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High-Resolution Typing of *Leptospira interrogans* Strains by Multispacer Sequence Typing

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Leptospirosis is a worldwide zoonosis which is responsible for the typical form of Weil's disease. The epidemiological surveillance of the *Leptospira* species agent is important for host prevalence control. Although the genotyping methods have progressed, the identification of some serovars remains ambiguous. We investigated the multispacer sequence typing (MST) method for genotyping strains belonging to the species *Leptospira interrogans*, which is the main agent of leptospirosis worldwide. A total of 33 DNA samples isolated from the reference strains of *L. interrogans* serogroups Icterohaemorrhagiae, Australis, Canicola, and Grippotyphosa, which are the most prevalent serogroups in France, were analyzed by both the variable-number tandem-repeat (VNTR) and MST methods. An MST database has been constructed from the DNA of these reference strains to define the MST profiles. The MST profiles corroborated with the VNTR results. Moreover, the MST analysis allowed the identification at the serovar level or potentially to the isolate level for strains belonging to *L. interrogans* serovar Icterohaemorrhagiae, which then results in a higher resolution than VNTR (Hunter-Gaston index of 0.94 versus 0.68). Regarding *L. interrogans* serogroups Australis, Canicola, and Grippotyphosa, the MST and VNTR methods similarly identified the genotype. The MST method enabled the acquisition of simple and robust results that were based on the nucleotide sequences. The MST identified clinical isolates in correlation with the reference serovar profiles, thus permitting an epidemiological surveillance of circulating *L. interrogans* strains, especially for the Icterohaemorrhagiae serogroup, which includes the most prevalent strains of public health interest.

Leptospirosis is a worldwide zoonosis with high mortality and morbidity rates in humans (1). The current reports estimate that more than 500,000 severe cases occur annually throughout the world (2). Recent outbreaks have increased with the rainfall (2, 3) and the urbanization associated with the conditions of slum living (2, 4, 5). In humans, the infection ranges from a mild form to a severe infection with fever, jaundice, and renal failure (Weil's disease), which leads to death in 5 to 10% of all patients with leptospirosis (4, 6, 7). In animals, leptospirosis can cause reproductive failure, with abortion and infertility in cattle and acute febrile illness with renal and hepatic failure in dogs (8).

Leptospirosis is caused by spirochetes, which belong to the family *Leptospiraceae*. Actually, two classifications of *Leptospira* spp. coexist, with one based on serological analyses and the other on genomic classification. The serologic classification of *Leptospira* spp. is based on the expression of the lipopolysaccharide (LPS) antigens and has been used to define more than 300 serovars (1). The DNA-DNA hybridization data have been used to classify the genus *Leptospira* into 21 genomospecies, including saprophytic, intermediate, and pathogenic species (1, 7, 9, 10).

The identification of a *Leptospira* species isolate has important implications for both diagnosis and epidemiology. During the serological diagnosis, the identification permits the establishment of the serogroup responsible for the infection. Moreover, the serogroup identification enables the determination of the animal reservoir and the prevalence of serogroup according to geographic regions (4), which can then be used to plan updates to the vaccine components (11, 12).

The serological reference test, which is the microscopic agglutination test (MAT), is a specific and complex procedure performed by reference laboratories. This method is a serogroup-specific test rather than a serovar-specific test, because cross-reactions between different serovars from the same serogroup can occur (8, 13), which means that the serovar identification is uncertain, thereby limiting its power as an epidemiological tool.

