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# Application of Multilocus Variable-Number Tandem-Repeat Analysis for Molecular Typing of the Agent of Leptospirosis

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**Leptospirosis is a worldwide-distributed zoonosis, endemic in tropical areas. Epidemiologic investigations of leptospirosis still rely on tedious serological identification tests. Recently, molecular typing systems based on variable-number tandem-repeat (VNTR) analysis have been described and have been used to identify *Leptospira interrogans* strains. Although *L. interrogans* is the most common *Leptospira* species encountered in human infections around the world, other pathogenic species, such as *Leptospira kirschneri* and *Leptospira borgpetersenii*, are also frequently associated with human leptospirosis. In this study, we aimed to extend multilocus VNTR analysis (MLVA) identification of strains to species other than *L. interrogans*. We designed primers for VNTR loci found in *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*. The discriminatory power of the redefined primers was evaluated on collection strains and then on clinical strains. We also carried out a retrospective study on 156 strains isolated from patients and animals from New Caledonia, an area of high endemicity in the South Pacific. Our results show that this simple PCR-based MLVA typing technique is a powerful methodology for the epidemiology of leptospirosis.**

Leptospirosis is an infectious disease caused by spirochetes belonging to the genus *Leptospira*. It is considered the most widespread zoonosis in the world (37). With large outbreaks occurring during the last decade in, for example, Nicaragua (36), Brazil (15), and India (39), leptospirosis is now recognized as an important emerging infectious disease. However, its prevalence is still underestimated due to the poor quality of surveillance data, the highly diverse clinical manifestations of the disease that make diagnosis difficult, and the low sensitivity of the standard diagnostic tests (21).

Typing of the agent of leptospirosis is based on the serological identification of circulating strains. There are over 200 recognized serovars of pathogenic leptospires currently clustered into antigenically related serogroups (14). Maintenance hosts serve as the reservoirs of serovars belonging to the same serogroup (e.g., rats are the maintenance hosts for serovars of the Ballum and Icterohaemorrhagiae serogroups) (17). Serogroups are identified using the microagglutination test (MAT), and serovars are identified using the cross-adsorption agglutination test. These methods are tedious, as live cultures of collection strains must be maintained for use as antigens and thus require lab facilities that are not usually available in regions in which the incidence of the disease is the highest. Moreover, the interpretation of the results is complicated by the frequent cross-reactions that occur between serogroups. The results are also difficult to standardize because they depend on the biologist operating the microscope (17). Genotypic classification is now replacing this phenotypic classification. However, although the serological classification has no taxonomic value, establishing the infecting serogroup or serovar is useful for identifying reservoirs and for developing preventive strategies (21). Thus, serogroup and serovar classifications still coexist with molecular classifications of *Leptospira*. Although serogroup classification is not related to molecular classification, serovars can be characterized by different molecular typing methods, such as restriction fragment length polymorphism (26, 34), randomly amplified polymorphic DNA (30), and pulsed-field gel electrophoresis (PFGE) (10, 11). However, these molecular methods have certain drawbacks: they require large amounts of DNA or may not always be sufficiently discriminating or reproducible.

Recently, multilocus variable-number tandem-repeat (VNTR) analysis (MLVA), a molecular method used for the DNA fingerprinting of a wide range of pathogenic bacterial species, such as *Mycobacterium tuberculosis*, *Bacillus anthracis*, and *Yersinia pestis* (for a review, see reference 18), has been proposed for typing *Leptospira interrogans* strains (19, 33). The discriminative power of the method in *L. interrogans* depends on the VNTR loci: MLVA is useful for identifying serovars (19) or for discriminating strains within the same serovar (33).

Most molecular genotyping methods for typing *Leptospira* strains have been developed using *L. interrogans*. Indeed, *L. interrogans* is the most frequently reported *Leptospira* species, with the *L. interrogans* serogroup Icterohaemorrhagiae representing more than half of the leptospires encountered in human infections. However, other pathogenic *Leptospira* species, such as *Leptospira kirschneri* and *Leptospira borgpetersenii*, which are mainly associated with infections in animals, are also found in humans.

In this study, we aimed to improve the MLVA described by Majed et al. (19) to allow any pathogenic species of *Leptospira* and, thus, any leptospiral isolates to be typed without previous knowledge of the species. We tested newly defined primers on 99 collection strains belonging to *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* species and validated them on clinical

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TABLE 1. VNTR loci proposed for MLVA of *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*

| Locus | Primers (sequence [5′–3′]) | PCR product size (bp) for[a]: | | |
|---|---|---|---|---|
| | | *L. interrogans* | *L. kirschneri* | *L. borgpetersenii* |
| VNTR-4 | 4a_bis (AAGTAAAAGCGCTCCCAAGA)<br>4b_bis (ATAAAGGAAGCTCGGCGTTT) | 425 + 34*n* | 425 + 34*n* | 425 + 34*n* |
| VNTR-7 | 7a_bis (GATGATCCCAGAGAGTACCG)<br>7b (TCCCTCCACAGGTTGTCTTG) | 299 + 46*n* | 299 + 46*n* | No product |
| VNTR-10 | 10a_bis (GAGTTCAGAAGAGACAAAAGC)<br>10b_bis (ACGTATCTTCATATTCTTTGCG) | 420 + 46*n* | 347 + 46*n* | 333 + 46*n* |
| VNTR-Lb4 | Lb4a (AAGAAGATGATGGTAGAGACG)<br>Lb4b (ATTGCGAAACCAGATTTCCAC) | No product | No product | 573 + 60*n* |
| VNTR-Lb5 | Lb5a (AGCGAGTTCGCCTACTTGC)<br>Lb5b (ATAAGACGATCAAGGAAACG) | 668 + 39*n* | 668 + 39*n* | 722 + 36*n* |

[a] The PCR product size is the size of the VNTR flanking region plus *n* (repeat size), with *n* being the number of repeats.

strains. We carried out a study of leptospirosis in New Caledonia, a tropical region of high endemicity in the South Pacific, to show that MLVA is a powerful epidemiological method.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** We obtained 99 reference strains belonging to 53 serovars of *L. interrogans*, 23 serovars of *L. kirschneri*, and 23 serovars of *L. borgpetersenii* from the collection maintained by the National Reference Laboratory for *Leptospira* at the Institut Pasteur, Paris, France. We included in the study 34 human and animal strains from different geographical origins (Saint Petersburg, Russia, Croatia, Guadeloupe, and other locations). In addition, we studied 156 clinical isolates (143 and 13 isolates recovered from patients and animals, respectively) that were collected from 1989 to 2001 throughout the whole territory of New Caledonia.

