Human *Leptospira* Isolates Circulating in Mayotte (Indian Ocean) Have Unique Serological and Molecular Features


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Leptospirosis is one of the most widespread zoonoses in the world. However, there is a lack of information on circulating *Leptospira* strains in remote parts of the world. We describe the serological and molecular features of leptospires isolated from 94 leptospirosis patients in Mayotte, a French department located in the Comoros archipelago, between 2007 and 2010. Multilocus sequence typing identified these isolates as *Leptospira interrogans*, *L. kirschneri*, *L. borgpetersenii*, and members of a previously undefined phylogenetic group. This group, consisting of 15 strains, could represent a novel species. Serological typing revealed that 70% of the isolates belonged to the serogroup complex Mini/Sejroe/Hebdomadis, followed by the serogroups Pyrogenes, Grippotyphosa, and Pomona. However, unambiguous typing at the serovar level was not possible for most of the strains because the isolate could belong to more than one serovar or because serovar and species did not match the original classification. Our results indicate that the serovar and genotype distribution in Mayotte differs from what is observed in other regions, thus suggesting a high degree of diversity of circulating isolates worldwide. These results are essential for the improvement of current diagnostic tools and provide a starting point for a better understanding of the epidemiology of leptospirosis in this area of endemicity.

Leptospirosis is an emerging zoonosis with a worldwide distribution that may affect millions of people annually (1). Leptospirosis is expected to become more important due to global climate changes and rapid urbanization in developing countries where slum settlements have produced the conditions for epidemic rat-borne transmission of the disease. Leptospirosis is also an endemic disease in some rural regions because of the exposure to a large number of animal reservoirs (17, 19).

Leptospirosis is caused by infection with certain strains of *Leptospira*, a genus currently consisting of nine pathogenic species subdivided into more than 200 serovars (5). Isolation of clinical isolates is important, because establishing the causal serovar is the first step toward (i) improving diagnostic tools, (ii) identifying animal reservoirs, and (iii) generating control strategies (eradication of reservoirs, vaccine development, etc.). However, there is a lack of information on circulating *Leptospira* strains worldwide. This is particularly true in certain regions, like Africa. A better understanding of the epidemiology of leptospirosis therefore requires the isolation of *Leptospira* strains, which is challenging because the organisms are found in the bloodstream during a few days (first week after the onset of symptoms) or transiently in the urine (17).

Mayotte is a small island (374 km²) with a population of 186,452 (July 2007 census), 53% of whom are under age 20 (23). The yearly population growth is estimated at 3.1%. Forty percent of the population are foreigners, most of them illegal immigrants from the Comoros Islands. One-fourth of the population has no direct access to water and depends on neighbors, public water fountains, or rivers for water for drinking, bathing, and laundry. In 2009, 30% of households were farmers (23). Risk factors for infections by leptospires in Mayotte are multiple. Due to the poor living conditions, people walk barefoot, are often engaged in agricultural or gardening activities, and have frequent contact with rivers or streams. Cattle are not confined to prairies but circulate freely alongside earthen streets, fields, and rivers. A retrospective study performed between 1984 and 1989 estimated the annual leptospirosis incidence in Mayotte at 3.83 cases per 100,000 individuals (16). Since 2007, surveillance of leptospirosis on the island has been reinforced, and the annual incidence rate was estimated to be higher than 8 patients per 100,000 individuals (3). By using multilocus VNTR (variable number of tandem repeats) analysis (MLVA), pulsed-field gel electrophoresis (PFGE), and *ligB* sequencing, we previously detected the existence of potentially new pathogenic *Leptospira* genotypes among isolates from 22 patients (3). The high success rate of isolation by culture allowed the typing of additional isolates. In the present study, leptospires isolated from blood samples from 94 patients were characterized by *rrs* sequencing, multilocus sequence typing (MLST), and microscopic agglutination test (MAT) using rabbit antisera and monoclonal antibodies.

