***Leptospira*-rat-human relationship in Luzon, Philippines**

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19 **Abstract**

20

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

36 humans particularly in the Luzon islands.

37

38 **Keywords.** *Leptospira*; rat; human; PFGE; *flaB*-PCR; 23S rDNA (*rrl*)-PCR; *gyrB*

39 sequence analysis; geographic information system (GIS); pathogenicity; hamster

40

1. Introduction

Leptospirosis, a zoonosis common in tropical and sub-tropical countries, affects millions of people annually [1]. Rodents, specifically rats, are the most important reservoirs of this infection. 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 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

75 genetic and serological characterization of rat isolates in the Philippines [17]. The study was
76 carried out in two areas, Metro Manila and Laguna, and we isolated 50 *Leptospira* strains
77 from 46 wild rats. Based on *gyrB* sequencing and PFGE analysis, four groups of rat isolates
78 were found: *L. interrogans* serovar Manilae; serovar Losbanos; and serogroup Grippotyphosa;
79 and, *L. borgpetersenii* serogroup Javanica. Our previous study showed that there was a
80 difference in geographic distribution among *Leptospira* species and serovar/serogroup [17].
81 The association between rat isolates and human isolates were not analyzed in the said study.
82 However, those rat isolates caused hematuria, icterus, and pulmonary hemorrhage, which are
83 the symptoms of severe human leptospirosis, and led to the death of golden Syrian hamsters
84 [18]. From these results, it was expected that these two *Leptospira* serovars and two
85 serogroups are circulating among rats, and that the rats may be one of the possible
86 transmission sources of leptospirosis in the Philippines.

87 The present study was carried out in order to clarify the relation between rat and human
88 *Leptospira* isolates in the Philippines. We successfully obtained clinical isolates from
89 leptospirosis patients and new isolates from wild rats during the same period. Then, we
90 investigated the serotype, genotype, and geographic distribution of the isolates, and evaluated
91 the *Leptospira*-rat-human relationship in the Philippines, particularly in Luzon. Moreover, we

92 compared the PFGE patterns and the *gyrB* sequences of the isolates in this study with those of
93 previous isolates from rats.

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

96

97 **2. Materials and methods**

98

99 *2.1 Isolation of leptospires*

100 *2.1.1 Humans*

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

107

108 *2.1.2 Wild rats*

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

122

123 2.2 DNA extraction

124 For DNA extraction, a confluent culture (approximately 1×10^8) of isolates was
125 harvested by centrifugation at 16,000 x g for 3 min. Genomic DNA was extracted using the

126 Illustra Bacterial GenomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK)
127 following the protocol for Gram-negative bacteria.

128

129 2.3 *Typing of isolates*

130 2.3.1 23S rDNA (*rrl*)-polymerase chain reaction (PCR)

131 Woo *et al.* [20] reported that the 23S rDNA (*rrl*)-PCR was capable of detecting all
132 *Leptospira* species. For 23S rDNA-PCR, the primers, *rrl*-F (5'-
133 GACCCGAAGCCTGTCGAG -3') and *rrl*-R (5'- GCCATGCTTAGTCCCGATTAC-3') were
134 used. Each PCR solution (50 µl) consisted of 1× ExTaq Buffer (Takara, Otsu, Shiga, Japan),
135 100 µM each dNTP, 0.25 µM of each universal primer, 100 ng of extracted DNA and 1.25
136 units of Ex Taq™ HS DNA polymerase (Takara, Otsu, Shiga, Japan). Amplification was
137 carried out in a thermal cycler (Program Temp Control System PC-320, ASTEC Co. Ltd.,
138 Fukuoka, Japan) under the following conditions: 30 cycles of 94°C for 20 sec, 54°C for 30
139 sec, 72°C for 1 min and a final extension at 72°C for 6 min. PCR amplification was confirmed
140 by electrophoresis on 1.5% agarose gels.

141

142 2.3.2 *Flagellin B gene (flaB)-PCR*

Kawabata *et al.* [21] previously reported that *flaB*-PCR was capable of detecting pathogenic *Leptospira* strains. For *flaB*-PCR, the primers, L-flaB-F1 (5'-CTCACCGTTCTCTAAAGTTCAAC-3') and L-flaB-R1 (5'-TGAATTCGGTTTCATATTGTC-3') were used. The preparation of PCR solution, DNA amplification, and electrophoresis were done in the same way as that for 23S rDNA PCR described above.

