Comparative Analysis of *Leptospira* Strains Isolated from Environmental Soil and Water in the Philippines and Japan

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There have been few reports on the epidemiological analysis of environmental *Leptospira* isolates. This is probably because the isolation of leptospires from the environment was usually unsuccessful due to the overgrowth of contaminants and the slow growth of *Leptospira*. In this study, we collected a total of 88 samples of soil and water from three sites: Metro Manila and Nueva Ecija, Philippines (an area where *Leptospira* is now endemic), and Fukuoka, Japan (an area where *Leptospira* was once endemic). We succeeded in isolating *Leptospira* from 37 samples by using the novel combination of five antimicrobial agents reported in 2011. The frequencies of positive isolation of *Leptospira* in the Philippines and Japan were 40 and 46%, respectively. For *Leptospira*-positive samples, five colonies from each sample were isolated and analyzed by pulsed-field gel electrophoresis (PFGE).

The isolates from each area showed their respective characteristics in phylogenetic trees based on the PFGE patterns. Some isolates were closely related to each other across borders. Based on 16S rRNA gene-based phylogenetic analysis, four isolates in Fukuoka were identified as a pathogenic species, *L. alstonii*; however, its virulence had been lost. One isolate from Nueva Ecija was identified as the intermediate pathogenic species *Leptospira licerasiae*. Most of the isolates from the environment belonged to nonpathogenic *Leptospira* species. We also investigated the strain variation among the isolates in a puddle over 5 months. We demonstrated, using PFGE analysis, that *Leptospira* survived in the wet soil on dry days and appeared in the surface water on rainy days. These results showed that the soil could be a reservoir of leptospires in the environment.

*Leptospira* species are aerobic, spiral, Gram-negative bacteria. The organisms infect the host renal tubules and are shed into the environment via urination, where they can survive in moist soil and surface water for up to several months (1, 2). Humans and other animals become infected, mainly through their skins and mucous membranes, when they encounter a leptospire-contaminated environment (3). Due to this correlation between organisms in the environment and disease transmission, the isolation of leptospires from the environment is important for epidemiological studies, as well as for the prevention and control of the disease. However, isolation from environmental samples is challenging due to the slow growth of leptospires and the overgrowth of coexisting microorganisms. We previously reported a novel combination of antimicrobial agents (sulfamethoxazole, trimethoprim, amphotericin B, fosfomycin, and 5-fluorouracil [STAFF]) for selective isolation of leptospires from contaminated samples (4). This cocktail, after being incorporated into *Leptospira* growth medium, inhibited the growth of contaminants and successfully isolated leptospires in environmental samples.

In 1915, Inada and Ido discovered spirochetes and specific antibiotics in the blood of Japanese coal miners with infectious jaundice (Weil’s disease) in Fukuoka, Japan (5). Now this organism is known as *Leptospira interrogans* strain Icter No.1. Until 1960, more than 200 deaths of leptospirosis were reported every year in Japan. Most of them were farmers working in rice fields. After 1960, the mechanization of agriculture was introduced, and farmers started using rubber boots while working in the field. At the same time, an inactivated vaccine against pathogenic *Leptospira* was developed. For these reasons, the number of patients with leptospirosis in Japan decreased dramatically (6). In 2011, only 31 cases were reported. On the other hand, many people have been infected with *Leptospira* among Asian countries located in tropical areas, such as India, Thailand, Indonesia, Philippines, etc. (6). In the Philippines, the incidence of patients was closely related to rainfall. Outbreaks usually occurred during the rainy season (June to November) and just after the rainy season in flood-prone areas (6).

The present study was carried out in order to find out whether the environmental distribution of *Leptospira* species accounts for the difference of the current status of leptospirosis between Japan and the Philippines. We collected soil and water samples in both countries and succeeded in isolating leptospires from these samples by using the STAFF combination of antimicrobial agents. When the sample was culture positive, five *Leptospira* colonies from each sample were isolated by using the single colony isolation method and were analyzed by pulsed-field gel electrophoresis (PFGE). 16S rRNA gene-based phylogenetic analysis was performed for all isolates with the 20 previously described genomospecies in the genus *Leptospira*, and the genomospecies of the isolates were deduced.

