2010); however, Marikina City was not included in their sampling sites. A link between infectious agent reservoirs and humans has been established for leptospirosis (Matthias et al. 2008); thus a biosurveillance study of infection in feral rats in Marikina would provide a basis for the assessment of the existing status of leptospiral infection that could potentially affect local residents. Using gene-based detection techniques, a model biosurveillance protocol for leptospiral infection on feral rats could be validated in the Marikina area.

Two gene markers widely used in the detection and identification of pathogenic leptospires are *lipL32,* which encodes for an outer membrane protein and present only in pathogenic strains, and *gyrB,* which encodes for the beta subunit of DNA gyrase and used for the identification and/or differentiation of closely related strains (Haake et al. 2000, Villanueva et al. 2010). These genes were used independently in previous local studies involving human isolates (Comandante et al. 2009) and reservoir infections (Villanueva et al. 2010). A combination of these two genes in a binary format would make possible the rapid detection of pathogenic leptospires in reservoir hosts without the need for serology and other pathogenicity tests. Thus, this study was aimed to test the utility of the binary gene markers *lipL32* and *gyrB* in the detection and identification of leptospires infecting feral rats in Marikina City.

MATERIALS AND METHODS

The procedures on animal care and use implemented in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Manila - National Institutes of Health.

Sample collection

Fifteen traps set with partially burnt copra as bait were installed per sampling session in the wet section of Marikina City Public Market (GPS location: 14° 37' 59" N, 121° 5' 46" E; Fig. 1) and residential areas of Barangay Malanday, Marikina City (GPS location: 14° 38' 55" N, 121° 5' 42" E; Fig. 1). A total of eight consecutive sampling sessions were done in the entire collection period (i.e., September 5 to October 27, 2010). The captured rats were sacrificed by carbon dioxide inhalation and

