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Original article

Leptospira-rat-human relationship in Luzon, Philippines

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Abstract

Leptospirosis is a zoonotic infection that is caused by the pathogenic species of *Leptospira*. Rats are the most important reservoirs of these organisms. Our study aimed to characterize *Leptospira* isolates from humans and rats and elucidate the *Leptospira*-rat-human relationship in Luzon, Philippines. Forty strains were isolated from humans and rats. The isolates were confirmed to be *Leptospira* and pathogenic through *rrl*- and *flaB*-PCR, respectively. Around 73% of the isolates were found to be lethal to hamsters. Serotyping showed that there were mainly three predominant leptospiral serogroups in the study areas namely Pyrogenes, Bataviae, and Grippotyphosa. Gyrase B gene sequence analysis showed that all the isolates belonged to *Leptospira interrogans*. Most had 100% similarity with serovar Manilae (15/40), serovar Losbanos (8/40), and serogroup Grippotyphosa (8/40). Strains from each group had highly identical pulsed-field gel electrophoresis patterns and were further grouped as A (Pyrogenes, 14), B (Bataviae, 8), and C (Grippotyphosa, 10). Results further revealed that similar serotypes were isolated from both humans and rats in the same areas. It is suggested that these three predominant groups with highly similar intra-group PFGE patterns may have been primarily transmitted by rats and persistently caused leptospirosis in humans particularly in the Luzon islands.

Keywords: *Leptospira*; Rat; Human; PFGE; *gyrB* sequence analysis; Geographic information system (GIS); Pathogenicity

1. Introduction

Leptospirosis, a zoonosis common in tropical and subtropical countries, affects millions of people annually [1]. Rodents, specifically rats, are the most important reservoirs of *Leptospira*. They maintain *Leptospira* in their kidneys, continuously shed the organisms in their urine, and contaminate the environment (i.e., water and soil). Humans and other animals become infected, mainly through their skin and mucous membranes, when they come in contact with a leptospire-contaminated environment [2].

The close relationship among *Leptospira*, rats, and humans has been described in several studies [3–5]. However, there have been very few reports characterizing the relationship between rat and human isolates using serologic or molecular epidemiologic methods. It is probably due to difficulties in the simultaneous isolation of *Leptospira* from rats and humans.

To perform epidemiologic studies on leptospirosis, serovar/serogroup identification of isolates is essential. However, it is not routinely performed in most laboratories because there are more than 250 pathogenic serovars organized into 24 serogroups based on antigenic relatedness, and the maintenance of large

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panels of reference antisera and live antigens is required [6]. In order to overcome these problems, some molecular techniques discriminating *Leptospira* isolates for epidemiological studies were developed. Most of them can only identify until the species level (FAFLP, RFLP, 16S rRNA sequence analysis, etc.). MLST has been reported to be useful for phylogenetic study [7]. Some researchers reported that pulsed-field gel electrophoresis (PFGE) is an alternative method for the identification of *Leptospira* serovars [7–9]. PFGE has the ability of differentiating between strains of the same serovars that belong to different *Leptospira* species [10]. PFGE was often used to compare *Leptospira* isolates in previously reported epidemiological studies. Methods often used in identifying the species of *Leptospira* are DNA–DNA hybridization and 16S rRNA gene (*rrs*) sequence analysis. However, there have been several reports that used the housekeeping enzyme, DNA gyrase B subunit gene (*gyrB*), for identification of *Leptospira* isolates [11–13]. Slack et al. [13] reported that the nucleotide divergence of *gyrB* was greater than 16S rRNA (3.5–16.1% vs. 0.1–1.4%).