**MATERIALS AND METHODS**

Isolation procedure of *Leptospira* species from environmental samples.

The collection of environmental samples was performed in three sites,....
Metro Manila and Nueva Ecija, Philippines, and Fukuoka, Japan, from July 2010 to November 2011. Metro Manila is the most densely populated area in the Philippines. Samples were collected from pools of water in the markets or in the roadsides, uncovered drainage system, canals, and rivers. Nueva Ecija, located north of Metro Manila, is a province where majority of the population are engaged in agricultural activities. Soil and water samples were collected from irrigation areas, pools of water where water buffaloes bathe, dry canals, ponds, moist soil beside the pools of water, and an artificial lake. Fukuoka is the prefecture where Inada and Ido discovered leptospires from patients suffering from an acute febrile illness with jaundice in 1915. Soil and water samples were collected in the campus of Kyushu University, Fukuoka City.

Soil and water samples (approximately 10 g and 10 ml, respectively) were collected in sterile 15-ml screw-cap tubes. The moisture content of the 15 soil samples was determined by placing a known quantity of the sample (~5 g) in a dry glass dish, and then the sample was dried at 200°C for 2 h until the weight remained constant (7). To the tubes containing the rest of the soil samples, 10 ml of sterile water was added, followed by mixing. All of the tubes were kept in a vertical position for 1 h to allow the sediments to settle. Then, 2.0 ml of supernatant from the sample was added to 2.5 ml of 2 × concentrated Korthof’s medium (8) supplemented with 500 µl of 10×-concentrated STAFF (sulfamethoxazole, 400 µg/ml; trimethoprim, 200 µg/ml; amphotericin B, 50 µg/ml; fosfomycin, 4 mg/ml; 5-fluorouracil, 1 mg/ml) (4). These tubes were incubated at 30°C and checked daily by dark-field microscopy for the presence of Leptospira, which was confirmed by observing their characteristic thin helical structures with prominent hooked ends and motility. Samples were considered as negative if no leptospires were detected after 28 days of incubation. When Leptospira had been observed microscopically, the cultures were filtered using a 0.2-µm-pore-size membrane filter, and 0.5 ml of the filtrate was added to new tubes containing 4.5 ml of fresh Korthof’s medium without STAFF.

For single-colony isolation, solid medium was prepared by the incorporation of 1% (wt/vol) agar in liquid Korthof’s medium. The bacteria in the liquid culture medium were counted and diluted to ~10⁶ cells/ml. The solid medium was inoculated by spreading 0.1 ml of diluted bacterial culture evenly over the surface of the medium with a glass spreader. The inoculated plate was sealed with tape, followed by incubation at 30°C. The plates were observed daily for the appearance of subsurface colonies, which took an average of 5 to 14 days. A single colony was picked up with Pasteur pipette, transferred into fresh liquid Korthof’s medium, and incubated at 30°C.

Enzyme digestion and PFGE. The bacterial suspension was mixed with a melted 1% SeaKem Gold Agarose (Lonza, Rockland, ME) at a concentration of 10⁹ cells/ml. The bacteria-agarose mixture was immediately dispensed into the wells of a disposable plug mold (Bio-Rad Laboratories Inc., Hercules, CA), transferred into fresh liquid Korthof’s medium, and stored at -20°C until the enzyme digestion for 16 h. The enzyme digestion was carried out with 10 units of SmaI (Toyobo Co., Ltd., Osaka, Japan) and 20 units of NcoI (New England BioLabs, Ipswich, MA). DNA bands were visualized on a UV transilluminator and photographed with a red filter.

Fingerprint patterns were analyzed using GelCompar II software (Applied Maths, Inc., Austin, TX). Dendrograms were created by UPGMA (unweighted pair-group method with arithmetic averages) cluster analysis based on the Dice band-based coefficient. Band comparison settings of 1.2% optimization and 1% position tolerance were used.

DNA extraction. For DNA extraction, a confluent culture of isolates was harvested by centrifugation (16,000 × g, for 3 min) at 4°C. Genomic DNA was extracted using the Illustra Bacteria GenomicPrep Mini spin kit (GE Healthcare, Buckinghamshire, United Kingdom) according to the protocol designated for Gram-negative bacteria.