All strains were cultured at 30°C in EMJH liquid medium (8, 12) and grown for up to 1 month to stationary phase. A MAT was carried out using standard methods using rabbit hyperimmune sera raised against the 23 live antigens (provided by the WHO Collaborating Center for Leptospirosis, Institut Pasteur, Paris, France) representing the main pathogenic serogroups of *Leptospira* spp.

**VNTR primer design.** The nucleotide sequences of both the large and the small chromosomes, CI and CII, respectively, of *L. interrogans* serovar Lai strain 56601 (32), *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (24), and *L. borgpetersenii* serovar Hardjobovis strain L550 (B. Adler, personal communication) were analyzed using Tandem Repeats Finder software (1) and the Tandem Repeats Database (http://minisatellites.u-psud.fr/) (7). Primer pairs within the coding sequences flanking the VNTR loci were designed.

**DNA manipulation.** Genomic DNA of *Leptospira* was isolated using the phenol-chloroform method as previously described (28). The DNA was amplified using *Taq* polymerase (Amersham) under the following conditions: one denaturation cycle at 94°C for 5 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min; and a final elongation at 72°C for 10 min. The amplified products were analyzed by 1% agarose gel electrophoresis. The sizes of the amplicons were estimated by comparison to a 100-bp ladder (Invitrogen). When confirmation of species was needed, we used 16S rRNA gene sequencing or LFB1 real-time assay as previously described (23, 29). PCR products were directly sequenced by Genome Express (Meylan, France) using the PCR primers. Sequences were aligned and analyzed using MEGA3 software (16).

**Data analysis.** The copy number of the repeats of each VNTR locus was deduced from the sequencing data and band sizes of the amplified products. Bionumerics software package, version 4.0 (Applied Maths, Sint-Martens-Latem, Belgium), was used for clustering analyses of the data using the categorical coefficient and the unweighted-pair group method using average linkages clustering parameters. The polymorphism indices of individual and grouped VNTRs were calculated using Nei's diversity index (25).

## RESULTS

**Primer design for VNTR analysis of *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii*.** A previously described MLVA typing strategy has been developed for strains belonging to *L. interrogans* (19, 33). In this study, we aimed to develop this methodology for typing other pathogenic *Leptospira* species, in particular *L. kirschneri* and *L. borgpetersenii*, to allow us to type isolates found in natural populations without having any previous knowledge of their species.

The analysis of a subset of *L. kirschneri* and *L. borgpetersenii* strains showed that none of the seven primer pairs used for *L. interrogans* typing (VNTR-4, VNTR-7, VNTR-9, VNTR-10, VNTR-11, VNTR-19, and VNTR-23) (19) worked with these species (data not shown). Among the 44 primer pairs previously defined from the genome of *L. interrogans* serovar Lai (19), three (VNTR-32, VNTR-33, and VNTR-42) allowed the amplification of 23 serovars of *L. kirschneri*. However, the discrimination of the amplified products was poor (VNTR-32, 8 types; VNTR-33 and VNTR-42, 2 types among the 23 serovars of *L. kirschneri*), and therefore, we kept none of these markers for further analysis.

In our previous study (19), the primers were designed from the intergenic regions flanking the VNTR loci of *L. interrogans* serovar Lai. In the present study, we used the nucleotide sequences of the two available *L. interrogans* genomes (24, 32) and designed primers from the coding regions of putative genes flanking the VNTR loci. We considered that the nucleotide sequences within the coding sequences would be better conserved than those within the intergenic regions and that the location of the genes may be conserved in species closely related to *L. interrogans*. Majed et al. (19) showed that only three markers (VNTR-7, VNTR-10, and VNTR-19) were needed to identify serovars of *L. interrogans*, and thus, we focused on redefining the primer pairs for amplifying these loci.

With the new primer pairs, VNTR-7 and VNTR-10 were successfully amplified in the 53 and 23 serovars of *L. interrogans* and *L. kirschneri*, respectively (Tables 1 and 2). The VNTR-10 locus could also be amplified from the 18 serovars of *L. borgpetersenii* analyzed in our study. VNTR-10 is located within the *rfb* locus that encodes enzymes involved in lipopolysaccharide biosynthesis. However, as the coding sequences were located too far from VNTR-19, we were unable to design new primers for this locus. Analyses of the results of Majed et al. (19) obtained with the seven primer pairs showed that replacing VNTR-19 with VNTR-4 resulted in a similar discrimination level among *L. interrogans* serovars. Thus, we designed new primer pairs for VNTR-4, and the 23 serovars of *L.*

TABLE 2. Results obtained for the 99 collection strains analyzed by the MLVA method