Several genotyping methods have been developed to tentatively identify the isolates to the serovar level. Pulsed-field gel electrophoresis (PFGE) (14, 15) is a standardized method that can identify 90% of the pathogenic *Leptospira* species serovars (15, 16). Moreover, the discrimination of certain pathogenic serovars, such as Pomona, Icterohaemorrhagiae, and Copenhageni, is still difficult by PFGE (14, 17). Multilocus variable-number tandem-repeat (VNTR) analysis (18) was part of the routine analysis for the diagnosis of *Leptospira* species genotypes. This method has been standardized and requires only simple PCR equipment. The VNTR analysis enables the serovar identification of causal agents that belong to the *Leptospira interrogans*, *Leptospira kirschneri*, and *Leptospira borgpetersenii* species. However, its resolution is dependent on the agarose component of the electrophoresis gel, and the analysis of nearby fragment sizes can occasionally be ambiguous (19). Moreover, the differentiation of certain serovars, such as between *L. interrogans* serovars Icterohaemorrhagiae and Copen-

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TABLE 1 MST primers used for the PCR amplification and sequencing of *L. interrogans* isolates

Spacer	ORF upstream (F) and downstream (R) of spacer sequence[a]	Position and nucleotide sequence (5′→3′)	PCR product size (bp)[a]
MST1 F	Hypothetical protein	210612 ACG GGA GAA TCT GTC AAA GG	207–263
MST1 R	Hypothetical protein	210837 AAA TTC ACT GAT TAC CTC CAC CA	
MST3 F	4-Diphosphocytidil-2-*C*-methyl-D-erythriol kinase	3792625 ACG ATC GTG CCA ATG TTT TT	361–375
MST3 R	Transcriptional regulator	3793059 AAA ATT CAA ACC GGC AAA TG	
MST4 F	Hypothetical protein	3656262 GGT TTA TTT GAA ATC ATG AAA ACT TCT	323–328
MST4 R	Hypothetical protein	3656586 TAT CTT TTA TTC AAT CTT GTG AAT CC	
MST7 F	Inhibitor of MCP methylation	2448482 AAA AGG CCT TCT TGG GTT TA	392–393
MST7 R	Hypothetical protein	2448875 GAT CAA CGG TCC GTA AAA CG	
MST9 F	Bifunctional 3,4-dihydroxy-2-butanone 4-phosphate	1155922 TTT GAA AAA CGA CTG AGG TCA A	236–237
MST9 R	Hypothetical protein	1156167 TCG ATA TCG GCA CAA AAT CA	

[a] ORF, open reading frame; F, forward; R, reverse. Based on the *L. interrogans* serovar Lai strain Lai 56601 genome sequence reference (GenBank accession no. AE010300.2).

hageni or between *L. interrogans* serovars Bratislava and Australis, remains difficult (20). Recently, a genotyping method based on the partial sequences of some housekeeping genes, known as multilocus sequence typing (MLST), has been developed for a phylogenetic analysis of *Leptospira* species isolates (21, 22). MLST produced nonambiguous, specific results with a good resolution. However, MLST demonstrated an inability to distinguish certain isolates at the serovar level, such as for Icterohaemorrhagiae and Copenhageni (23).

Recently, multispacer sequence typing (MST) has emerged as a new genotyping method, which is based on the comparison of the nucleotide sequences of several intergenic regions. The spacers seem to be more variable than coding genes, because they were less submitted to selection pressure (24). MST has been developed to genotype several pathogens, including *Rickettsia conorii* (24), *Bartonella quintana* (25), and *Mycobacterium tuberculosis* (26). MST produced reproducible and robust results with a high discriminatory power compared with that of MLST (24). Moreover, MST permitted the phylogeographical lineages to be defined (26), which is of great interest in epidemiologic studies. In this study, we investigated the MST for *L. interrogans* species genotyping, particularly for the Icterohaemorrhagiae, Canicola, Australis, and Grippotyphosa serogroups, which are the most prevalent serogroups in France (12, 27–30). The results of MST genotyping have been subsequently compared with the results of the VNTR analysis used as the reference molecular typing method.