FlaB-PCR. Kawabata et al. (9) reported that the 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′-TGAATTCGGTTTCTATATTGCC-3′) were used. Each PCR solution (50 µl) consisted of 1× Ex Taq buffer (TaKaRa), 100 µM concentrations of each deoxyribonucleoside triphosphate, 0.25 µM concentrations of each universal primer, 100 ng of extracted DNA, and 1.25 U of Ex Taq HS DNA polymerase (TaKaRa). Amplification was carried out in a thermal cycler (Program Temp Control System PC-320; ASTEC Co., Ltd., Fukuoka, Japan) under the following conditions: 30 cycles of 94°C for 20 s, 54°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 6 min. PCR amplification was confirmed by electrophoresis on 1.5% agarose gels.

16S rRNA gene sequencing and phylogenetic analysis. In order to differentiate the species of the leptospiral isolates, 16S rRNA gene sequences were analyzed (10). An internal portion of the 16S rRNA gene (~1,480 bp) was amplified by PCR with a bacterial universal primer set (P16S-8UA [5′-AGAGTTTGATCATCGTCAG-3′] and P16S-1485R [5′-TACGGYTACCTTGTTACGACT-3′]). Amplification was performed under the following conditions: 30 cycles of 96°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. After confirmation of the amplification of the 16S rRNA gene on 1% agarose gels, the PCR products were purified by using the Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI), and the sequence was determined using a 3130 genetic analyzer (Applied Biosystems). The sequences of the other Leptospira species used for alignment and for calculating levels of homology were obtained from GenBank. Multiple sequence alignments of DNA sequences were performed using CLUSTAL W software (11). Phylogenetic distances were calculated using the neighbor-joining method (12). A phylogenetic tree was constructed using TREEVIEW software (13).

Identification of serogroups of isolates. Serogroups of the isolates presuming to be pathogenic or intermediate pathogenic Leptospira were identified by microscopic agglutination test (MAT) using a panel of anti-Leptospira rabbit sera for 23 serogroups, including Icterohaemorrhagiae, Australis (serovar Bratislava), Hebdomadis, Djasiman, Louisiana, Sejroe, Grippotyphosa, Celledoni, Javanica, Pomona, Shermani, Canicola, Australis (serovar Bratislava), Hebdomadis, Djasiman, Louisiana, Sejroe, Grippotyphosa, Celledoni, Javanica, Pomona, Shermani, Canicola, Sarmin, Manhao, Semaranga (serovar Patoc), Tarassovi, Auftumalis, Panama, Mini, Cinopteri, Sejroe (serovar Hardjo), Pyrogenes (serovar Manilae), and Bataviae (serovar Losbanos).

Pathogenicity test of isolates in golden Syrian hamsters. The pathogenicity of the isolates was tested in 4-week-old male golden Syrian hamsters (Japan SLC, Inc., Shizuoka, Japan). The hamsters were inoculated intraperitoneally with 10⁷ of Leptospira isolates in 1 ml of phosphate-buffered saline and then observed for 28 days. Hamsters inoculated with Korthof’s medium only were used as negative controls. Blood and kidney samples from dead or sacrificed hamsters were cultured.

Animal experiments were reviewed and approved by the Ethics Committee on Animal Experiment at the Faculty of Medical Sciences, Kyushu University. The experiments were carried out under the conditions stipulated by the Guidelines for Animal Experiments of Kyushu University and law 105 and notification 6 of the Government of Japan.
RESULTS

Isolation of leptospires from water and soil samples. A total of 60 samples were collected in the Philippines, and the spirochetes, which showed thin helical structures with prominent hooked ends and characteristic motility, were found by dark-field microscopy in the cultures of 26 samples. Subsequently, 2 of the 26 positive samples were found to contain only \emph{Leptonema} species by 16S rRNA gene sequence determination and phylogenetic analysis. Therefore, 24 (40%) were found to be \emph{Leptospira} positive: 8 in Metro Manila and 16 in Nueva Ecija. In Japan, 28 samples were collected, and 13 (46%) were found to be positive. The results are presented in Table 1.