2.3.3 Gyrase B gene (*gyrB*)-PCR and sequence analysis

The DNA gyrase B subunit gene (*gyrB*) of all the *Leptospira* isolates from humans and rats was amplified using the primer set UP1TL (CAYGCNCGNGGNAARTTYGA) and UP2rTL (TCNACRTCNGCRTCNGTCAT) [22] under the following conditions: 30 cycles of denaturing at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 90 seconds. Final extension is at 72°C for 10 minutes. Sequencing of *gyrB* was done using the aforementioned primer set and LgyrF (GGTCTTTCCGGAGAAGATG) and LgyrR (GAATTTGAATTTGAGGTTGAGG) [12]. Sequence analysis was done based on a previous study [17]. Briefly, 3130 genetic analyzer (Applied Biosystems, Foster City, CA) was used to analyze the sequences. The alignment of sequences, neighbor-joining phylogenetic tree

construction, and bootstrap analysis were performed by using version 7.1 software package of Lasergene (DNASTAR Madison, WI). All the *gyrB* sequences of the *Leptospira* isolates in this study and 4 strains in the previous study (i.e., K64, K37, K6, and K5) [17] have been deposited and assigned DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank accession numbers AB968314 to AB968358.

2.3.4 *NotI* enzyme digestion and pulsed-field gel electrophoresis analysis

Pulsed-field gel electrophoresis (PFGE) was carried out based on the method previously described [23]. The patterns of leptospiral DNA digested with *NotI* were analyzed using GelCompar II software (Applied Maths, Inc., Austin, TX). Dendrograms were created by UPGMA cluster analyses based on the Dice band-based coefficient. Band comparison settings of 1.2% optimization and 1% position tolerance were used. The fingerprint patterns of the isolates in this study were compared with that of previous isolates from the Philippines such as strains LT 398 [24] and K64 [17] (serogroup Pyrogenes, serovar Manilae); strains LT 101-69 [24] and K37 [17] (serogroup Bataviae, serovar Losbanos); strains UP-BL-FR13 (Yanagihara Y, et al, unpublished data) and K5 [17] (serogroup Grippytyphosa); and, strain K6 (serogroup Javanica serovar Javanica) [17].

177

178 2.3.5 Serotyping

179 Serotyping (serogroup) of *Leptospira* isolates from humans and rats was done
180 through microscopic agglutination test (MAT) using monoclonal and polyclonal antibodies
181 raised against several *Leptospira* serovars [25]. Mouse monoclonal antibodies were raised
182 against 4 previously isolated leptospires from the Philippines [17] namely *L. interrogans*
183 serogroup Pyrogenes serovar Manilae (strain K64), serogroup Bataviae serovar Losbanos
184 (strain K42), serogroup Grippotyphosa (strain K5), and *L. borgpetersenii* serogroup Javanica
185 serovar Javanica (strain K6). The monoclonal antibodies were developed based on the method
186 by Masuzawa et al [26] using P3-X63-Ag8-U1 mouse myeloma cells. Rabbit polyclonal
187 antibodies, on the other hand, were raised against *L. interrogans* serovar Manilae (strains LT
188 398 and K64), serovar Losbanos (strains LT 101-69 and K37), serovar Icterohaemorrhagiae
189 (strain Ictero No. 1), serovar Pyrogenes (strain Pyrogenes), serovar Canicola (strains Hond
190 Utrecht and Moulton), *L. interrogans* (strain UP-BL-FR13), *L. borgpetersenii* serovar Poi
191 (strain Poi), and *L. biflexa* serovar Patoc (strain Patoc 1) based on a method specified in the
192 WHO Guidance [6] with some modifications [18].

193

2.4 Pathogenicity of isolates in golden Syrian hamsters

The pathogenicity of 29 selected *Leptospira* isolates was tested using four-week-old male golden Syrian hamsters as previously reported with some modifications [17-18]. Briefly, hamsters were subcutaneously injected with 1 ml of 10^7 and 10^5 of each strain and the animals were observed for 21 days. Moribund and surviving hamsters were sacrificed using sevoflurane (Maruishi Pharmaceutical, Japan).

2.5 Geographic Information System (GIS)

The addresses of the patients as well as the trapping sites of the rats were obtained and plotted on the map using the GIS software, ArcGIS.