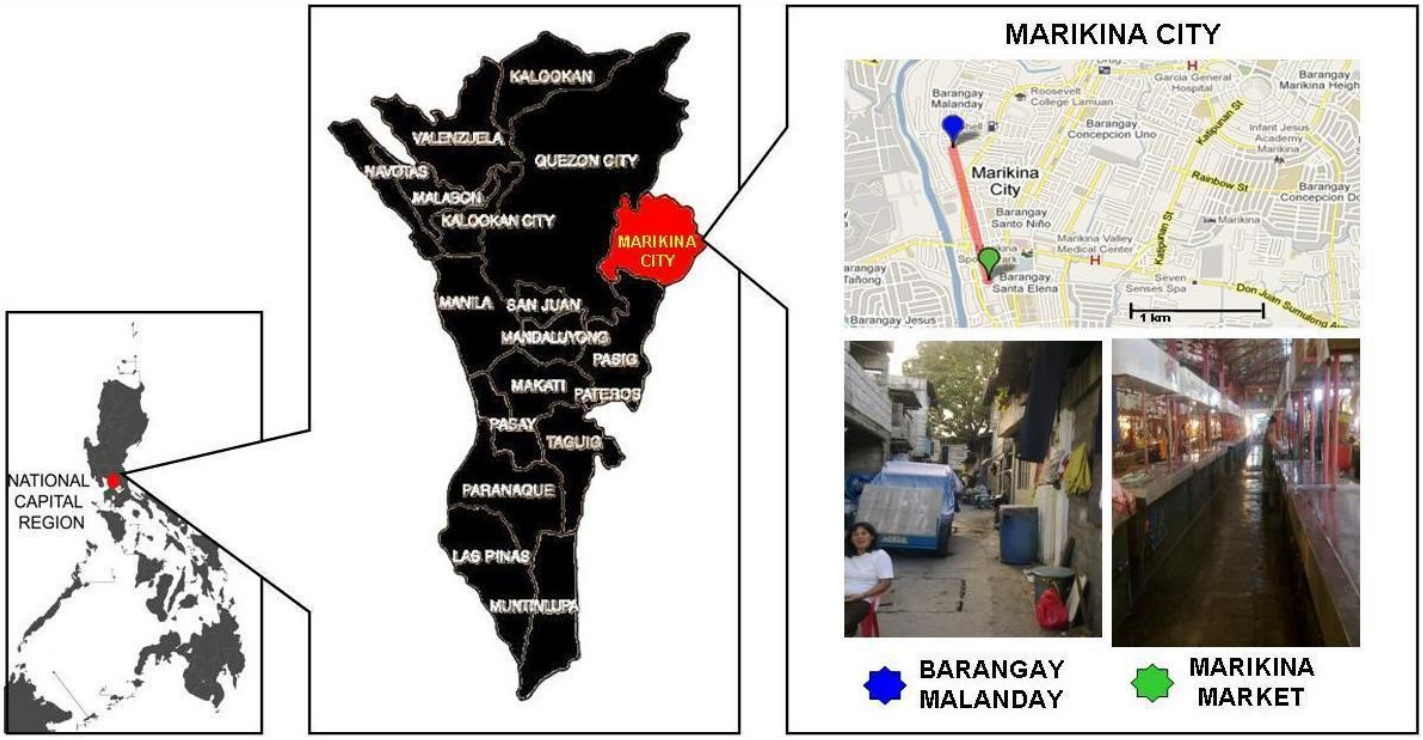

Figure 1. Map of the relative locations of the Marikina Public Market (GPS location: 14° 37' 59" N, 121° 5' 46" E) and Barangay Malanday (GPS location: 14° 38' 55" N, 121° 5' 42" E). Colored markers show the exact location of the trapping sites. Source of map: Google Maps.