PFGE analysis of \emph{Leptospira} isolates. We hypothesized that a \emph{Leptospira}-positive sample might contain more than one \emph{Leptospira} strain. Therefore, single colony isolation was done on the 37 positive bacterial cultures using solid medium. When the subsurface colonies appeared, five single colonies were picked up from each plate, and cultured in fresh liquid Korthof’s medium. All of the isolates were analyzed by PFGE.

Of the 37 \emph{Leptospira}-positive samples, 29 showed identical fingerprint patterns among five isolates. However, 3 of the 37 \emph{Leptospira}-positive samples showed two or three kinds of fingerprint pattern among five isolates (Fig. 1). One sample, ES-1, had two PFGE patterns (Fig. 1A), and two samples, ES-56 (Fig. 1B) and ES-96 (Fig. 1C), contained at least three strains showing PFGE patterns different from each other. Isolates from five samples showed no band of NotI-restricted DNA; however, the fingerprint patterns were seen by using Smal or PacI (data not shown). Five isolates from each sample showed the same Smal or PacI restriction pattern, but the band patterns were different among the five samples. Accordingly, a total of 42 isolates was obtained from 37 \emph{Leptospira}-positive samples.

The NotI restriction patterns of the 37 isolates (23 from the Philippines and 14 from Japan) and reference strains of particular \emph{Leptospira} serogroups were compared by phylogenetic tree analysis. In all, 35 different PFGE patterns were obtained from the 37 isolates. The discrimination power of PFGE with NotI was 95%. The phylogenetic tree based on NotI restriction patterns separated the environmental isolates into seven clusters (clusters A to G) (Fig. 2).

16S rRNA gene sequence determination and phylogenetic analysis. For the purpose of species differentiation, almost full-length 16S rRNA gene sequences of 42 isolates were determined. The phylogenetic tree was constructed based on 16S rRNA gene sequences of isolates and type strains of 20 \emph{Leptospira} genomospecies, deposited in GenBank (Fig. 3). Four isolates, i.e., MS267, MS306, MS311, and MS316, were organized in the clade of pathogenic \emph{Leptospira} species and showed the highest 16S rRNA gene sequence similarity to \emph{L. alstonii}. One isolate, MS383, was placed within the radiation of intermediate pathogenic \emph{Leptospira} species and was mostly related to \emph{L. licerasiae}. The other 37 isolates were grouped into the clade of saprophytic \emph{Leptospira} species. Thirty-

<table>
<thead>
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<th>Area</th>
<th>Water</th>
<th>Soil</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metro Manila, Philippines</td>
<td>8/39</td>
<td>0/0</td>
<td>8/39</td>
</tr>
<tr>
<td>Nueva Ecija, Philippines</td>
<td>13/18</td>
<td>3/3</td>
<td>16/21</td>
</tr>
<tr>
<td>Fukuoka, Japan</td>
<td>10/16</td>
<td>3/12</td>
<td>13/28</td>
</tr>
<tr>
<td>Total</td>
<td>31/73</td>
<td>6/15</td>
<td>37/88</td>
</tr>
</tbody>
</table>

![FIG 1 PFGE patterns of Leptospira isolated from three environmental water samples: ES-1 (A), ES-56 (B), and ES-96 (C). Chromosomal DNA of isolates was digested with the NotI restriction endonuclease. Lanes: M, bacteriophage lambda concatamer molecular size marker; 1 to 5, PFGE patterns of five Leptospira strains isolated from each sample.](http://aem.asm.org/ on October 24, 2017 by guest)
FIG 2 Phylogenetic tree based on PFGE fingerprint patterns showing the positions of 37 isolates (23 from the Philippines and 14 from Japan) and 19 reference strains. The patterns of isolates from environmental samples were categorized into seven clusters (clusters A to G). Similarity between patterns was calculated by using the Dice index. The data were sorted by the UPGMA method. Band comparison settings of 1.2% optimization and 1% position tolerance were used.
one isolates, five isolates, and one isolate were presumed to be *L. yanagawae*, *L. meyeri*, and *L. vanthielii*, respectively.