2.6 Human and Animal Ethics

All experiments involving human samples and animals were reviewed and approved by the Ethical and Technical Review Boards and the Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Manila. For human samples, the patients or their guardian were given both verbal and written explanation of the study in

English or the vernacular. After thorough explanation, informed consents were obtained, and samples were collected.

3. Results

3.1 Isolation and 23S rDNA PCR of leptospires from human patients and rats

In the present study carried out from 2010 to 2011, a total of 40 strains of leptospires were obtained: 27 from humans and 13 from rats (Table 1). One out of 26 patients had positive blood and urine cultures so the total number of human isolates obtained was 21 strains from blood and 6 strains from urine. Leptospires were isolated from the urine in the bladder (5 isolates), empty urinary bladders (2 isolates), and kidneys (6 isolates) of 9 out of 56 trapped rats. Four of these rats had positive kidney and urine cultures. Based on their morphological characteristics [19], 4 of the 9 culture-positive rats were *Rattus norvegicus*, 2 were *R. tanezumi*, while the 3 were not identified due to missing information. All the 40 isolates from humans and rats were confirmed to be leptospires under the dark-field microscope because they showed the characteristic thin helical structures with prominent

hooked ends and motility. Furthermore, all the isolates were determined to be *Leptospira* species by the presence of 482 base pair bands in 23S rDNA PCR (data not shown).

3.2 *flaB*-PCR of the isolates

The *flaB*-PCR assay produced positive PCR products with a size of 793 bp for all 40 isolates from humans and rats (data not shown). These results suggested that all the isolates were pathogenic.

3.3 PFGE, *gyrB* sequence analysis, and identification of serogroups of *Leptospira* isolates

The *NotI* restriction patterns of the 40 *Leptospira* isolates in this study and the seven strains previously isolated in the Philippines were compared by phylogenetic tree analysis. As shown in Fig. 1, the band patterns of the 40 isolates were categorized into four groups: A, B, C, and others. The number of isolates belonging to each group was 14 (35%), 8 (20%), 8 (20%), and 10 (25%), respectively. The isolates which belonged to Groups A, B or C accounted for 75% of the total number of isolates in from 2010-2012, suggesting a high frequency of isolation of leptospires belonging to these 3 groups.

242 Gyrase B gene sequence analysis revealed that all the isolates belonged to *L.*
243 *interrogans* (Fig. 2). Strains with Group A PFGE pattern, except PI-17, had identical
244 sequences with strains LT 398 and K64 of serovar Manilae (38%). Group B and C strains had
245 identical *gyrB* sequences with serovar Losbanos and serogroup Grippotyphosa, respectively
246 (both 20%). It is interesting to note that strains belonging to Group C had identical sequences
247 to Group A except for one-base substitution. Strains belonging to the others group had similar
248 sequences with serovar Icterohaemorrhagiae (2 strains) and serovar Canicola (1 strain).
249 Strains PI-35 and 43 had sequence similar (99.9%) to serovar Pyrogenes. There is 1 base
250 difference between these 2 strains and serovar Pyrogenes strain Salinem. Strains PI-14, 34, 40,
251 and 42 were similar to serovar Naam. However, BLAST search revealed that these 6 strains
252 had 100% identical *gyrB* sequences to different serovars belonging to different serogroups.
253 Therefore, these strains could not be identified until the serovar level (Fig. 2).

254 The serogroup of each isolate was identified by MAT (Table 2, Fig. 1, Fig. 2).
255 Together with the PFGE results in Fig. 1 and *gyrB* sequence results in Fig. 2, group A was
256 composed of strains belonging to serogroup Pyrogenes. Group B strains reacted with antisera
257 for serogroup Bataviae. And, all the isolates in Group C were identified as serogroup
258 Grippotyphosa.

It is noteworthy that the 3 predominant groups contained both human and rat isolates. In other words, human and rat isolates in each group had highly similar fingerprint patterns. The PFGE patterns of Group A isolates were indistinguishable to that of strain K64 (serogroup *Pyrogenes* serovar *Manilae*), which we isolated from rats in 2006 and categorized into ‘group A’ in our previous study [17]. Similarly, Group B and C strains showed the indistinguishable PFGE patterns of strain K37 (serogroup *Bataviae* serovar *Losbanos*) and strain K5 (serogroup *Grippotyphosa*) isolated from rats in 2006 and categorized into ‘group B’ and ‘group C’ in our previous report [17], respectively (Fig. 1). Moreover, the fingerprint pattern of Group A strains was also identical to that of strain LT 398 (serogroup *Pyrogenes* serovar *Manilae*), which was a reference strain isolated from a rat in Manila in 1957 [24]. Likewise, Group B strains were found to be identical to strain LT 101-69 (serogroup *Bataviae* serovar *Losbanos*) isolated from a rat before 2006 [24], and Group C strains were considered to be indistinguishable to strain UP-BL-FR13 (serogroup *Grippotyphosa*) isolated from rats in 2000 (Fig. 1).