| Species | Serogroup | Serovar | Strain | Country | Source | Result for locus[a]: | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | VNTR-4 | VNTR-7 | VNTR-10 | VNTR-Lb4 | VNTR-Lb5 |
| *L. interrogans* | Australis | Australis | Ballico | Australia | Human | 1 | 6 | 14 | | |
| *L. interrogans* | Australis | Bangkok | Bangkok-D92 | Thailand | Dog | 7 | 9 | 18 | | |
| *L. interrogans* | Australis | Bratislava | Jez-Bratislava | Czechoslovakia | Hedgehog | 1 | 6 | 13 | | |
| *L. interrogans* | Australis | Fugis | Fudge | Malaysia | Human | 1 | 7 | 13 | | |
| *L. interrogans* | Australis | Jalna | Jalna | Czechoslovakia | Mouse | 4 | 6 | 9 | | |
| *L. interrogans* | Australis | Muenchen | Munchen C90 | Germany | Human | 1 | 10 | 9 | | |
| *L. interrogans* | Autumnalis | Autumnalis | Akiyami A | Japan | Human | 3 | 12 | 9 | | |
| *L. interrogans* | Autumnalis | Carlos | C-3 | Philippines | Toad | 1 | 6 | 1 | | |
| *L. interrogans* | Autumnalis | Mooris | Moores | Malaysia | Human | 3 | 14 | 7 | | |
| *L. interrogans* | Bataviae | Bataviae | Van Tienen | Indonesia | Human | 1 | 10 | 6 | | |
| *L. interrogans* | Canicola | Benjamini | Benjamin | Indonesia | Human | 2 | 4 | 8 | | |
| *L. interrogans* | Canicola | Bindjei | Bindjei | Indonesia | Human | 6 | 9 | 10 | | |
| *L. interrogans* | Canicola | Broomi | Patane | Australia | Human | 1 | 5 | 14 | | |
| *L. interrogans* | Canicola | Canicola | Hond Utrecht IV | The Netherlands | Dog | 1 | 10 | 3 | | |
| *L. interrogans* | Canicola | Jonsis | Jones | Malaysia | Human | 7 | 11 | 12 | | |
| *L. interrogans* | Canicola | Kuwait | 136/2/2 | Kuwait | Rat | 0 | 1 | 1 | | |
| *L. interrogans* | Canicola | Portlandvere | MY 1039 | Jamaica | Human | 1 | 10 | 3 | | |
| *L. interrogans* | Canicola | Schueffneri | Vleermuis 90C | Indonesia | Bat | 1 | 8 | 2 | | |
| *L. interrogans* | Canicola | Sumneri | Sumner | Malaysia | Human | 5 | 14 | 16 | | |
| *L. interrogans* | Djasiman | Djasiman | Djasiman | Indonesia | Human | 5 | 1 | 13 | | |
| *L. interrogans* | Djasiman | Gurungi | Gurung | Malaysia | Human | 1 | 10 | 7 | | |
| *L. interrogans* | Grippotyphosa | Grippotyphosa | Andaman | Andaman Islands | Human | 0 | 11 | 6 | | |
| *L. interrogans* | Grippotyphosa | Liangguang | 1880 | China | Rat | 0 | 0 | 1 | | |
| *L. interrogans* | Grippotyphosa | Muelleri | RM2 | Malaysia | Rat | 2 | 3 | 7 | | |
| *L. interrogans* | Grippotyphosa | Valbuzzi | Valbuzzi | Australia | Human | 2 | 1 | 1 | | |
| *L. interrogans* | Hebdomadis | Hebdomadis | Hebdomadis | Japan | Human | 1 | 4 | 5 | | |
| *L. interrogans* | Hebdomadis | Kremastos | Kremastos | Australia | Human | 23 | 0 | 2 | | |
| *L. interrogans* | Icterohaemorrhagiae | Birkini | Birkin | Brazil | Human | 4 | 8 | **16** | | |
| *L. interrogans* | Icterohaemorrhagiae | Copenhageni | Fiocruz L1-130 | Holland | Human | 2 | **1** | **7** | | **6** |
| *L. interrogans* | Icterohaemorrhagiae | Gem | Simon | Sri Lanka | Human | 1 | 6 | 9 | | |
| *L. interrogans* | Icterohaemorrhagiae | Icterohaemorrhagiae | RGA | Belgium | Human | 2 | **1** | 7 | | |
| *L. interrogans* | Icterohaemorrhagiae | Lai | Lai | China | Human | 5 | **3** | **2** | | **6** |
| *L. interrogans* | Icterohaemorrhagiae | Naam | Naam | Indonesia | Human | 8 | 7 | 7 | | |
| *L. interrogans* | Icterohaemorrhagiae | Smithi | Smith | Malaysia | Human | 6 | 2 | **10** | | |
| *L. interrogans* | Icterohaemorrhagiae | Tonkini | LT 96-68 | Vietnam | Human | **2** | 6 | **11** | **5** | **5** |
| *L. interrogans* | Louisiana | Lanka | R 740 | Sri Lanka | Human | 1 | 12 | 5 | | |
| *L. interrogans* | Pomona | Kennewicki | LT 1026 | United States | Bovine | 5 | **0** | 10 | | |
| *L. interrogans* | Pomona | Pomona | Pomona | Australia | Human | 2 | 0 | 14 | | |
| *L. interrogans* | Pyrogenes | Abramis | Abraham | Malaysia | Human | 6 | 4 | 11 | | |
| *L. interrogans* | Pyrogenes | Biggis | Biggs | Malaysia | Human | 5 | 0 | 11 | | |
| *L. interrogans* | Pyrogenes | Camlo | LT 64-67 | Vietnam | Human | 3 | 10 | 7 | | |
| *L. interrogans* | Pyrogenes | Guaratuba | An 7705 | Brazil | Opossum | 2 | 0 | 14 | | |
| *L. interrogans* | Pyrogenes | Manilae | LT 398 | Philippines | Rat | 0 | 3 | 8 | | |
| *L. interrogans* | Pyrogenes | Pyrogenes | Salinem | Indonesia | Human | 3 | 10 | 13 | | |
| *L. interrogans* | Pyrogenes | Robinsoni | Robinson | Australia | Human | 0 | 0 | 14 | | |
| *L. interrogans* | Ranarum | Evansi | 267-1348 | Malaysia | Water | 0 | 3 | 12 | | |
| *L. interrogans* | Sejroe | Geyaweera | Geyaweera | Sri Lanka | Human | 1 | 13 | 5 | | |
| *L. interrogans* | Sejroe | Haemolytica | Marsh | Malaysia | Human | 1 | 13 | 2 | | |
| *L. interrogans* | Sejroe | Hardjo | Hardjoprajitno | Indonesia | Human | 2 | 9 | **14** | | |
| *L. interrogans* | Sejroe | Jin | A81 | China | Human | 0 | 7 | **13** | | |
| *L. interrogans* | Sejroe | Ricardi | Richardson | Malaysia | Human | 5 | 3 | 11 | | |
| *L. interrogans* | Sejroe | Romanica | LM 294 | Romania | *Mus musculus* | 3 | 2 | 11 | | |
| *L. interrogans* | Sejroe | Wolffi | 3705 | Indonesia | Human | 3 | 2 | 11 | | |
| *L. kirschneri* | Australis | Ramisi | Musa | Kenya | Human | **1** | 5 | 4 | | |
| *L. kirschneri* | Autumnalis | Bim | 1051 | Barbados | Dog | 1 | **1** | 7 | | **5** |
| *L. kirschneri* | Autumnalis | Lambwe | Lambwe | Kenya | Rat | **0** | 1 | 8 | | |
| *L. kirschneri* | Bataviae | Djatzi | HS 26 | Puerto Rico | Human | 0 | 5 | 12 | | |
| *L. kirschneri* | Bataviae | Bafani | Bafani | Puerto Rico | Human | **2** | 2 | 9 | | |
| *L. kirschneri* | Canicola | Galtoni | LT 1014 | Argentina | Cow | 1 | 1 | 16 | | |
| *L. kirschneri* | Canicola | Kamituga | Kamituga | Zaire | Human | 1 | 4 | 4 | | |
| *L. kirschneri* | Cynopteri | Cynopteri | 3522C | Indonesia | Bat | 0 | **6** | 9 | | |
| *L. kirschneri* | Djasiman | Agogo | Agogo | Ghana | Human | 1 | 1 | 10 | | |
| *L. kirschneri* | Grippotyphosa | Grippotyphosa | Moskva V. | Russia | Human | **3** | 2 | 11 | | |
| *L. kirschneri* | Grippotyphosa | Ratnapura | Wumalasena | Sri Lanka | Human | 1 | **7** | 6 | | |
| *L. kirschneri* | Grippotyphosa | Valbuzzi | Duyster | ND[b] | ND | 0 | 2 | **9** | | |
| *L. kirschneri* | Grippotyphosa | Vanderhoedeni | Kipod 179 | Israel | Hedgehog | 2 | 2 | 12 | | |
| *L. kirschneri* | Hebdomadis | Kabura | Kabura | Zaire | Human | 1 | **5** | 10 | | |
| *L. kirschneri* | Hebdomadis | Kambale | Kambale | Zaire | Human | 0 | 4 | 16 | | |
| *L. kirschneri* | Icterohaemorrhagiae | Bogvere | LT 60-69 | Jamaica | Rat | 0 | 6 | 12 | | |
| *L. kirschneri* | Icterohaemorrhagiae | Dakota | Grand River | United States | Water | | 1 | 6 | | |