**Distribution of Leptospira in the study sites.** The results of PFGE fingerprint pattern and 16S rRNA gene sequence determination of 42 isolates are summarized in Table 2. Strains presumed to be *L. alstonii* were all isolated only in Fukuoka, and they belonged to PFGE cluster A. Isolate MS383 (*L. licerasiae*) and *L. vanthielii* belonged to PFGE clusters B and C, respectively. The majority (67%) of strains isolated in Metro Manila belonged to *L. yanagawae* and PFGE cluster F, and the strains belonging to this
group were also found to be distributed in Nueva Ecija and Fukuoka. In contrast, 16 of 18 strains (89%) isolated in Nueva Ecija were presumed to be *L. yanagawae*; however, they had various PFGE patterns belonging to four clusters (D, E, F, and G). In particular, the isolates belonging to PFGE cluster G were obtained only in this area. On the other hand, 15 strains isolated in Fukuoka were grouped into various genomospecies. Of these, the strains presumed to be *L. alstonii* and *L. vanthieli* were isolated only in Fukuoka. Two strains belonging to *L. yanagawae* and PFGE cluster D (strains MS324 and MS336) were closely related to the two strains, MS173 and MS372, which were isolated in Nueva Ecija, suggesting the trans-border distribution of this genomospecies (Fig. 2).

**Characterization of *L. alstonii* and *L. licerasiae* strains.** (i) *flaB*-PCR of the isolates. The *flaB*-PCR assay produced positive PCR products for the four isolates that showed the highest 16S rRNA gene sequence similarity to *L. alstonii* (Table 2). The results from *flaB*-PCR and 16S rRNA gene sequence determination suggested that isolates MS267, MS306, MS311, and MS316 were pathogenic *Leptospira*. The other 38 isolates, however, were determined to be negative by *flaB*-PCR, including MS383, which is presumed to be an intermediate pathogenic *Leptospira* (*L. licerasiae*) (Table 2).

(ii) Serogroups of the isolates. MAT was performed for serological identification of the five isolates presumed to be pathogenic or intermediate pathogenic *Leptospira*. Positive agglutination for strains of *L. alstonii* was observed: MS267 and MS306 with Bratislava antisera, MS311 with Javanica antisera, and MS316 with Grippotyphosa antisera. The PFGE patterns of these four isolates were similar to that of *L. borgpetersenii* strain Poi, the reference strain of serogroup Javanica. No agglutination of MS383 (*L. licerasiae*) was observed with any of the panel of antisera for 23 serogroups.

(iii) Pathogenicity of isolates in golden Syrian hamsters. To examine the pathogenicity of the five isolates, groups consisting of five hamsters were inoculated intraperitoneally with $10^7$ of each

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Sample</th>
<th>Sampling region</th>
<th>Sampling mo, yr</th>
<th>Genomospecies</th>
<th><em>flaB</em>-PCR result</th>
<th>PFGE cluster</th>
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</thead>
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<td>MS146</td>
<td>Water</td>
<td>ES-1</td>
<td>Metro Manila</td>
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<td><em>L. yanagawae</em></td>
<td>−</td>
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<td>Metro Manila</td>
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<td><em>L. yanagawae</em></td>
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<td>Fukuoka</td>
<td>June, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
<td>F</td>
</tr>
<tr>
<td>MS285</td>
<td>Water</td>
<td>ES-56</td>
<td>Fukuoka</td>
<td>June, 2011</td>
<td><em>L. meyeri</em></td>
<td>−</td>
<td>F</td>
</tr>
<tr>
<td>MS286</td>
<td>Water</td>
<td>ES-56</td>
<td>Fukuoka</td>
<td>June, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
<td>F</td>
</tr>
<tr>
<td>MS287</td>
<td>Water</td>
<td>ES-56</td>
<td>Fukuoka</td>
<td>June, 2011</td>
<td><em>L. meyeri</em></td>
<td>−</td>
<td>E</td>
</tr>
<tr>
<td>MS290</td>
<td>Water</td>
<td>ES-57</td>
<td>Fukuoka</td>
<td>June, 2011</td>
<td><em>L. meyeri</em></td>
<td>−</td>
<td>No band</td>
</tr>
<tr>
<td>MS296</td>
<td>Water</td>
<td>ES-58</td>
<td>Fukuoka</td>
<td>July, 2011</td>
<td><em>L. vanthieli</em></td>
<td>−</td>
<td>C</td>
</tr>
<tr>
<td>MS306</td>
<td>Soil</td>
<td>ES-73</td>
<td>Fukuoka</td>
<td>July, 2011</td>
<td><em>L. alstonii</em></td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>MS311</td>
<td>Soil</td>
<td>ES-75</td>
<td>Fukuoka</td>
<td>July, 2011</td>
<td><em>L. alstonii</em></td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>MS316</td>
<td>Soil</td>
<td>ES-76</td>
<td>Fukuoka</td>
<td>July, 2011</td>
<td><em>L. alstonii</em></td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>MS324</td>
<td>Water</td>
<td>ES-79</td>
<td>Fukuoka</td>
<td>August, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
<td>D</td>
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<tr>
<td>MS326</td>
<td>Water</td>
<td>ES-80</td>
<td>Fukuoka</td>
<td>August, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
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<td>MS331</td>
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<td>ES-82</td>
<td>Fukuoka</td>
<td>August, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
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<td>MS336</td>
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<td>ES-83</td>
<td>Fukuoka</td>
<td>August, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
<td>D</td>
</tr>
<tr>
<td>MS341</td>
<td>Water</td>
<td>ES-84</td>
<td>Fukuoka</td>
<td>August, 2011</td>
<td><em>L. yanagawae</em></td>
<td>−</td>
<td>F</td>
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</table>
isolate/ml and observed for 28 days. No hamster showed any symptoms of leptospirosis or died after infection. Blood and kidney samples from hamsters sacrificed on day 28 postinfection were cultured. Leptospires were not recovered from any of the infected hamsters.