The remaining ten isolates were grouped into three serogroups namely: *Canicola*, *Icterohaemorrhagiae*, and non-group A *Pyrogenes*. Seven of these belonged to serogroup

Pyrogenes and showed four PFGE patterns that were distinct from the fingerprints of strains in Group A, which are also under the Pyrogenes group.

Results also showed that both *R. norvegicus* and *R. tanezumi* harbored serovar Manilae and serogroup Grippotyphosa. Interestingly, 1 *R. norvegicus* harbored both Manilae and Losbanos in its kidney and urine, respectively. Our results showed that no particular rat species harbored specific serovar or serogroup.

3.4 Pathogenicity of the isolates in golden Syrian hamsters

To examine the pathogenicity of 29 selected isolates, hamsters were inoculated subcutaneously with 10^5 or 10^7 of each isolate and were observed for 21 days. Thirteen of 18 isolates (72%) from humans and 8 of 11 from rats (73%), regardless of serotype and dose, were found to be lethal to hamsters. The animals infected with the isolates became moribund from the 6th to the 12th day after infection. Sick hamsters were observed to have ruffled fur, decreased food and water intake, continuous decrease in body weight until moribundity, and decreased mobility with some having prostration.

3.5 Distribution of Leptospira in the study sites

Geographic distributions of culture-positive patients and rats were analyzed using geographic information system (GIS). The patients' addresses as well as the areas where rats were trapped were plotted on the map (Fig. 3) using ArcGIS software.

Rat and human isolates that belonged to Group A (serogroup *Pyrogenes*) were found to be mainly isolated in Metro Manila (National Capital Region). It is noteworthy that the strains in Group A isolated from 4 patients with leptospirosis were also found to be in the same area where leptospires from the kidneys and/or urine of 8 rats were also isolated. Group B strains (serogroup *Bataviae*) were found to be isolated only in Metro Manila and its surrounding areas (i.e., Laguna and Cavite). In the City of Manila, both rat and human isolates in Group B were obtained. In contrast, all strains of Group C (serogroup *Grippytyphosa*) were isolated in Pangasinan and Nueva Ecija (Regions I and III, respectively), both are located north of Metro Manila. The strains belonging to non-group A *Pyrogenes* showed a wide geographic distribution. An important finding with regards to the distribution of the strains is that those strains belonging to Group A had high fingerprint pattern similarity whereas those strains belonging to non-group A *Pyrogenes* showed diverse PFGE patterns. The two rat isolates that were obtained in Iloilo City of Panay Island, located in the Visayas, were found to be indistinguishable by PFGE, and were identified, through MAT, as serogroup

Icterohaemorrhagiae. Sequence analysis of the *gyrB* gene of these 2 isolates showed 100% identity to that of serovar Icterohaemorrhagiae.

4. Discussion

There are several previous reports on the dominant clone of *Leptospira* associated with outbreaks of human leptospirosis based on partial *rrs* and *secY* sequences [27], MLST [28], arbitrarily primed PCR [29], and PFGE [7]. In this study, we analyzed 27 *Leptospira* isolates from blood and urine of patients with leptospirosis, and 13 isolates from rats. We found that all of these isolates belonged to *L. interrogans* based on *gyrB* sequence analysis (Fig. 2). Results also revealed that there were three predominant groups: serovars Manilae and Losbanos, and serogroup Grippotyphosa (Figs. 1 and 2). The isolates of each predominant group showed highly identical PFGE patterns (Group A, B, and C in Fig. 1). The results of PFGE and serotyping of the isolates were in congruence with the results of *gyrB* sequencing.

A very important finding in this study is that the isolates of each human leptospiral group was also obtained from rats either in the same year or a year apart and some were in the same areas. We also showed that the fingerprint patterns of each three *Leptospira* groups were

identical with that of previous isolates from rats in 2006 [17]. Moreover, the fingerprint patterns of reference strains isolated before 2006 (i.e., strains LT 398 and LT 101-69) were also found to be similar with that of each of the three *Leptospira* groups. This is the first study demonstrating that the three predominant *Leptospira* groups have been circulating among rats during the past decades or more than half a century particularly in the biggest island of the Philippines, Luzon.