*Continued on facing page*

TABLE 2—*Continued*

| Species | Serogroup | Serovar | Strain | Country | Source | Result for locus[a]: VNTR-4 | VNTR-7 | VNTR-10 | VNTR-Lb4 | VNTR-Lb5 |
|---|---|---|---|---|---|---|---|---|---|---|
| *L. kirschneri* | Icterohaemorrhagiae | Mwogolo | Mwogolo | Zaire | Human | 0 | **1** | 6 | | |
| *L. kirschneri* | Icterohaemorrhagiae | Ndahambukuje | Ndahambukuje | Zaire | Human | 0 | 4 | | | |
| *L. kirschneri* | Icterohaemorrhagiae | Ndambari | Ndambari | Zaire | Human | 0 | 1 | 12 | | |
| *L. kirschneri* | Pomona | Kunming | K 5 | China | *Apodemus chevrieri* | 0 | **4** | 16 | | |
| *L. kirschneri* | Pomona | Mozdok | 5621 | Russia | Field vole | 0 | 1 | 4 | | |
| *L. kirschneri* | Pomona | Tsaratsovo | B 81/7 | Bulgaria | Harvest mouse | 0 | 1 | 4 | | |
| *L. borgpetersenii* | Ballum | Castellonis | Castellon3 | Spain | Wood mouse | 1 | | **1** | 4 | 6 |
| *L. borgpetersenii* | Celledoni | Anhoa | LT 90-68 | Vietnam | Human | 2 | | | 12 | 4 |
| *L. borgpetersenii* | Celledoni | Whitcombi | Whitcomb | Malaysia | Human | | | | 5 | 5 |
| *L. borgpetersenii* | Hebdomadis | Jules | Jules | Zaire | Human | **0** | | **1** | **4** | 6 |
| *L. borgpetersenii* | Hebdomadis | Nona | Nona | Zaire | Human | 1 | | 1 | 4 | 6 |
| *L. borgpetersenii* | Hebdomadis | Worsfoldi | Worsfold | Malaysia | Human | | | | 5 | 5 |
| *L. borgpetersenii* | Javanica | Ceylonica | Piyasena | Sri Lanka | Human | **2** | | 1 | 4 | **6** |
| *L. borgpetersenii* | Javanica | Dehong | De10 | China | *Suncus murinus* | 2 | | **1** | 4 | 6 |
| *L. borgpetersenii* | Javanica | Poi | Poi | Italy | Human | 2 | | 1 | 5 | 5 |
| *L. borgpetersenii* | Javanica | Yaan | 80-27 | China | *Crocidura* | 2 | | 1 | **2** | 6 |
| *L. borgpetersenii* | Mini | Mini | Sari | Italy | Human | | | 1 | 5 | 6 |
| *L. borgpetersenii* | Pyrogenes | Hamptoni | Hampton | Malaysia | Human | 2 | | | **6** | **5** |
| *L. borgpetersenii* | Sejroe | Hardjobovis | Sponselee | Holland | Bovine | 2 | | 1 | 5 | **4** |
| *L. borgpetersenii* | Sejroe | Istrica | Bratislava | Czechoslovakia | Wood mouse | 2 | | 1 | 3 | 5 |
| *L. borgpetersenii* | Sejroe | Sejroe | M84 | Denmark | Mouse | 2 | | **1** | 3 | 5 |
| *L. borgpetersenii* | Tarassovi | Gengma | M48 | China | Pig | 2 | | 1 | 5 | **5** |
| *L. borgpetersenii* | Tarassovi | Kanana | Kanana | Kenya | Gerbil | 2 | | 1 | 4 | 6 |
| *L. borgpetersenii* | Tarassovi | Tarassovi | perepelitsin | Russia | Human | | | **1** | 9 | 6 |
| *L. borgpetersenii* | Autumnalis | Srebana | 1409/69 | Bulgaria | *Sorex araneus* | 0 | | | | 2 |
| *L. borgpetersenii* | Bataviae | Moldaviae | 1114-2 | Russia | ND | 0 | | 1 | 10 | 6 |
| *L. borgpetersenii* | Pyrogenes | Kwale | Julu | Kenya | Human | 0 | | **1** | 7 | 6 |
| *L. borgpetersenii* | Sejroe | Balcanica | 1627 Burgas | Bulgaria | Human | | | 1 | **8** | 6 |
| *L. borgpetersenii* | Tarassovi | Guidae | RP29 | Brazil | Pig | | | 1 | **9** | 6 |

[a] If no value is given, no PCR product was obtained. Boldface type indicates that the PCR product was sequenced.
[b] ND, not determined.