(iv) Dynamic change of the pathogenic isolates in four environmental sites. As already stated, genetically pathogenic leptospires, i.e., L. alstonii, were isolated from a water sample (ES-54) or from soil samples (ES-73, ES-75, and ES-76) from four sites in Fukuoka, Japan. To clarify whether the pathogenic leptospires colonize and survive for a long period or not, water or soil samples were collected from the four sites and cultured again after 5 months. We found that the three samples from the three sites, where the samples ES-54, ES-73, and ES-75 were collected, contained no Leptospira 5 months after the first isolation. On the other hand, the soil sample from the site where sample ES-76 was collected contained leptospires. The five isolates from ES-76 showed the same PFGE pattern closely related to that of MS316 (data not shown). This isolate (MS355) was flaB-PCR positive and showed 99.9% similarity in the 16S rRNA gene sequence to strain MS316 of L. alstonii serovar Grippotyphosa. However, only 10% of agglutination of MS355 was observed with antisera for serovar Grippotyphosa.

Influence of moisture content and pH of the soil samples. The moisture content of 15 soil samples ranged from 3.5 to 42.8%. A total of 67% of the samples with moisture content of ≥20% were Leptospira positive, whereas only 23% were found to be positive in the sample with a moisture content of <20% (Fig. 4). The pH value ranged from 6.2 to 7.2. Three of five samples with a pH of 6.2 were found to be positive. This result suggested that a pH of >6.2 (up to 7.2) had no influence on the distribution and survival of Leptospira in the environmental soil.

Persistence of Leptospira in a certain site. The water sample, ES-55, collected from a rain puddle on 30 June 2011 contained Leptospira strain MS280 (the PFGE pattern of MS280 was referred to as pattern I). This rain puddle usually dried up within a few days in the absence of rain. We expected that the soil of the puddle could be a reservoir of Leptospira. Therefore, the soil was collected from both the surface and 3 cm below the surface after 3 days of drought on 16 July 2011. The moisture contents of these soil samples were 3.5 and 11.1%, respectively. Only the soil sample from a 3-cm depth was found to be Leptospira positive. PFGE analysis of the isolates revealed two fingerprinting patterns: I and II (Fig. 5B).

After 5 months, the soil sample from the 3-cm depth (7.8% of the moisture content) was collected from the same site again. The sample was found to be positive again and had two PFGE patterns, I and III (Fig. 5C). Thus, the isolate referred to as PFGE pattern I was found not only in the rain puddle but also in the soil and survived for at least 5 months.

DISCUSSION
To understand the dynamic changes in the epidemiology of leptospirosis, isolation of Leptospira from not only the human and animal sources but also the environmental water and soil is essential. Previously, there have been few reports on the epidemiological analysis of environmental isolates. This is probably because the isolation of leptospires from environmental samples was usually unsuccessful due to the overgrowth of contaminants and the slow growth of Leptospira. In the present study, the leptospires were isolated from environmental samples at a high rate by using a combination of five antimicrobial agents (STAFF) (4).