Isolation results showed that one human leptospirosis patient had leptospires in his blood and urine, and 4 rats had these organisms in their kidneys and urine. The leptospires obtained from the blood and urine of the patient had 100% identical *gyrB* sequences and similar band patterns, and were classified as belonging to Group B (serovar Losbanos). The *Leptospira* isolates from the kidneys and urine of 3 of the 4 rats had similar band patterns to Group A (serovar Manilae). However, one interesting finding in our study was that 1 of the 4 rats harbored different types of *Leptospira* in its kidney and urine. The isolate from the kidney had 100% *gyrB* sequence identity and similar fingerprint patterns with Group A (serovar Manilae) while that from the urine was identical to Group B (serovar Losbanos). We have also reported this phenomenon in our previous study [17], wherein 4 rats harbored different types of *Leptospira* (i.e., serovar Manilae and serogroup Javanica). Furthermore, results

showed that serovar Manilae and serogroup Grippotyphosa were isolated from rats regardless of their species (i.e., *R. norvegicus* and *R. tanezumii*).

Geoprocessing techniques such as geographic information system (GIS) are important tools in health studies especially in determining the spatial distribution of certain diseases [30]. In our study, GIS was used to plot the distribution of *Leptospira* isolates. It was found that the *Leptospira* groups were unequally distributed in Philippines, to be specific, Group A strains (except for one isolate, PI-44) were isolated in Metro Manila; Group B strains were obtained in Metro Manila and its surrounding areas; and, Group C strains were collected in Pangasinan and Nueva Ecija, located north of Metro Manila. Strains identical to human isolates were always obtained from rats trapped in the same areas where leptospirosis patients lived. It is assumed that leptospirosis is not usually caused by human-to-human transmission, therefore, these three predominant *Leptospira* groups are considered to be transmitted from rats to humans. These findings revealed that the dominant *Leptospira* strains are maintained among the wild rats and occasionally transmitted to humans through the urine of reservoir hosts (i.e., rats) or urine-contaminated environment. The reason why the three dominant groups were selected in Luzon is unclear, however, these groups may have some selective advantage, such as high bacterial load in the urine of rats, increased resistance to

360 desiccation, and high ability to invade susceptible animals or humans.

361 It is interesting to know the details about the transmission of *Leptospira* organisms
362 in rat colonies. We hypothesize that there might be horizontal or vertical transmission among
363 the rats. Aside from these transmission routes, the selected groups could have been widely
364 spread by regional flooding. This is because each group was distributed over a wide area.
365 Further study, however, is needed to assess the relationship between the distribution of a
366 *Leptospira* group and the area of flooding as well as the possibility of horizontal or vertical
367 transmission, especially among the animal reservoirs.

368 It was found that the PFGE patterns of Groups A and C strains were similar to each
369 other, and the number of different fragments between these strains was only four. DNA
370 Gyrase B gene sequencing results revealed that Group C strains had almost similar sequences
371 (except for one base substitution) with Group A strains. This was a similar phenomenon that
372 we observed in our previous study [17]. Based on these results, strains belonging to these
373 groups may ‘probably be related.’ According to geographic distribution analysis, the strains of
374 these two groups (A and C) were isolated in different regions (Metro Manila; and Pangasinan
375 and Nueva Ecija, respectively). Although they were different serogroups and from different
376 regions they could, however, be genetically related.

377 In our previous study in 2006 [17], serogroup Javanica strains were isolated from
378 rats in Metro Manila and Laguna, which is located on the southwest of Metro Manila, and
379 these strains were categorized as the fourth predominant serogroup (group D). But, in the
380 present study, no *Leptospira* were isolated belonging to serogroup Javanica. This
381 phenomenon was also observed in the study by Amilasan, et al [16] after the great flood that
382 occurred in the Philippines in 2009. It is unclear why strains belonging to serogroup Javanica
383 were not predominantly isolated anymore. However, there are at least three possibilities.
384 Firstly, strains belonging to this serogroup may still be dominant but we might have missed
385 isolating them due to the limited number of rats that were trapped in our current study.
386 Secondly, Ellinghausen-McCullough-Johnson-Harris (EMJH) medium was possibly essential
387 to isolate strains belonging to serogroup Javanica. Although there was no sufficient evidence,
388 the strains of serovar Javanica tended to favor EMJH medium over Korthof's medium, as we
389 previously reported [17]. In the present study, we only used Korthof's medium to culture the
390 samples. Thirdly, serogroup Javanica could be predominant only transiently. Lau et al. [31]
391 reported the change of predominant serovars of human *Leptospira* isolates between 2004 and
392 2010 in American Samoa. They described that leptospiral serovar epidemiology can be