*kirschneri* were successfully amplified. These new primers for VNTR-4 also allowed 17 of the 23 *L. borgpetersenii* strains belonging to different serovars to be amplified.

DNA sequencing of the amplified products showed that the nucleotide sequences of the repeats of VNTR-4, VNTR-7, and VNTR-10 are well conserved among *L. interrogans*, *L. kirschneri*, and *L. borgpetersenii* species (Table 3). However, the size of the regions flanking VNTR-4 and VNTR-7 is conserved among *Leptospira* species, whereas the regions flanking VNTR-10 are shorter in *L. kirschneri* and *L. borgpetersenii* than in *L. interrogans* (Table 1). Consequently, the patterns obtained for the VNTR-10 locus must be carefully interpreted for the correct number of repeats. MLVA revealed that genetic diversity among *L. interrogans* serovars was higher than among *L. kirschneri* serovars (Table 3). As the analyzed *L. kirschneri* and *L. interrogans* serovars represent 88% and 64%, respec-

TABLE 3. Characteristics of VNTR loci studied

| Locus | Species | Repeat consensus sequence | Diversity index[a] |
|---|---|---|---|
| VNTR-4 | *L. interrogans* | TCGAGCGCCCATAGAAGCGAGACGCTGAGTTACT | 0.83 |
| | *L. kirschneri* | TCGAGCGCCAATAGAAGCGAGACACTGAGTTACT | 0.61 |
| | *L. borgpetersenii* | TCGAGCGCCCATAGAAGCGAGACGCTGAGTTACT | ND |
| VNTR-7 | *L. interrogans* | GTGGGAACTCTCACAAATTGAGATTTTACAGATGAACTTTTCAAAT | 0.91 |
| | *L. kirschneri* | GTGGGAACTCTCACAAATCGAGGATTTTACAGATGAACTTTTCAAAT | 0.75 |
| | *L. borgpetersenii* | No PCR product | |
| VNTR-10 | *L. interrogans* | GTGGGAACTCACACAATTTGCGGGTTTTACAGTTAAATTTAGAAAT | 0.92 |
| | *L. kirschneri* | GTGGGAACTCACACAATTTCCGGGTTTGACAGTTGAACCTATAAT | 0.88 |
| | *L. borgpetersenii* | GTGGGAACCCACGCAATTTCCGGGTTTGACAGTTGAACTTAGAAAT | 0.31 |
| VNTR-Lb4 | *L. interrogans* | No PCR product | |
| | *L. kirschneri* | No PCR product | |
| | *L. borgpetersenii* | AGTTGGATTTGAATCGGAGATGATATTTTCTTTGTTTACGATTTGTG AATGTTCTTCCGG | 0.83 |
| VNTR-Lb5 | *L. interrogans* | TGATAACCGCCACCGCCTTGTCCAGGTCCACGATTTCCC | ND |
| | *L. kirschneri* | TGATAACCGCCGCCCTGTCCAGGACCACGATTGCCC | ND |
| | *L. borgpetersenii* | TGATAACCGCCACCTTGTCCGGGACCACGATTTCCT | 0.56 |

[a] Nei diversity index (25) calculated for 53 serovars of *L. interrogans*, 23 serovars of *L. kirschneri*, and 23 serovars of *L. borgpetersenii*. ND, not done (PCR product obtained, but pattern was monomorphic, so VNTR locus was not considered for further analysis).

tively, of the serovars described by Brenner et al. in 1999 (3), our finding should not be biased by our sample collection. The genetic diversity revealed by MLVA was much lower in *L. borgpetersenii*, with the 23 analyzed serovars representing 55% of the serovars described by Brenner et al. (3).

The combination of the VNTR-4, VNTR-7, and VNTR-10 loci was useful for typing *L. interrogans* (multilocus diversity index, 0.98) and *L. kirschneri* (multilocus diversity index, 0.95). We were unable to differentiate only four *L. interrogans* strain pairs and two *L. kirschneri* strain pairs using these three VNTRs. The two strains within each pair usually belong to the same serogroup (*L. interrogans* serovars Copenhageni and Icterohaemorrhagiae from serogroup Icterohaemorrhagiae, *L. interrogans* serovars Romanica and Wolffi from serogroup Sejroe, *L. interrogans* serovars Canicola and Portlandvere from serogroup Canicola, *L. kirschneri* serovars Tsaratsovo and Mozdok from serogroup Pomona). The strains within the two other pairs belong to different serogroups (*L. interrogans* serovar Pomona from serogroup Pomona and *L. interrogans* serovar Guaratuba from serogroup Pyrogenes, *L. kirschneri* serovar Kambale from serogroup Hebdomadis and *L. kirschneri* serovar Kunming from serogroup Pomona).

Primers for the VNTR-4 and VNTR-10 successfully amplified the 23 collection strains of *L. borgpetersenii*. However, VNTR-4 was poorly discriminatory and VNTR-10 was monomorphic; indeed, sequencing of the VNTR-10 amplicons revealed that the difference in amplicon size was due to variation in the length of the VNTR flanking regions rather than in the copy number of repeats (data not shown). Consequently, we needed to find *L. borgpetersenii* strain-specific VNTRs. The *L. borgpetersenii* serovar Hardjobovis strain L550 whole-genome sequence has been analyzed using Tandem Repeats Finder software (B. Adler, personal communication). As no VNTR was detected using the criteria used for the VNTR search in *L. interrogans* (19), we chose new criteria: repeat size, 15 to 140 bp; number of repeats, 2 to 12; sequence homology between repeats, >75%. Among the 13 VNTR regions matching these criteria, seven were selected because the PCR products were easily resolved on agarose gel. We tested the seven primer pairs on a subset of *L. borgpetersenii* strains, retaining two pairs (VNTR-Lb4 and VNTR-Lb5) for further analysis of *L. borgpetersenii*. The combination of the VNTR-10, VNTR-Lb4, and VNTR-Lb5 loci was useful for typing *L. borgpetersenii* strains (multilocus diversity index, 0.88). VNTR-Lb5 could also be amplified from *L. interrogans* and *L. kirschneri* but gave monomorphic patterns among *L. interrogans* (6 repeats) and *L. kirschneri* (5 repeats) strains. The VNTR-Lb5 locus is located in the coding region of *infB1*, a gene encoding a putative translation initiation factor.