**MATERIALS AND METHODS**

**Diagnostic PCR.** Data reporting on leptospirosis relies on positive PCR diagnosis of leptospirosis by the laboratory of the Hospital Centre of May-
TABLE 1 Laboratory results of leptospirosis testing and incidence per year in Mayotte, France, from 2007 to 2010

<table>
<thead>
<tr>
<th>Year</th>
<th>No. of PCRs</th>
<th>No. of positive results</th>
<th>% positivity</th>
<th>No. of isolates (%)</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>142</td>
<td>16</td>
<td>11.9</td>
<td>425</td>
<td>8.6</td>
</tr>
<tr>
<td>2008</td>
<td>400</td>
<td>38</td>
<td>9.5</td>
<td>20 (52.6)</td>
<td>19.8</td>
</tr>
<tr>
<td>2009</td>
<td>671</td>
<td>84</td>
<td>12.5</td>
<td>37 (44)</td>
<td>42.4</td>
</tr>
<tr>
<td>2010</td>
<td>1,310</td>
<td>58</td>
<td>4.4</td>
<td>33 (56.9)</td>
<td>28.4</td>
</tr>
<tr>
<td>Total</td>
<td>2,523</td>
<td>196</td>
<td>7.8</td>
<td>94</td>
<td>25.1</td>
</tr>
</tbody>
</table>

a Autochthonous cases.

b Number of cases per 100,000 persons.

otté (CHM), the only laboratory on the island with diagnostic capacities for leptospirosis. Total genomic DNA was extracted from plasma collected in EDTA tubes using a MagNaPure Compact instrument (Roche Molecular Diagnostics). Herpes simplex virus 1 (HSV-1) DNA was added to the samples before DNA extraction as an internal control. Real-time PCR assays were performed to amplify the genes frb1 (21), lipL32 (18), and rrs (28) until 2009 and the genes rrs (28) and lipL32 (29) from 2009 to 2010.

Strain isolation from blood and culture conditions. Plasma from about 3 ml of heparinized blood of patients was transferred into EMJH liquid medium (10, 13), and cultures were incubated at 30°C for 3 months (14, 24). This justifies a distinct species status for the strains of group B.

Identification of a new species and undescribed sequence types. Partial sequencing of the 5' variable region of the 16S rRNA gene (20, 25) indicated that the strains belonged to the pathogenic species Leptospira interrogans (8/94), L. kirschneri (22/94), and L. borgpetersenii (49/94). In 15 cases, we did not find 16S rRNA sequences that matched our sequences in the NCBI database. The highest 16S rRNA gene (rrs) sequence similarity (4 out of 280 bp of the variable region were different) was found for L. borgpetersenii reference strains. These 15 L. borgpetersenii-like strains were designated “L. borgpetersenii group B” strains (see Table S1 in the supplemental material).

RESULTS AND DISCUSSION

Leptospirosis in Mayotte from 2007 to 2010. During the period from 2007 to 2010, a total of 2,752 blood samples from 2,523 patients were analyzed by PCR for the diagnosis of acute leptospirosis (Table 1). A total of 198 cases of leptospirosis were confirmed, of which two were imported from neighboring islands Anjouan (Comoros island) and Madagascar. Therefore, the total number of autochthonous confirmed cases was 196, which corresponds to an overall annual incidence of 25 cases per 100,000 persons. In the Indian Ocean, reported annual incidence ranges from 6.4 per 100,000 population in Reunion Island (4, 6, 9) to more than 40 per 100,000 population in the Andaman islands and Seychelles (27, 31). Despite reinforced surveillance, the incidence of leptospirosis in Mayotte is certainly still underestimated for a variety of reasons, which include difficulty in PCR testing all suspected cases and results of false-negative PCR due to early therapy with antibiotics or blood sampling late in the disease. Nevertheless, this estimated incidence of the disease in Mayotte is among the highest in French overseas territories.

Isolation of human Leptospira isolates in Mayotte from 2007 to 2010. Culture of leptospires was carried out from the blood of PCR positive samples (3) whenever possible and sent to the National Reference Center (NRC) for Leptospirosis (Institut Pasteur) for typing. In this study, isolates from 94 patients, which correspond to 48% of autochthonous confirmed cases between 2007 and 2010, were further analyzed (Table 1).

The patients with leptospirosis were aged 13 to 78 years, with a mean age of 32.1 years. Males represented 73.4% of the patients. This is consistent with data from other countries where leptospirosis is endemic (30, 31). The infections occurred almost exclusively during the rainy season, i.e., November to May (91% of the examined cases). Of the 94 hospitalized patients, 11 were admitted to the intensive care unit. Three patients did not survive (see Table S1 in the supplemental material).