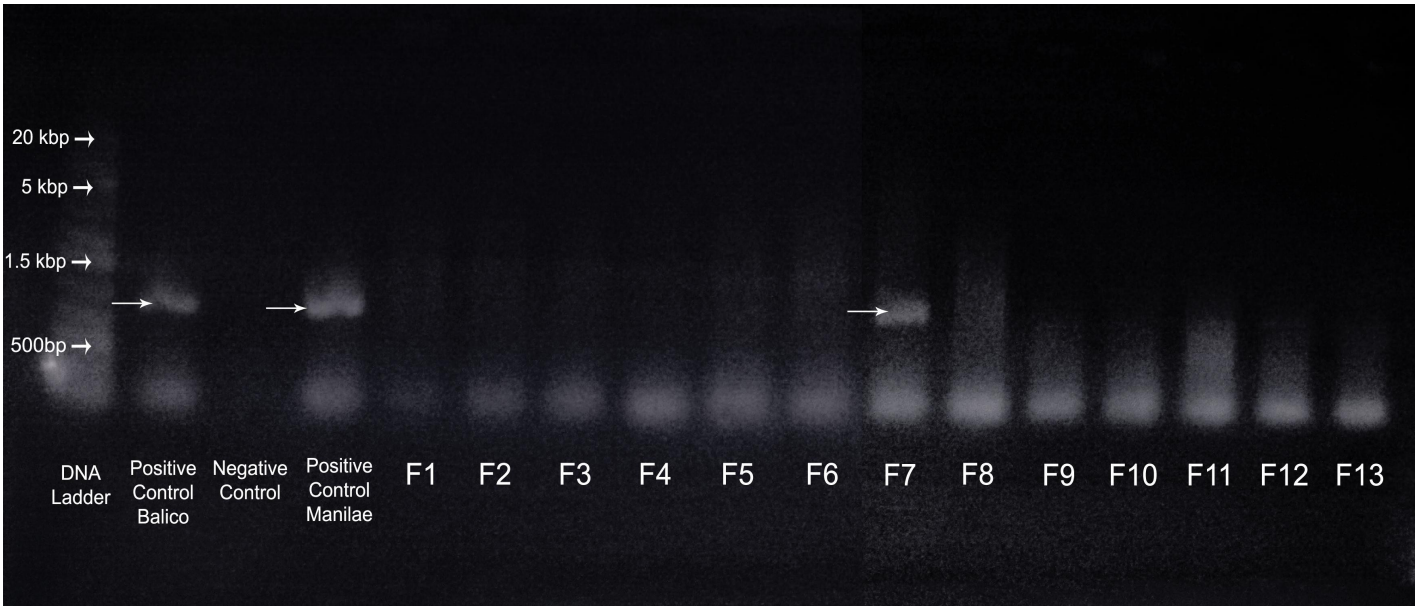

Figure 2. Amplified products from the *lipL32* PCR run. Positive controls used DNA template extracted from *Leptospira interrogans* serovars Balico and Manilae. Lanes F1 to F13 correspond to DNA extracted from formalin-preserved samples. Sample F7 shows ~800 bp amplification product similar to positive controls (shown by arrow).

dissected aseptically on site. The right kidneys were isolated and preserved in 4% formalin. About five cubic millimeters of the cortical region of the left kidneys were cut and preserved in RNALater® as positive control for the sample treatment.

DNA extraction

Formalin preserved kidneys were processed after 24 to 48 hours to minimize DNA fragmentation. DNA was extracted using QIAamp DNA FFPE Tissue Kit while DNA from RNALater® preserved samples was extracted using QIAGen DNEasy Blood and Tissue Kit. Crude DNA extracts were subjected to agarose gel electrophoresis for quality assessment.

Detection of *Leptospira* spp. DNA in kidney samples

DNA extracts from *Leptospira interrogans* serovars Balico and Manilae obtained from the Infectious Disease Section of the Philippine General Hospital (IDS-PGH) were used as positive controls for the subsequent assays. The PCR amplification targeting *gyrB* was done using the modified primers from Villanueva et al. (2010) with the following sequences (LgyrF: 5'-GGTCTTTCCGGAGAAGATG-3' and UP2rTL: 5'-TCNACRTCNGCRTCNGTCAT-3'). The PCR conditions were: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 40 seconds, annealing at 53°C for 40 seconds, and extension at 72°C for 1 minute; and final extension at 72°C for 5 minutes. The PCR amplification targeting *lipL32* was done utilizing the primers of Haake et al. (2000) with the following sequences (lipL32F: 5'-CTAAGTTCATACCGT GATTT-3' and lipL32R: 5'-ATTACT-TAGTCGCGTCAGAA-3'). The PCR conditions were: initial denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 40 seconds, annealing at 53°C for 30 seconds, extension at 72°C for 1 minute; and final extension at 72°C for 5 minutes. Five µL of the PCR products were subjected to agarose gel electrophoresis for visualization. The amplicons were sent to Macrogen Inc., Korea for sequencing.

Identification of *Leptospira* spp. in positive samples

Gene sequences from amplicons were queried using the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database. Sequence alignment was done using MEGA5 (Tamura et al. 2011) to validate the sequence identity and locate sites of polymorphism. The accession numbers of DNA sequences of the isolates submitted to the GenBank are the following: *Leptospira interrogans* serovar Balico (JQ013523, JQ013520), *Leptospira interrogans* serovar Manilae (JQ013522, JQ013519), sample F7 (JQ013521, JQ013518).

RESULTS

A total of twenty-seven rats were captured from the sampling sessions conducted. All the rats were identified as *Rattus norvegicus* by the Zoological Division of the Philippine National Museum. Ten samples (40.88 ± 1.11 cm, head to tail length) were captured from the Wet Section of Marikina Public Market and seventeen (37.75 ± 5.07 cm, head to tail length) from the residential area of Barangay Malanday.

Detection of the presence of *Leptospira* spp. in rat samples

DNA extracts from the two tissue preservation protocols, formalin and RNALater® were compared for their quality and suitability as template for PCR. The RNALater® preserved tissue samples yielded intact DNA with little to no fragmentation while extracts from formalin-preserved samples fixed at a maximum of 24 hours showed some degree of fragmentation (i.e., evidenced by the smearing of DNA in agarose gel electrophoresis). The presence of high molecular weight DNA in all the samples from the two tissue preservation protocols indicate their utility as template for PCR, although formalin-preserved samples were subsequently used with preference for biosafety considerations during sample collection.