**Validation of MLVA on clinical strains.** We tested the ability of the five VNTR loci selected (VNTR-4, VNTR-7, VNTR-10, VNTR-Lb4, and VNTR-Lb5) to type *Leptospira* strains on 34 clinical strains belonging to seven serogroups (Icterohaemorrhagiae, Canicola, Pomona, Grippotyphosa, Autumnalis, Sejroe, and Ballum) (Table 4). From the results obtained from the collection strains, the amplification of VNTR-7 and VNTR-10 suggested that 31 of the 34 clinical strains belonged to either *L. interrogans* or *L. kirschneri*. Similarly, the failure to amplify VNTR-19 suggested that the clinical strains of the serogroup Grippotyphosa belonged to *L. kirschneri*. Finally, the failure to amplify VNTR-7 together with the successful amplification of VNTR-Lb4 and VNTR-Lb5 suggested that the clinical strains from serogroups Sejroe and Ballum belonged to *L. borgpetersenii* (Table 4). We confirmed this result by 16S rRNA gene sequencing (29) or LFB1 real-time assay (23) (data not shown).

Among the five *L. kirschneri* strains from Guadeloupe (French West Indies islands) identified as *L. kirschneri* serovar Bogvere by PFGE, two genotypes were identified by MLVA, irrespective of the host from which they were isolated. Similarly, among the four *L. kirschneri* strains identified as *L. kirschneri* serovar Grippotyphosa by PFGE (Tables 2 and 4), only one strain had a multilocus VNTR pattern identical to the Moskva V type strain of serovar Grippotyphosa. Other strains of the serovar Grippotyphosa showed new VNTR patterns or patterns similar to those of the *L. kirschneri* serovars Valbuzzi and Vanderhoedeni. This finding suggests that a high heterogeneity exists among serovars identified as Grippotyphosa by PFGE.

Other clinical isolates (e.g., strains of the serogroup Grippotyphosa from Saint Petersburg and strains of the serogroup Pomona from Croatia) also showed VNTR patterns that were not recorded among the 99 studied collection strains from the 225 officially described serovars.

Clinical strains isolated from the same geographic area and belonging to the same serogroup shared a common VNTR pattern (e.g., strains of serogroups Icterohaemorrhagiae, Grippotyphosa, and Canicola from Russia and strains of serogroup Pomona from Croatia). Among the five strains belonging to the serogroup Canicola isolated in Russia, only one strain belonged to *L. interrogans* serovar Schueffneri; this strain was the only one isolated in Far Eastern Russia.

**Epidemiologic investigation of leptospirosis in New Caledonia.** We validated MLVA as a useful tool for typing *Leptospira* spp. by conducting a study in New Caledonia, a region of high endemicity. We subjected 156 *Leptospira* strains isolated between 1989 and 2001 to VNTR analysis using the VNTR-4, VNTR-7, and VNTR-10 markers. There are five serogroups in New Caledonia, and 66% of the strains belonged to the serogroup Icterohemorrhagiae (Table 5). Serogroup Sejroe was found only in animals (deer and swine), whereas serogroup Pomona was found in human and animals. The VNTR-4, VNTR-7, and VNTR-10 loci were successfully amplified from strains from serogroups Icterohaemorrhagiae, Pomona, and Pyrogenes, and the VNTR-4, VNTR-10, VNTR-Lb4, and VNTR-Lb5 loci were amplified from strains from serogroups Ballum and Sejroe, suggesting that they belong to *L. borgpetersenii* species. Only one serovar was found among strains belonging to serogroups Icterohaemorrhagiae, Pomona, Ballum, and Sejroe (Table 5). Among the nine human isolates belonging to serogroup Pyrogenes, two distinct genotypes were identified. Serovars could not be deduced from these two genotypes, as they were not present among the 99 analyzed collection strains, suggesting that serovars not yet identified may exist in New Caledonia.

## DISCUSSION

MLVA has proved a powerful tool for identifying *L. interrogans* serovars (19, 33). In this study, we extended this molec-