MATERIALS AND METHODS

Selection of MST spacers. The complete genome sequences of *L. interrogans* serovar Lai strain Lai 56601 (GenBank accession no. AE010300.2) and *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 (GenBank accession no. AE016823.1) were investigated by the EMBOSS software (http://www.emboss.sourceforge.net). The intergenic spacers were further extracted from the reference genomes using Script software. The homologous spacer comparisons were analyzed by the Difseq software in EMBOSS. Then, an NCBI BLAST of these homologous spacers was performed to detect the differences between these spacer sequences. The spacers were selected using the following criteria: (i) sequence length of ≤400 bp, (ii) software script-filtered range of sequence similarity of 70 to 99% between *L. interrogans* genomes, and (iii) a difference between *L. interrogans* serovar Lai and Copenhageni sequences of ≥5 bp. The spacer homology between the *L. interrogans* genomes was further ensured by the presence of the same homologous genes upstream and downstream of the spacers. A dot plot was constructed for each spacer to visualize the type of genetic events that were responsible for the spacer sequence heterogeneity, i.e., insertion, deletion, or mutation. The PCR primers for the spacers were designed using the Primer3 software program (Infobiogen, Evry, France) and are indicated in Table 1.

***Leptospira* sp. strains.** The development of MST for *L. interrogans* species was conducted by the amplification and sequencing of spacers using DNA samples from 33 *Leptospira* sp. reference strains provided by the National Reference Center and WHO Collaboration Center for Leptospirosis (Institut Pasteur, Paris, France) and the Laboratoire des Leptospires. These strains, which are listed in Table 2 , belonged to four serogroups (*L. interrogans* serogroups Icterohaemorrhagiae, Australis, Canicola, and Grippotyphosa) that were chosen for their high prevalence in France (27).

The DNA was also extracted from 35 clinical isolates, including 25 isolates from human patients with leptospirosis (isolated by the University Hospitals of Pointe à Pitre in Guadeloupe and Fort de France in Martinique and the National Reference Center of Leptospirosis as part of the national surveillance of leptospirosis) and 10 isolates that were isolated from rats (*Rattus norvegicus*) trapped in five areas inside or in the suburb of the town of Lyon (France) in 2012. These captures were facilitated by collaboration with several pest control operations. Captures and euthanasia of rats were performed in accordance with the European Union legislation (directive 86/609/EEC) and conducted by two of us, authorized to perform the rodent experiments (authorization no. 69-080931 by the "Préfecture du Rhône"). Rats are considered invasive mammals in France, and rat samples are material for which no ethical committee approval has been required. Beforehand, all specimens yielded a PCR-positive result with a cycle threshold (C_T) inferior to 31 cycles using a specific pathogenic *Leptospira* species TaqMan real-time PCR kit (TaqVet PathoLept, LSI, Lissieu, France).

VNTR analysis. The *Leptospira* sp. reference strains and clinical specimens were genotyped using the VNTR method (18). The PCRs were performed in a final volume of 50 μl containing the following: 35 μl of H_2O, 5 μl of 10× buffer (Qiagen, Courtaboeuf, France), 2 μl of 25 mM $MgCl_2$, 1 μl of deoxynucleoside triphosphates (dNTPs), 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 1.5 U of HotStarTaq DNA polymerase (Qiagen), and 12 ng of target DNA. The samples were amplified as previously described (18). After a 4-hour 1.8% agarose MS-8 gel electrophoresis (Conda Pronadisa, Madrid, Spain), the amplified products were analyzed, and the sizes of the amplicons were defined by comparison with a 100-bp DNA ladder (Promega France, Lyon, France), with the size of the reference strain used as a control (18).

MST analysis. The MST PCRs were performed in a final volume of 50 μl containing 35 μl of H_2O, 5 μl of 10× buffer (Qiagen), 2 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs (Qiagen), 1 μl of forward primer (10 μM), 1 μl of reverse primer (10 μM), 1.5 U of HotStarTaq DNA polymerase (Qiagen), and 12 ng of target DNA. The PCRs were performed according to the following program: 15 min of enzyme activation at 95°C, followed by 40 cycles consisting of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min and a 10-min final elongation step at 72°C. After controlling the size of the