The DNA extracts from the twenty-seven formalin-preserved samples were tested in PCR and one sample (i.e., sample F7 from Barangay Malanday) showed positive amplification for *lipL*32 primers indicating the presence of leptospiral DNA (Figure 2). The amplicon size was in agreement with the positive control (IDS-PGH sample *L.interrogans* serovar Balico). The *lipL32* PCR, while providing information on the presence of *L.interrogans*, also indicates that the infecting species is pathogenic (i.e., *lipL32* being present only in pathogenic species). The same set of samples analyzed by PCR for *gyrB* yielded consistent results for amplification with only sample F7 giving a positive result relative to control (Figure 3).

Identification of detected *Leptospira* spp.

Sequence analysis of the *lipL32* gene of the detected *Leptospira* spp. (JQ013521) using NCBI BLAST showed 100% sequence similarity with several representative *Leptospira interrogans* strains. Although the detected *Leptospira* spp. in sample F7 was found to have sequence hits corresponding to the pathogenic species *Leptospira interrogans,* the exact strain and serovar are not definable from the available sequence information from the database.

Meanwhile, the *gyrB* sequence amplified from sample F7 (JQ013518) showed 99% similarity to the high scoring hits in NCBI BLAST comprising of sequences from representatives of *Leptospira interrogans* strains. This result was in agreement with the *lipL32* analysis, thus validating the species identity of the detected leptospire. The alignment of *gyrB* hits and the query sequence showed multiple polymorphisms indicating the detected leptospire to be a different strain from those represented in the GenBank (Figure 4).

DISCUSSION

The positive detection of an infecting *Leptospira* spp. in a captured rat in Marikina City suggests a possibility that some rats within the area might be reservoirs for the pathogen. The specific identification of the infected rat (Sample F7) in Barangay Malanday suggests some localization factors at play, more so that there are more rats captured in the Barangay compared to the Wet Section of the Marikina Public Market. The regular clean-up programs in the Public Market could have limited the breeding site of the rats in comparison with the Barangay area. Thus, this study provides a basis for future investigations to fully assess the prevalence of emerging leptospiral infection in reservoirs within the city and the risk this poses to the local residents.