TABLE 4. Clinical strains used for validation of the MLVA method

| Serogroup[a] | Serovar[b] | Strain | Yr isolated | Location | Source | Result for locus: | | | | | Serovar deduced[c] | Species |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | VNTR-4 | VNTR-7 | VNTR-10 | VNTR-Lb4 | VNTR-Lb5 | | |
| Grippotyphosa | ND[d] | SP-1 | 2003 | Saint Petersburg, Russia | Human | 1 | 6 | 8 | ND | ND | Unidentified serovar | *L. kirschneri* |
| Grippotyphosa | ND | SP-2 | 1988 | Saint Petersburg, Russia | Human | 1 | 6 | 8 | ND | ND | Unidentified serovar | *L. kirschneri* |
| Grippotyphosa | ND | SP-3 | 1981 | Northwest Russia | Human | 1 | 6 | 8 | ND | ND | Unidentified serovar | *L. kirschneri* |
| Grippotyphosa | ND | SP-4 | 1972 | Northwest Russia | Human | 1 | 6 | 8 | ND | ND | Unidentified serovar | *L. kirschneri* |
| Grippotyphosa | ND | SP-5 | 1959 | Northwest Russia | Human | 1 | 6 | 8 | ND | ND | Unidentified serovar | *L. kirschneri* |
| Icterohaemorrhagiae | ND | SP-6 | 2004 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Icterohaemorrhagiae | ND | SP-7 | 1999 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Icterohaemorrhagiae | ND | SP-8 | 1994 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Icterohaemorrhagiae | ND | SP-9 | 1994 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Icterohaemorrhagiae | ND | SP-10 | 1994 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Icterohaemorrhagiae | ND | SP-11 | 1992 | Saint Petersburg, Russia | Human | 2 | 1 | 7 | ND | ND | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Canicola | ND | SP-12 | 2000 | Saint Petersburg, Russia | Human | 1 | 10 | 3 | ND | ND | Canicola or Portlandvere | *L. interrogans* |
| Canicola | ND | SP-13 | 2000 | Saint Petersburg, Russia | Human | 1 | 10 | 3 | ND | ND | Canicola or Portlandvere | *L. interrogans* |
| Canicola | ND | SP-14 | 1939 | Far Eastern Russia | Human | 1 | 8 | 2 | ND | ND | Shueffneri | *L. interrogans* |
| Canicola | ND | SP-15 | 2000 | Saint Petersburg, Russia | Human | 1 | 10 | 3 | ND | ND | Canicola or Portlandvere | *L. interrogans* |
| Canicola | ND | SP-16 | 1992 | Saint Petersburg, Russia | Human | 1 | 10 | 3 | ND | ND | Canicola or Portlandvere | *L. interrogans* |
| Pomona | ND | 318 | 1950 | Croatia | Human | NA[e] | 1 | 10 | ND | ND | Unidentified serovar | *L. interrogans* |
| Pomona | ND | 327 | 1977 | Croatia | Cat | 3 | 1 | 10 | ND | ND | Unidentified serovar | *L. interrogans* |
| Pomona | ND | 328 | 1975 | Croatia | Hare | NA | 1 | 10 | ND | ND | Unidentified serovar | *L. interrogans* |
| Pomona | ND | 329 | 1974 | Croatia | Calf | NA | 1 | 10 | ND | ND | Unidentified serovar | *L. interrogans* |
| Grippotyphosa | Grippotyphosa | 2 002 297 | 2002 | Croatia | Human | NA | 2 | 12 | ND | ND | Vanderhoedeni | *L. kirschneri* |
| Grippotyphosa | Grippotyphosa | 2 002 306 | 2002 | Croatia | Human | NA | 2 | 11 | ND | ND | Grippotyphosa | *L. kirschneri* |
| Sejroe | Istrica | M18 | 2000 | Croatia | *Mus musculus* | 2 | NA | 1 | 3 | 5 | Sejroe or Istrica | *L. borgpetersenii* |
| Sejroe | Istrica | M1 | 2000 | Croatia | *Mus musculus* | NA | NA | 1 | 3 | 5 | Sejroe or Istrica | *L. borgpetersenii* |
| Icterohaemorrhagiae | Bogvere | 2003-09-419 | 2003 | Guadeloupe | Rat | 0 | 6 | 12 | ND | ND | Bogvere | *L. kirschneri* |
| Icterohaemorrhagiae | Bogvere | 2003-09-420 | 2003 | Guadeloupe | Rat | 0 | 6 | 14 | ND | ND | Bogvere (other genotype) | *L. kirschneri* |
| Icterohaemorrhagiae | Bogvere | 2004-02-144 | 2004 | Guadeloupe | Human | 0 | 6 | 14 | ND | ND | Bogvere (other genotype) | *L. kirschneri* |
| Icterohaemorrhagiae | Bogvere | 2003-05-347 | 2003 | Guadeloupe | Rat | 0 | 6 | 12 | ND | ND | Bogvere | *L. kirschneri* |
| Icterohaemorrhagiae | Bogvere | 2004-11-277 | 2004 | Guadeloupe | Human | 0 | 6 | 12 | ND | ND | Bogvere | *L. kirschneri* |
| Icterohaemorrhagiae | ND | R4 | 2000 | Guadeloupe | Rat | 2 | 1 | 7 | ND | ND | Copenhageni or Ictero | *L. interrogans* |
| Ballum | ND | 2002 10 110 | 2002 | Guadeloupe | Rat | 1 | NA | 1 | 4 | 6 | 6 possible serovars | *L. borgpetersenii* |
| Autumnalis | Bim | 971 | 1990's | Barbados | Human | 1 | 1 | 7 | ND | ND | Bim | *L. kirschneri* |
| Grippotyphosa | Grippotyphosa | 2000 11 449 | 2000 | France | Human | NA | 2 | 9 | ND | ND | Valbuzzi | *L. kirschneri* |
| Grippotyphosa | Grippotyphosa | Marigot | 1967 | West Africa | Human | NA | 2 | 5 | ND | ND | Unidentified serovar | *L. kirschneri* |

[a] Serogroup identification was performed by MAT.
[b] Serovar identification was performed by PFGE using NotI restriction enzyme.
[c] Serovar deduced from VNTR analysis; serovars which have been confirmed by PFGE are underlined.
[d] ND, not determined.
[e] NA, no PCR product was obtained.
[f] Unidentified serovar means the VNTR pattern was not encountered among the 99 serovars of the collection strains tested.

TABLE 5. Results of MLVA performed with *Leptospira* strains isolated in New Caledonia (1989 to 2001)

| Serogroup[a] | Host (no. of isolates) | Result for locus: | | | | | % of isolates | Serovar[b] | Species |
|---|---|---|---|---|---|---|---|---|---|
| | | VNTR-4 | VNTR-7 | VNTR-10 | VNTR-Lb4 | VNTR-Lb5 | | | |
| Icterohaemorrhagiae | Human (104), dog (1) | 2 | 1 | 7 | ND[c] | ND | 67 | Copenhageni or Icterohaemorrhagiae | *L. interrogans* |
| Pomona | Human (21), swine (6), deer (3) | 2 | 0 | 13 | ND | ND | 19 | Pomona | *L. interrogans* |
| Pyrogenes | Human (8) | 2 | 6 | 11 | ND | ND | 5 | Unidentified serovar | *L. interrogans* |
| Pyrogenes | Human (1) | 1 | 6 | 11 | ND | ND | 1 | Unidentified serovar | *L. interrogans* |
| Ballum | Human (9) | 2 | NA[d] | 1 | 4 | 6 | 6 | Castellonis | *L. borgpetersenii* |
| Sejroe | Swine (1), deer (2) | 1 | NA | 0 | 5 | 4 | 2 | Hardjobovis | *L. borgpetersenii* |

[a] Serogroup identified by MAT.
[b] Serovar deduced from VNTR analysis.
[c] ND, PCR not done.
[d] NA, PCR done but no PCR product obtained.

ular approach to other pathogenic species of *Leptospira* by defining new primer pairs and selecting new VNTR loci. As a first step, we suggest using VNTR-4, VNTR-7, and VNTR-10 for discriminating between the serovars of *Leptospira* strains. This first screen discriminates about 92% and 90% of *L. interrogans* and *L. kirschneri* serovars, respectively. As a second step, for strains in which only VNTR-10 is amplified, we propose further analysis with primer pairs for VNTR-Lb4 and VNTR-Lb5 markers. This second screen discriminates about 60% of *L. borgpetersenii* serovars.