affected by change in distribution and/or abundance of animal reservoirs, introduction of new serovars through transport of animals or natural migration, or environmental changes.

Although all the isolates belong to pathogenic serogroups, not all of the strains caused moribundity in hamsters. Most of the human (72%) and rat isolates (73%) were found to be pathogenic to hamsters. It is common knowledge that pathogenic leptospires lose their virulence after being in vitro subcultured many times (more than 10 times). However, in this study, all strains that we used for the pathogenicity test were in vitro passaged less than 10 times. The virulence of *Leptospira* isolates may probably have been lost during transit in the environment or may depend on interaction with unknown host factor/s.

In conclusion, we demonstrated a high level of intra-group PFGE pattern similarity and *gyrB* sequence identity among the *Leptospira* isolates from humans and rats in Luzon, Philippines. It is suggested that there are mainly three dominant *Leptospira* groups circulating among rats, and these may have persistently caused leptospirosis in humans over the years particularly in this island. The findings in this study will be very crucial in the development of vaccines, at least in the Philippines, which will greatly contribute to the prevention and control of leptospirosis.

410 **Acknowledgments**

411

412 We would like to thank Bobby Crisostomo, Hideko Kameyama, Naomi Hidaka,
413 laboratory aides at DMM-CPH-UP Manila, staff of the Philippine Carabao Center in Nueva
414 Ecija, market officers, and vendors for their technical support in this study. We are also deeply
415 indebted to the patients and their guardians for their willingness to cooperate in this study. We
416 would also like to thank the Japan Science and Technology Agency (JST) and Japan
417 International Cooperation Agency (JICA) through their Science and Technology Research
418 Partnership for Sustainable Development (SATREPS) program for giving us the grant to
419 carry out this study. All the opinions and statements in this paper are solely that of the authors
420 and were not, in any way, influenced by the funding agencies nor by the institutions with
421 which the authors are affiliated.

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References

- [1] Abela-Ridder B, Sikkema R, Hartskeerl RA. Estimating the burden of human leptospirosis. *Int J Antimicrob Agents* 36 (2010) S5-7.
- [2] Adler B, de la Peña Moctezuma A. *Leptospira* and leptospirosis. *Vet Microbiol* 140 (2010) 287-296.
- [3] Saravanan R, Rajendran P, Thyagarajan SP, Smythe LD, Norris MA, Symonds ML, et al. *Leptospira autumnalis* isolated from a human case from Avadi, India, and the serovar's predominance in local rat and bandicoot populations. *Ann Trop Med Parasitol* 94 (2000) 503-6.
- [4] Koizumi N, Muto M, Tanikawa T, Mizutani H, Sohmura Y, Hayashi E, et al. Human leptospirosis cases and the prevalence of rats harbouring *Leptospira interrogans* in urban areas of Tokyo, Japan. *J Med Microbiol* 58 (2009) 1227-1230.

- 440 [5] Thaipadungpanit J, Wuthiekanun V, Chierakul W, Smythe LD, Petkanchanapong W,
441 Limpaboon R, et al. A dominant clone of *Leptospira interrogans* associated with an outbreak
442 of human leptospirosis in Thailand. PLoS Negl Trop Dis 1 (2007) e56.
443
- 444 [6] World Health Organization and International Leptospirosis Society. Human leptospirosis:
445 Guidance for diagnosis, surveillance and control. World Health Organization and International
446 Leptospirosis Society, Geneva, 2003.
447
- 448 [7] Galloway RL, Levett PN. Application and validation of PFGE for serovar identification of
449 *Leptospira* clinical isolates. PLoS Negl Trop Dis 4 (2010) e824.
450
- 451 [8] Herrmann JL, Bellenger E, Perolat P, Baranton G, Saint Girons I. Pulsed-field gel
452 electrophoresis of *NotI* digests of leptospiral DNA: a new rapid method of serovar
453 identification. J Clin Microbiol 30 (1992) 1696–1702.
454
- 455 [9] Romero EC, Blanco RM, Galloway RL. Application of pulsed-field gel electrophoresis for
456 the discrimination of leptospiral isolates in Brazil. Lett Appl Microbiol 48 (2009) 623–627.