The analyses of the *lipL32* query sequence, while showing

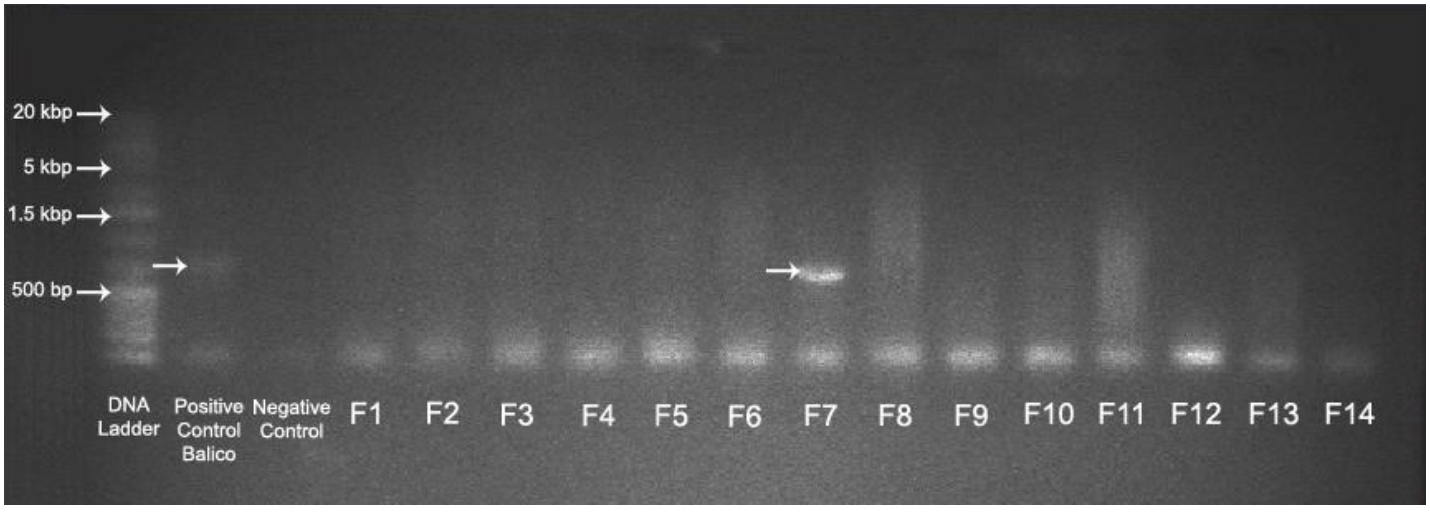

Figure 3. Amplified products from the *gyrB* PCR run. Positive controls used DNA template extracted from *Leptospira interrogans* serovar Balico. Lanes F1 to F13 correspond to DNA extracted from formalin-preserved samples. Sample F7 shows ~543 bp amplification product similar to positive control (shown by arrow).

100% similarity with several strains from the GenBank, did not contain substantial sequence polymorphism to differentiate strains within the species. This shows the highly conserved nature of the *lipL32* gene within the species consistent with those reported in several studies (Haake et al. 2000, Levett et al. 2005). In addition, some of the sequences deposited in the GenBank yielding 100% similarity with the query sequence were in fact, obtained from human isolates. Thus, the similarity of the *lipL32* sequences of human and rat isolates lends additional data to support the actual observation of inter-host transfer.

Analysis of the *gyrB* sequence allowed differentiation of the detected *L. interrogans* from the other strains in the GenBank. However, there is no representative strain sequence in the GenBank that exactly matches the query sequence. It is