TABLE 2 MST analysis of 33 *L. interrogans* reference strains

L. interrogans serogroup	*L. interrogans* serovar	Strain	Origin	No. of genotypes by MST typing/profile		
				MST1	MST3	MST9
Icterohaemorrhagiae	Birkini	Birkini	Malaysia	3	1	1
Icterohaemorrhagiae	Mwogolo	Korea	Korea	3	1	1
Icterohaemorrhagiae	Lai	Lai 56601	China	2	5	2
Icterohaemorrhagiae	Copenhageni	M20	Denmark	4	6	3
Icterohaemorrhagiae	Mankarso	Mankarso	Indonesia	3	3	4
Icterohaemorrhagiae	Icterohaemorrhagiae	CHU Réunion	La Réunion (France)	4	10	3
Icterohaemorrhagiae	Naam	Naam	Indonesia	3	2	1
Icterohaemorrhagiae	Icterohaemorrhagiae	R1		4	7	3
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	Belgium	4	8	3
Icterohaemorrhagiae	Copenhageni	Shibaura 9	Japan	4	9	3
Icterohaemorrhagiae	Smithi	Smith	Malaysia	4	4	5
Icterohaemorrhagiae	Icterohaemorrhagiae	Verdun	France	4	8	3
Icterohaemorrhagiae	Copenhageni	Wijinberg	The Netherlands	4	6	3
Icterohaemorrhagiae	Copenhageni	Fiocruz L1-130	Brazil	4	8	3
Australis	Muenchen	Munchen C90	Germany	5	11	6
Australis	Jalna	Jalna	Czechoslovakia	5	11	6
Australis	Fugis	Fudge	Malaysia	3	4	5
Australis	Bratislava	Jez-Bratislava	Czechoslovakia	6	3	3
Australis	Bangkok	Bangkok-D92	Thailand	1	2	3
Australis	Australis	Ballico	Australia	6	3	3
Canicola	Benjamini	Benjamin	Indonesia	9	2	11
Canicola	Bindjei	Bindjei	Indonesia	14	4	1
Canicola	Broomi	Patane	Australia	7	3	7
Canicola	Canicola	Hond Utrecht IV	The Netherlands	8	12	3
Canicola	Jonsis	Jones	Malaysia	9	14	2
Canicola	Kuwait	136/2/2	Kuwait	11	13	8
Canicola	Portlandvere	MY 1039	Jamaica	8	12	7
Canicola	Schueffneri	Vleermuis 90C	Indonesia	9	14	2
Canicola	Sumneri	Sumner	Malaysia	12	15	1
Grippotyphosa	Grippotyphosa	Andaman	Andaman Islands	8	2	3
Grippotyphosa	Liangguang	1880	China	10	16	9
Grippotyphosa	Muelleri	RM 2	Malaysia	5	4	3
Grippotyphosa	Valbuzzi	Valbuzzi	Australia	13	17	10

amplified fragments on 1.5% agarose gel electrophoresis (Electran agarose DNA pure grade; VWR, Fontenay sous Bois, France), the PCR products were sequenced with the BigDye Terminator sequencing kit using the 3730XL DNA analyzer (Applied Biosystems, Saint Aubin, France). The sequences were edited using the ChromasPro program and aligned using NPS Multalin multiple alignment (http://npsa-pbil.ibcp.fr). Direct visual examination of the edited alignments was also conducted to reduce the risk of alignment errors.

Reproducibility and discriminatory power. The stability of MST typing was evaluated on two serial cultures of *L. interrogans* reference isolates and on clinical isolates. The discrimination power of MST was evaluated and compared with that of the VNTR method using the Hunter-Gaston index, which was estimated as

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{s} n_j (n_j - 1)$$

where N is the total number of isolates in the sample population, S is the total number of types described, and n_j is the number of isolates belonging to the jth type (31).

Nucleotide sequence accession numbers. All of the spacer sequences obtained were deposited in GenBank under accession numbers KF420439 to KF420480.

RESULTS

Selection of MST spacers. A total of 200 spacers were edited by the comparison of the two *L. interrogans* genome sequences according to the previous criteria, from which 16 spacers were selected.