457

458 [10] Galloway RL, Levett PN. Evaluation of a modified pulsed-field gel electrophoresis

459 approach for the identification of *Leptospira* serovars. Am J Trop Med Hyg 78 (2008)

460 628-632.

461

462 [11] Kawabata H, Sakakibara S, Imai Y, Masuzawa T, Fujita H, Tsurumi M, et al. First record

463 of *Leptospira borgpetersenii* isolation in the Amami Islands, Japan. Microbiol Immunol 50

464 (2006) 429-434.

465

466 [12] Masuzawa T, Okamoto Y, Une Y, Takeuchi T, Tsukagoshi K, Koizumi N, et al.

467 Leptospirosis in squirrels imported from United States to Japan. Emerg Infect Dis 12 (2006)

468 1153-1155.

469

470 [13] Slack AT, Symonds ML, Dohnt MF, Smythe LD. Identification of pathogenic *Leptospira*

471 species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B

472 encoding gene. BMC Microbiol 6 (2006) 95.

473

- 474 [14] Yanagihara Y, Villanueva SY, Yoshida S, Okamoto Y, Masuzawa T. Current status of
475 leptospirosis in Japan and Philippines. *Comp Immunol Microbiol Infect Dis* 30 (2007)
476 399-413.
- 477
- 478 [15] McCurry J. Philippines struggles to recover from typhoons. *Lancet*. 374 (2009) 1489.
- 479
- 480 [16] Amilasan AT, Ujiie M, Suzuki M, Salva E, Belo MC, Koizumi N, et al. Outbreak of
481 leptospirosis after flood, the Philippines, 2009. *Emerg Infect Dis* 18 (2012) 91-94.
- 482
- 483 [17] Villanueva SY, Ezoe H, Baterna RA, Yanagihara Y, Muto M, Koizumi N, et al.
484 Serologic and molecular studies of *Leptospira* and leptospirosis among rats in the Philippines.
485 *Am J Trop Med Hyg* 82 (2010) 889-898.
- 486
- 487 [18] Villanueva SY, Saito M, Tsutsumi Y, Segawa T, Baterna RA, Chakraborty A, et al. High
488 virulence in hamsters of four dominantly prevailing *Leptospira* serovars isolated from rats in
489 the Philippines. *Microbiology* 160 (2014) 418-428.
- 490

- 491 [19] Aplin KP, Brown PR, Jacob J, Krebs CJ, Singleton GR. Field methods for rodent studies
492 in Asia and the Indo-Pacific. Australian Centre for International Agricultural Research,
493 Canberra, 2003.
- 494
- 495 [20] Woo TH, Patel BK, Smythe LD, Symonds ML, Norris MA, Dohnt MF. Identification of
496 pathogenic *Leptospira* genospecies by continuous monitoring of fluorogenic hybridization
497 probes during rapid-cycle PCR. J Clin Microbiol 35 (1997) 3140-3146.
- 498
- 499 [21] Kawabata H, Dancel LA, Villanueva SY, Yanagihara Y, Koizumi N, Watanabe H.
500 *flaB*-polymerase chain reaction (*flaB*-PCR) and its restriction fragment length polymorphism
501 (RFLP) analysis are an efficient tool for detection and identification of *Leptospira* spp.
502 Microbiol Immunol 45 (2001) 491-496.
- 503
- 504 [22] Yamamoto S, Harayama S. PCR amplification and direct sequencing of *gyrB* genes with
505 universal primers and their application to the detection and taxonomic analysis of
506 *Pseudomonas putida* strains. Appl Environ Microbiol 61 (1995) 1104-1109.
- 507