In the Philippines, many people are infected with *Leptospira*. Outbreaks usually occur during the rainy season (June to November) and just after the rainy season in flood-prone areas [14–16], although there are unofficial reports of outbreaks occurring even during the dry season especially in rural areas (e.g., areas with rice fields). We previously reported about the genetic and serological characterization of rat isolates in the Philippines [17]. The study was carried out in two areas, Metro Manila and Laguna, and we isolated 50 *Leptospira* strains from 46 wild rats. Based on *gyrB* sequencing and PFGE analysis, four groups of rat isolates were found: *Leptospira interrogans* serovar Manilae; serovar Losbanos; and serogroup Grippotyphosa; and, *Leptospira borgpetersenii* serogroup Javanica. Our previous study showed that there was a difference in geographic distribution among *Leptospira* species and serovar/serogroup [17]. The association between rat isolates and human isolates were not analyzed in the said study. However, those rat isolates caused hematuria, icterus, and pulmonary hemorrhage, which are the symptoms of severe human leptospirosis, and led to the death of golden Syrian hamsters [18]. From these results, it was expected that these two *Leptospira* serovars and two serogroups are circulating among rats, and that the rats may be one of the possible transmission sources of leptospirosis in the Philippines.

The present study was carried out in order to clarify the relation between rat and human *Leptospira* isolates in the Philippines. We successfully obtained clinical isolates from leptospirosis patients and new isolates from wild rats during the same period. Then, we investigated the serotype, genotype, and geographic distribution of the isolates, and evaluated the *Leptospira*-rat-human relationship in the Philippines, particularly in Luzon. Moreover, we compared the PFGE patterns and the *gyrB* sequences of the isolates in this study with those of previous isolates from rats.

Knowledge on the *Leptospira*-rat-human relationship is very essential in formulating countermeasures (e.g., vaccine) to combat leptospirosis.

2. Materials and methods

2.1. Isolation of leptospires

2.1.1. Humans

Blood and urine samples were obtained from suspected leptospirosis patients referred by physicians to the Department of Medical Microbiology, College of Public Health, University of the Philippines Manila between 2010 and 2011. As previously reported [6,17], one to three drops of blood and urine were cultured in modified Korthof's medium supplemented with 10% rabbit serum, kept at 30 °C incubator, and checked weekly for leptospiral growth until 3 months.

2.1.2. Wild rats

From 2010 to 2011, wild rats were trapped in markets and office buildings in Metro Manila (cities of Manila and Makati) and Iloilo, and rice fields in Nueva Ecija. Wire-mesh traps with baits were left overnight. The trapped rats were sacrificed by carbon dioxide inhalation. Blood was collected through cardiac puncture and urine was aseptically collected by bladder puncture with a needle. Rat kidneys were aseptically removed and emulsified with a 2.5-ml syringe based on a previously reported method [17]. Urine in bladder or empty urinary bladder, and kidneys were cultured in 5-fluorouracil–containing modified Korthof's medium. These cultures were incubated overnight at 30 °C. For the bladder and kidney cultures, 500 μl of the supernatant was sub-cultured into 4 ml of fresh medium the next day and were kept at 30 °C. All the cultures were examined weekly for leptospiral growth for three months. Species of rats were identified based on morphological features such as the color of ventral and dorsal fur, tail length compared to the length of head and body, number of pairs of mammae (females), and weight [19].

2.2. DNA extraction

For DNA extraction, a confluent culture (approximately 1×10^8) of isolates was harvested by centrifugation at 16,000 × g for 3 min. Genomic DNA was extracted using the Illustra Bacterial GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK) following the protocol for Gram-negative bacteria.

2.3. Typing of isolates

2.3.1. 23S rDNA (rrl)-polymerase chain reaction (PCR)

Woo et al. [20] reported that the 23S rDNA (*rrl*)-PCR was capable of detecting all *Leptospira* species. For 23S rDNA PCR, the primers, rrl-F (5′- GACCCGAAGCCTGTCGAG -3′) and rrl-R (5′- GCCATGCTTAGTCCCGATTAC-3′) were used. Each PCR solution (50 μl) consisted of 1 × ExTaq Buffer (Takara, Otsu, Shiga, Japan), 100 μM dNTP, 0.25 μM of each universal primer, 100 ng of extracted DNA and 1.25 units of Ex Taq™ HS DNA polymerase (Takara, Otsu, Shiga, Japan). Amplification was carried out in a thermal cycler (Program Temp Control System PC-320, ASTEC Co. Ltd., Fukuoka,