The redefinition of the primer pairs for amplifying VNTR-4, VNTR-7, and VNTR-10 allowed us to type *L. interrogans* and *L. kirschneri* strains. This revealed that these two species are closely related, as suggested by DNA-DNA hybridization analysis and 16S rRNA gene sequencing (30) and by the recent description of *L. kirschneri* as a genomospecies. It also suggests that gene order is conserved among these two species, at least for the genes flanking these VNTR loci.

VNTR-10 was the only VNTR marker that could be used to identify *L. borgpetersenii*. VNTR-10 is located within the *rfb* locus, which encodes proteins involved in lipopolysaccharide O-antigen biosynthesis. The *rfb* locus has a different genetic organization in *L. interrogans* and *L. borgpetersenii* serovars (5, 6, 13). However, the gene organization around VNTR-10 seems to be conserved, irrespective of the serovar and the species considered.

Although searching for tandem repeats in the *L. borgpetersenii* genome revealed many fewer VNTRs than in the *L. interrogans* genomes, we identified two loci (VNTR-Lb4 and VNTR-Lb5) as useful markers.

MLVA of the *Leptospira noguchii* serovar Panama strain CZ214K shows that this strain harbors degenerate repeats at several VNTR loci (data not shown). The analysis of the whole genome of the saprophyte *Leptospira biflexa* strain Patoc I (M. Picardeau, C. Bouchier, C. Boursaux-Eude, and C. Médigue, unpublished data) using Tandem Repeats Finder showed that this strain does not have obvious VNTR loci, except within the *infB1* gene (corresponding to the locus VNTR-Lb5). Variations in the number of repeats at the VNTR-Lb5 locus does not change the coding sequence frame of *infB1*, irrespective of the species considered: *L. interrogans* (39-bp-long repeats), *L. kirschneri* (39-bp-long repeats), *L. borgpetersenii* (36-bp-long repeats), *L. noguchii* (two 36-bp-long repeats in strain CZ214K), or *L. biflexa* (six 42-bp-long repeats in strain Patoc I) (data not shown).

The analysis of *Leptospira* collection strains showed that unilocus genetic diversity is higher among serovars in *L. interrogans* than in *L. kirschneri* and *L. borgpetersenii* species. As our strain collection was isolated from different hosts and from different geographic origins, this should not be a biased finding. We determined the validity of the VNTR loci for *Leptospira* spp. typing by studying clinical strains isolated from different hosts and from different geographic origins (Table 4). The selected VNTR loci were useful for typing *L. interrogans* and *L. kirschneri* strains. However, we should test more strains and, if possible, more VNTR loci to validate the usefulness of MLVA for typing *L. borgpetersenii*.

MLVA also showed that there is a genetic diversity within a few serovars and that the serovars of the collection strains underestimate the variety of isolates in natural populations. For example, the clinical strains identified by PFGE as belonging to serovar Grippotyphosa showed different VNTR patterns, suggesting that these strains are genetically heterogeneous. However, the serovar determination of these strains was by PFGE and not by a serological method. PFGE is useful for characterizing leptospiral serovars (9), but discrepancies between PFGE and serological methods have also been shown for serovars belonging to serogroups Grippotyphosa, Pomona, and Pyrogenes (11). Strains of the serovar Bogvere isolated in Guadeloupe show two slightly different patterns, one of which is similar to that seen for the collection strain LT60-69. These strains are found either in humans or in rats. In contrast, we were unable to determine the serovars for *L. interrogans* strains of the serogroup Pomona by comparing the VNTR patterns for collection strains. Analysis of clinical strains belonging to the same serogroup also showed that only one serovar is found among strains isolated from the same geographic area. The strain isolated in Far Eastern Russia in 1939 is interesting because the strains of serovar Schueffneri (serogroup Canicola) have been described in Japan and Indonesia (20). This is consistent with the east Asian origin of this strain belonging to the serovar Schueffneri. This also reflects the high stability of the serovars and that few changes are observed over time and geographical distribution. This was also observed among *L. interrogans* strains analyzed by PFGE from French overseas territories (10).

We confirmed MLVA as a powerful method for epidemiological purposes through a retrospective study in New Caledonia, a region of high leptospirosis incidence located in the South Pacific (22) (e.g., in 1999, the incidence of leptospirosis in New Caledonia was 122 cases per 100,000 inhabitants). Leptospirosis is endemic in the South Pacific and is a veterinary health problem in Australia (38) and New Zealand (35). It has also been reported in Pacific archipelagos such as Fiji (4) and Vanuatu (27). Our study showed that MLVA can be easily used for the large-scale typing of clinical isolates. It also showed that, as seen throughout the world, the most encountered serogroup is Icterohaemorrhagiae, confirming that rats (*Rattus rattus*, *Rattus exulans*, and more rarely, *Rattus norvegicus*) are the main reservoirs of leptospirosis in New Caledonia. The occurrence of serogroups Ballum and Pomona in human isolates indicates that, as well as rodents, cattle-breeding and swine-breeding activities are important risk factors (2). For each serogroup, we found only one serovar. The only exception was one strain of the serogroup Pyrogenes, which had a slightly different VNTR pattern and may be a different genotype rather than a different serovar. The VNTR pattern of strains belonging to the serogroup Pyrogenes is not found among collection strains, suggesting that a new Pyrogenes serovar may be present in New Caledonia. Surprisingly, the *L. interrogans* serovar Australis is present in Australia, being one of the main serovars, and in French Polynesia and other South Pacific archipelagos, whereas it is rare in New Caledonia.

In conclusion, MLVA is useful for epidemiologic investigations of leptospirosis in regions of high endemicity. MLVA provides a suitable tool for identifying circulating genotypes of slow-growing bacteria such as *Leptospira* spp. Further improvements need to be made to the method so that MLVA can be applied directly to biological (serum, blood, or urine of human and animals) and environmental samples, thus avoiding culturing of the pathogen. This would allow epidemiological studies in developing countries where it is not always possible to culture *Leptospira* spp., allowing a better surveillance of this underestimated disease.

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