- 508 [23] Saito M, Villanueva SY, Chakraborty A, Miyahara S, Segawa T, Asoh T, et al.
509 Comparative analysis of *Leptospira* strains isolated from environmental soil and water in the
510 Philippines and Japan. Appl Environ Microbiol 79 (2013) 601-609.
511
- 512 [24] Kmety E, Dikken H. Classification of the species *Leptospira interrogans* and history of
513 its serovars. University Press, Groningen, 1993.
514
- 515 [25] Bourhy P, Collet L, Clement S, Huerre M, Ave P, Giry C, et al. Isolation and
516 characterization of new *Leptospira* genotypes from patients in Mayotte (Indian Ocean). PLoS
517 Negl Trop Dis 4 (2010) e724.
518
- 519 [26] Masuzawa T, Kumagai M, Shimizu T, Yanagihara Y. Classification of *Leptospira*
520 *interrogans* serovar Lai strain 017 by using monoclonal antibodies. J Clin Microbiol 26
521 (1998) 2332-2337.
522
523
524

525

526

527 [27] Rahelinirina S, Léon A, Harstskeerl RA, Sertour N, Ahmed A, Raharimanana C, et al.

528 First isolation and direct evidence for the existence of large small-mammal reservoirs of

529 *Leptospira* sp. in Madagascar. PLoS One 5 (2010) e14111.

530

531 [28] Bourhy P, Collet L, Lernout T, Zinini F, Hartskeerl RA, van der Linden H, et al. Human

532 *Leptospira* isolates circulating in Mayotte (Indian Ocean) have unique serological and

533 molecular features. J Clin Microbiol 50 (2012) 307-311.

534

535 [29] Pereira MM, Matsuo MG, Bauab AR, Vasconcelos SA, Moraes ZM, Baranton G, et al. A

536 clonal subpopulation of *Leptospira interrogans* sensu stricto is the major cause of

537 leptospirosis outbreaks in Brazil. J Clin Microbiol 38 (2000) 450-452.

538

539 [30] Barcellos C, Sabroza PC. Socio-environmental determinants of the leptospirosis outbreak

540 of 1996 in western Rio de Janeiro: a geographical approach. Int J Environ Health Res 10

541 (2000) 301-313.

542

543 [31] Lau CL, Skelly C, Smythe LD, Craig SB, Weinstein P. Emergence of new leptospiral

544 serovars in American Samoa - ascertainment or ecological change? BMC Infect Dis 12 (2012)

545 19.

Figure Legends

Fig. 1. PFGE patterns of the 40 *Leptospira* isolates in this study and the seven strains previously isolated in the Philippines using *NotI* restriction enzyme. Human isolates are highlighted in dark colors and rat isolates in faint color. Closed red circles indicate strains that were isolated before and in 2006. PFGE results of rat and human isolates showed similar PFGE patterns in each group. a, probable serovar based on *gyrB* sequencing. b, antiserum to the indicated serogroup which showed the highest titer to the isolate.

Fig. 2. Phylogenetic tree based on the *gyrB* sequences of the *Leptospira* strains isolated from humans and rats. All the isolates were determined to belong to *L. interrogans*. Strains written in bold fonts were the isolates obtained in the current study. Accession numbers of the isolates are written in parentheses. Numbers indicate bootstrap value with 1,000 replicates. Colors represent the PFGE groupings as in Fig. 1. Bar: nucleotide substitution of 0.9%. Sg: serogroup. Sv: serovar. Str: strain.

562 **Fig. 3.** Geographic distributions of culture-positive patients and rats. The addresses of the
563 patients and the sample collection sites of the rats were obtained and plotted in the map using
564 ArcGIS. Colors of the symbols represent the PFGE groupings similar to Fig. 1.

565

566

567 Table 1. Distribution of *Leptospira* isolates from humans and rats according to sample
 568 collection sites.

Sample collection sites	No. of isolates from		Total
	humans	Rats	
A. Luzon			
(1) National Capital Region (Metro Manila)	8	9	17
(2) Regions I/Ilocos Region (Pangasinan) and III/Central Luzon (Nueva Ecija)	10	2	12
(3) Region IV-A/CALABARZON (Laguna and Cavite)	6	0	6
(4) Region V/Bicol Region (Camarines Sur)	1	0	1
B. Visayas			

Region VI/Western Visayas			
	0	2	2
(Iloilo)			
C. Unknown address	2	0	2
Total	27	13	40

570 Table 2. Serotyping results of *Leptospira* isolates from humans and rats.

Serogroup	No. of isolates from		Total
	humans	rats	
Pyrogenes	13	8	21
Bataviae	7	1	8
Grippotyphosa	6	2	8
Canicola	1	0	1
Icterohaemorrhagiae	0	2	2
Total	27	13	40

571

Figure 1

Figure 2

Figure 3