Identification of a new species and undescribed sequence types. Partial sequencing of the 5' variable region of the 16S rRNA gene (20, 25) indicated that the strains belonged to the pathogenic species Leptospira interrogans (8/94), L. kirschneri (22/94), and L. borgpetersenii (49/94). In 15 cases, we did not find 16S rRNA sequences that matched our sequences in the NCBI database. The highest 16S rRNA gene (rrs) sequence similarity (4 out of 280 bp of the variable region were different) was found for L. borgpetersenii reference strains. These 15 L. borgpetersenii-like strains were designated “L. borgpetersenii group B” strains (see Table S1 in the supplemental material).

Currently, only one MLST scheme has been designed for the characterization of Leptospira strains of all the pathogenic species (2, 22). An updated MLST database, which contains the sequence data from 271 isolates from different geographical areas, was recently published (22). When this scheme was applied, satisfactory amplifications were obtained for all loci except for the icda locus, which was removed from our analysis. Sequence alignments of adk, lipL32, secY, and lipL41 indicated a high level of polymorphism in our data set, as 12 to 21% of nucleotidic sites were variable. Phylogenetic analysis of individual gene sequences as well as concatenated sequences of the five genes (i.e., adk, lipL32, rs2, secY, and lipL41) showed highly congruent results, with four well-separated sequence clusters corresponding to the species L. interrogans and L. kirschneri and two distinct clusters of L. borgpetersenii corresponding to L. borgpetersenii and L. borgpetersenii group B (Fig. 1). The average nucleotide divergence between L. borgpetersenii and L. borgpetersenii group B was approximately 10% based on the four protein-coding gene sequences. Based on the 95% average nucleotide identity value correlating with 70% DNA-DNA hybridization (14, 24), this justifies a distinct species status for the strains of group B. Genome sequencing of strains from this
phylogenetic group should confirm the existence of this new *Leptospira* species.

MLST analysis revealed a total of 16 different sequence types (STs) (Fig. 1; also, see Table S1 in the supplemental material). The most frequent ST identified was ST1 (*n* = 29), followed by ST8 (*n* = 13), ST5 (*n* = 9), ST2 (*n* = 8), and ST3 (*n* = 8). Interestingly, one strain introduced from the Comoros Islands, strain 200803703, exhibited another ST (ST9) with little divergence from ST6 and ST13 (Fig. 1). During the course of this study, the proportion of ST8 infections increased significantly (0 in 2007, 1 in 2008, 2 in 2009, and 10 in 2010), suggesting that this genotype is an emerging variant.

Minimum spanning tree analysis of the MLST profiles from strains in Mayotte compared to those previously published (Fig. 2A) yielded a number of observations. First, there was only one ST (ST2) of *L. interrogans* in Mayotte, which is closely related to one previously reported ST. Second, strains of *L. borgpetersenii* clustered in three unrelated clonal groups, including *L. borgpetersenii* group B (Fig. 2B). Finally, *L. kirschneri* isolates from Mayotte were all related among themselves and were also related to previously reported genotypes in a single clonal group. None of the STs from Mayotte strains have been previously defined (22) (Fig. 2A). The majority of MLST data that have been previously reported corresponded to strains of *L. interrogans* (174/271) from Asiatic countries (22) and did not overlap with the genotypes found in Mayotte (Fig. 2A). Overall, these results show that strains of *Leptospira* in Mayotte are related to but distinct from previously reported strains from other regions. Larger sampling is obviously needed to determine in more detail the geographic specificity of Mayotte genotypes.

**Identification of new serovars.** Serogrouping was first performed with rabbit antisera against reference serovars of the main serogroups. Serogroup *Mini*, including strains cross-reacting with serogroups *Sejroe* and *Hebdomadis*, represented the predominant serogroup in Mayotte (*n* = 66, 70.5%). Other *Leptospira* serogroups identified were *Pyrogenes* (*n* = 17), *Grippotyphosa* (*n* = 8), and *Pomona* (*n* = 3) (see Table S1 in the supplemental material). Serogroup *Icterohaemorrhagiae*, which is found to be the most prevalent serogroup in the majority of countries, was not detected in Mayotte. Prior to 2007, the most prevalent *Leptospira* serogroups in Mayotte determined by MAT were *Sejroe*, *Grippotyphosa*, and *Pyrogenes* (NRC for Leptospirosis, Institut Pasteur, unpublished data). However, the antigens used did not include local isolates, which could have led to false-negative results.