We collected the environmental samples at three sites, Metro Manila and Nueva Ecija, Philippines, and Fukuoka, Japan. The frequency of isolation from environmental water was highest for Nueva Ecija (72%), followed by Fukuoka (62%) and then Metro Manila (21%). The water samples from urban area of Metro Manila were cloudy, and some of them were foul-smelling. Leptospires were found to be unable to survive in such an environment. The moisture content of soil greatly influenced the frequency of positive isolation from the soil. It was suggested that leptospires would hardly survive in the soil with a moisture content of <20%. On the other hand, the pH values of all soil samples were higher than 6.2, and such a pH had no influence on the distribution and survival of Leptospira in the soil. Henry and Johnson (7) previously reported the percentage of Leptospira-positive soil sample decreased with increasing distance from the spring, and beyond 25 m no positive cultures were obtained from the soil with a moisture content of 24% and a pH of 5.4. These findings were consistent with our results.

The taxonomy of Leptospira has undergone substantial revision with the use of 16S rRNA sequence comparison and DNA-DNA reassociation studies (14). At the time of this writing, the genus Leptospira consists of 20 genomospecies. The phylogenetic tree constructed based on 16S rRNA gene sequences of the type strains of 20 Leptospira genomospecies gives three clades, i.e., pathogenic Leptospira, intermediate pathogenic Leptospira and nonpathogenic (or saprophytic) Leptospira. The amplification and sequencing of the 16S rRNA gene of isolates is capable of accurately identifying and differentiating Leptospira species (15).

In the present study, only four isolates from Fukuoka were identified as a pathogenic Leptospira species, i.e., L. alstonii. One isolate from Nueva Ecija was identified as an intermediate pathogenic Leptospira species, L. licerasiae. Most of the isolates from the environmental soil and water belonged to nonpathogenic Leptospira species. It is thought that the frequency of pathogenic or intermediate pathogenic Leptospira isolation from the environment is not high even in areas of endemicity such as Metro Manila.

We found that our three environmental samples contained more than one strains by using single colony isolation method and PFGE analysis. This result would provide the first evidence that a few strains of Leptospira occasionally live together in environmental water and soil. Thus, we recommend that at least five strains per
one environmental sample should be subcultured and analyzed by PFGE for accurate epidemiologic study.

The dendrogram based on the PFGE patterns separated the 37 environmental isolates into seven clusters. Four strains of *L. alstonii*, one strain of *L. licerasiae*, and one strain of *L. vanthiellii* belonged to three independent clusters. The other 31 strains, including *L. meyeri* and *L. yanagawae*, formed four clusters. Two of these clusters included both strains of *L. meyeri* and strains of *L. yanagawae*, and these two genomospecies contained a variety of PFGE patterns (Fig. 2). In the present study, the serogroups of non-pathogenic strains were not determined; however, the classification of clusters based on the PFGE fingerprint pattern might reflect serogroup relatedness rather than genetic relatedness, as previously reported.

Using NotI as the restriction enzyme is a standard method to perform the PFGE analysis for the isolates of leptospires (8, 16, 17). We found that 5 of 42 strains showed no band of NotI-restricted DNA; however, the fingerprint patterns were seen using other enzymes, such as SmaI or PacI. Although there are no reports of *Leptospira* strains without a NotI restriction site, some of the isolates that were not restricted by NotI were classified as *L. yanagawae* and *L. meyeri* by 16S rRNA gene sequencing. We recommend using SmaI or PacI to digest the DNA when the differentiated patterns cannot be obtained when digested with NotI. Incidentally, we also found that two *Leptonema* isolates showed a ladder of small fragments with sizes of <100 kbp, when digested with NotI (data not shown).