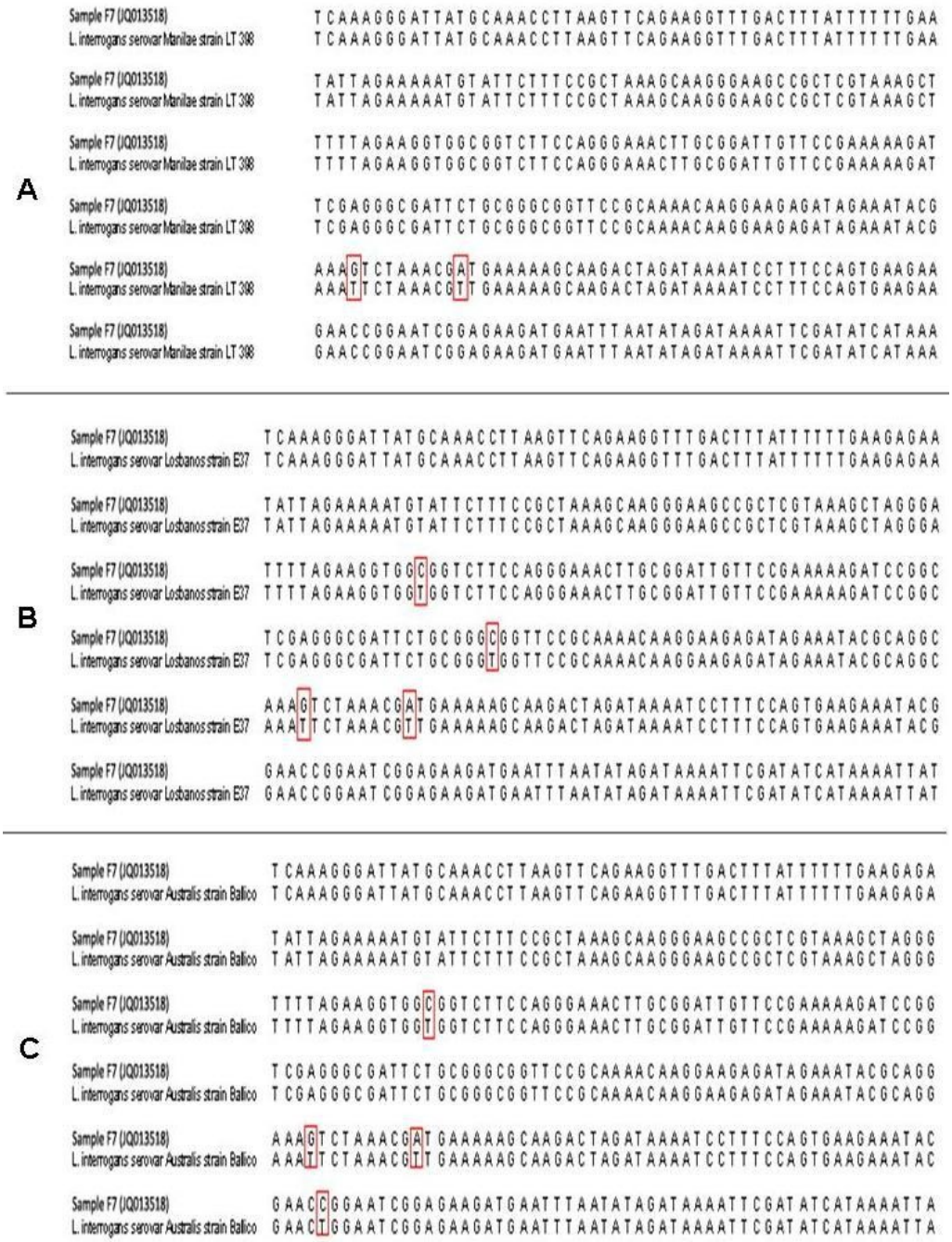

Figure 4. Sequence alignment of the query *gyrB* sequence with representative strains from the GenBank showing multiple polymorphisms along the partial sequence of the gene. Representative strains from the GenBank include A) *L. interrogans* strains LT-398, K1-59 and E5; B) *L. interrogans* L1-130, M20, RGA, Ictero I and E37; C) *L. interrogans* Balico.

likely that the detected *Leptospira interrogans* is a new strain first detected in feral rats in the Marikina City area, and thus requires further investigation. A comparison of the gene sequence with the closest match in the GenBank (i.e., *L. interrogans* strains LT-398, K1-59 and E5) showed the presence of two mutations (Figure 4), suggesting the strain could be novel. Further validation by sequencing a longer target and the use of MAT assay on the preserved tissue would warrant this finding. Similarly, a follow-up microbiological sampling of the leptospiral isolate would help establish the identity and potential risk of this emerging strain.

Here we report the utility of binary gene marker analysis using *lipL32* and *gyrB* in detecting pathogenic *L. interrogans* in community infectious agent reservoirs. The *lipL32* gene has been used locally by Comandante et al. (2009) to characterize pathogenic leptospiral species isolated from human samples (IDS-PGH) while Villanueva et al. (2010) utilized the *gyrB* gene in identifying lepstospiral species isolated from rat samples around Metro Manila. No study was done locally to explore the potential of the two gene combination for biosurveillance purposes. Thus, the findings in this study provide a basis for any subsequent biosurveillance protocols where binary gene markers will be employed: one gene to probe on pathogenicity and the other to probe on strain specificity.

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CONFLICTS OF INTERESTS

The authors have no conflicts of interests.

CONTRIBUTIONS OF THE INDIVIDUAL AUTHORS

VLAR and KJBA mainly conducted the experiments and wrote the manuscript and are equally responsible for the data of this paper; MMPB provided guidance and biological expertise in editing the manuscript; ECB provided guidance on animal handling, health and policies and provided biological expertise in editing the manuscript; and FMH provided laboratory support, guidance on the experiments and molecular biology expertise in editing the manuscript.

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