We then tested at least one representative strain of the predominant STs (ST1, ST2, ST3, ST5, ST6, and ST10) as defined by MLST (see above) against a panel of MAbs against serovars belonging to the serogroups *Grippotyphosa*, *Pomona*, *Mini/Sejroe/Hebdomadis* complex, *Ballum*, *Pyrogenes*, and *Icterohaemorrhagiae* (see Table S2 in the supplemental material). Agglutination patterns of isolates 200701203 (ST1), 200901122 (ST3), and 200801774 (ST6) were similar to those of *L. borgpetersenii* serovar *Mini* strain Sari and serovar *Kenya* strain Nijenga and *L. kirschneri* serovar *Grippotyphosa* type *Moskva* strain *Moskva*, respectively. However, the PFGE profiles obtained for 200701203 (ST1) and reference strain Sari were distinct (>3 band differences in NolI macrorestriction profiles) (3), suggesting that they may not belong to the same serovar (11, 12) or that they underwent genomic rearrangements not affecting the serovar status. This was also true for 200801774 (ST6) and reference strain *Moskva* (3). The STs of reference strains which were included in the study by Nalam et al. (22) were also distinct from the STs of our clinical isolates (data not shown), further suggesting that isolates from a given serovar may correspond to different genotypes. Isolates from Mayotte may therefore belong to new types of these serovars. The agglutination profiles of 200801925 (ST3) corresponded to those of the *L. kirschneri* reference strains, serovar *Kambale* strain *Kambale* and *Robinsoni* strain *Robinsons*. Again, the PFGE profiles obtained for 200901489 (ST2) and the reference strains were distinct (3). Thus, unambiguous typing is not possible for this
isolate. Most interestingly, 200901118 (ST10) was found to belong to either serogroup Pomona or the closely related serogroup Grippotyphosa. Typing with panels of MAbs reacting with these serogroups revealed a pattern most similar to that of serogroup Pomona, serovar Mozdok. However, this isolate was identified as *L. borgpetersenii*, while none of the reference serovars within the serogroups Pomona and Grippotyphosa belong to this species. Isolate 200901118 (ST10) could therefore be serovar Mozdok but belong to another species. A similar observation was previously reported for serovar Hardjo, which is present in both *L. interrogans* and *L. borgpetersenii* (7, 17).

Further analysis should include the use of the cross agglutinin absorption test (CAAT) to confirm that some of the circulating strains in Mayotte correspond to new serovars.

Little is known about the animal reservoirs of leptospires in Mayotte. At the beginning of the 1990s, zebus (the local bovine species), goats, and dogs were highly infected, with seroprevalences of 85% (34/40), 70% (7/10) and 83% (5/6), respectively (8). In a study on 284 zebu sera in 2009, 11.6% were positive (our unpublished data). Predominant serogroups were Mini (36%), Grippotyphosa (33%), and Pyrogenes (15%), which reflect our findings with human isolates, suggesting predominantly rural infection.

Although the number of strains was relatively low, we did not observe an apparent correlation between a specific genotype or serotype and the ability to cause severe disease (data not shown). Similarly, we were unable to associate distinct clonal types with the place of exposure to leptospirosis, as far as these have been documented. Since January 2010, investigations are being carried out for all confirmed cases of leptospirosis through a standardized questionnaire administered at the home of the patient, in order to collect data on environmental and behavior risk factors. Together

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**FIG 2** Minimum spanning trees of *Leptospira* strains based on allelic profiles of *adk*, *lipL32*, *rrs2*, *secY*, and *lipL41*. (A) Minimum spanning tree of 372 *Leptospira* strains, including strains from Mayotte and previously described strains (22). (B) Minimum spanning tree of *L. interrogans* (*n* = 142), *L. borgpetersenii* (*n* = 109), and *L. kirschneri* (*n* = 51) showing the lack of overlap between strains from Mayotte and the MLST database published by Nalam et al. (22). Open circles correspond to genotypes previously described by Nalam et al. (22), while colored circles correspond to strains found in Mayotte. Bold, plain, and dashed lines linking the circles correspond to one, two, or more allelic mismatches, respectively; note that links with more than two allelic mismatches are not reliable.
with the typing of strains, this should help us to improve our understanding of the epidemiology of leptospirosis in Mayotte and in other countries.

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REFERENCES