Unlike pathogenic *Leptospira*, little is known about the behavior or reservoirs of nonpathogenic (saprophytic) *Leptospira* in the environment. Henry and Johnson (7) reported leptospires that were most frequently associated with soils of high moisture, and the reservoir of the saprophytic *Leptospira* could be the soil. We investigated the strain changing of the isolates in a puddle during 5 months. It is noteworthy that the same strain, which was initially isolated from the water of the puddle, was consistently isolated from the soil 3 cm from the depth of the puddle (11.1% moisture content) even when the puddle had dried up. No leptoспорie was isolated from the surface of the ground (3.5% moisture content), possibly because it could not survive in such a dry condition. This suggested that saprophytic *Leptospira* survives in wet soil on dry days and appears in the surface water on rainy days. This is the first report that demonstrated soil as a possible reservoir of saprophytic *Leptospira* using PFGE analysis. In the Philippines, outbreaks of leptospirosis usually occur during the rainy season and just after the rainy season in flood-prone areas (6). In such areas, pathogenic *Leptospira* could appear from soil contaminated with the urine of infected animals and get mixed with flood water, causing a wide spread of the organisms. After a flood, the pathogens might survive in the soil of the entire flood area for several months, while the virulence of the pathogens might be lost in time, as described earlier.

The present study was also carried out to clarify the differences among isolates between Japan and the Philippines using PFGE analysis. The isolates of each area showed their own characteristics. In Metro Manila, majority (two-thirds) of the isolates were *L. yanagawae* belonging to PFGE cluster F. The isolates in Nueva Ecija showed various PFGE patterns belonging to four clusters.
The PFGE cluster G was unique in this area only. The isolates in Fukuoka were classified as a variety of genomospecies, including the unique species \textit{L. alstonii} and \textit{L. vanthieli}. However, some isolates, for example, four strains in cluster D (Fig. 2), were closely related to each other across borders.

It is well known that mammalian species excrete pathogenic leptospires in their urine and serve as reservoirs for their transmission (18). The pathogens are maintained in sylvatic and domestic environments by transmission among rodent species. In these reserves, infection produces chronic, asymptomatic carriage. The maintenance of leptospirosis in these populations is due to their continuous exposure to rodent reservoirs or to transmission within animal herds (18). In the present study, four strains of genetically pathogenic \textit{Leptospira} were isolated within an area 50 m in diameter. We believe these strains are maintained among small animals because no pathogenic isolates were obtained from the university campus outside this limited area. To determine the animal reservoirs, we tried to trap rats and mice but were unsuccessful.

Recently, only a small number of cases of leptospirosis have been reported in Japan. However, strains of genetically pathogenic \textit{Leptospira} strains were isolated in Fukuoka. Their virulence had already been lost, and three-quarters of the isolates were eliminated within 5 months. Trueba et al. (2) reported that a strain of \textit{L. interrogans} was able to remain motile for 110 days (pH 7.2) in distilled water and survived 347 days when incubated in a semisolid medium composed of distilled water and 0.5% purified agarose. It is hypothesized that pathogenic leptospirosis may be killed within 5 months after excretion into soil and water if some uncertain conditions are not satisfied. The ability of pathogenic \textit{Leptospira} to survive long in the environment seems to vary. Adler and coworkers (19) analyzed the genomes of two pathogenic species, \textit{L. borgpetersenii} and \textit{L. interrogans}. These researchers showed that \textit{L. borgpetersenii} lost the signal transduction function, and its survival outside a mammalian host was impaired, whereas \textit{L. interrogans} retained an environmental sensory function that facilitated disease transmission through water. We suggest that our \textit{L. alstonii} isolates could adapt in the environment only for a short period and lose their virulent phenotype in the environment outside of the body of a mammalian host. Leptospiroses are known to lose their virulent phenotype with prolonged \textit{in vitro} culture passages, and animal passage is needed to maintain their virulence. In the same way, the potential virulence of the pathogenic isolates was possibly lost during the thriving of leptospiroses in the environment after being excreted in the urine of reservoir animals.

In areas of endemicity, an enormous number of rodent reservoirs might maintain the virulence of pathogenic \textit{Leptospira} in their kidneys and intermittently excrete urine containing these virulent leptospiroses into the environment. It could be said that the control of rodents is one of the best ways to get rid of virulent leptospiroses in the environment, and this could be a reason for the dramatic decrease in the number of leptospirosis patients after 1960 in Japan. There is therefore an urgent need to improve sanitary conditions and control rodents in countries and areas where leptospirosis is endemic and prevalent.

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**REFERENCES**