Five spacers, MST1, MST3, MST4, MST7, and MST9, were then chosen based on the high number of genotypes identified *in silico*. The localization of these spacers was determined, and the primers were designed (Table 1).

First, the five spacers were tested on the DNA samples from 14 unrelated strains of *L. interrogans* serogroup Icterohaemorrhagiae. The nucleotide sequences obtained were analyzed and used to define several genotypes. The MST1, MST3, and MST9 spacers were more discriminating, resulting in three, 10, and five genotypes, respectively. A database was constructed based on the sequences derived from the selected spacer.

MST database. In the present study, the MST analysis focused on four serogroups that were previously analyzed by the VNTR method: *L. interrogans* serogroups Icterohaemorrhagiae, Australis, Canicola, and Grippotyphosa (18). A total of 33 reference strains were analyzed by MST, including 14 strains belonging to *L. interrogans* serogroup Icterohaemorrhagiae, six strains of serogroup Australis, nine strains of serogroup Canicola, and four strains of serogroup Grippotyphosa (Table 2).

Among the 33 reference strains, the genetic events observed in these spacers were mutations, insertions, and deletions, which were used to identify several genotypes for each spacer. For the MST1 spacer, 14 genotypes were defined, with sizes varying between 207 and 263 bp. The MST1 spacer presented 25 variable

regions, with 18 regions (94.7%) being single nucleotide mutations and one region (5.3%) being an insertion. Moreover, six regions were considered to be multivariable regions, containing one multiple nucleotide insertion region, one single nucleotide mutation, three deletion regions, and two regions containing a single nucleotide sequence insertion. For the MST3 spacer, 17 genotypes were defined based on 14 variable regions. The MST3 sequence size varied between 361 and 375 bp. A total of 12 regions (85.7%) were single nucleotide mutations, one region (7.1%) was a deletion, and one region (7.1%) was a nucleotide sequence insertion. This last region was located between the positions of 215 and 227 and comprised a poly(T) nucleotide sequence. The number of T nucleotides varied from 9 to 13 and allowed *L. interrogans* serogroup Icterohaemorrhagiae to be differentiated into five genotypes. For the MST9 spacer, 11 genotypes were defined. The MST9 sequence size varied between 236 and 237 bp. A total of 11 variable regions were identified, with 10 (90.9%) being single nucleotide mutations and one (9.1%) being an insertion.

The genotypes of these three spacers were combined to define the MST profile for each serovar. The MST genotype sequences were also checked to confirm that they were not coding regions and proved that the sequences targeted were effectively in spacer regions.

Comparison of MST typing with the VNTR reference method. The *Leptospira* sp. reference strains were analyzed by both the VNTR and MST methods.

As previously analyzed by Salaun et al. (18), the 33 *Leptospira* sp. strains analyzed in this study provided a unique VNTR profile, except for *L. interrogans* serovars Canicola and Portlandvere, which exhibited the same VNTR profile. Similarly, the strains belonging to the Copenhageni and Icterohaemorrhagiae serovars were not differentiated by the VNTR analysis.

A total of 24 VNTR locus combinations were defined among the 33 reference strains (Table 2), whereas 26 MST locus combinations were defined. MST enabled identification not only at the serogroup and serovar levels but also at the strain level in some cases.

In *L. interrogans* serogroup Icterohaemorrhagiae, including the eight serovars studied, MST permitted the identification of each serovar, except for *L. interrogans* serovars Birkini and Mwogolo. Moreover, for each serovar, a unique profile was defined for each strain, except for the strains M20 and Wijnberg, belonging to *L. interrogans* serovar Copenhageni, and the strains RGA and Verdun, belonging to *L. interrogans* serovar Icterohaemorrhagiae. In comparison, the VNTR analysis exhibited the same profile for the strains belonging to either *L. interrogans* serovar Icterohaemorrhagiae or Copenhageni. The index of discrimination of MST and VNTR methods was calculated among serovars of *L. interrogans* serogroup Icterohaemorrhagiae and found to be 0.94 and 0.68, respectively.

In *L. interrogans* serogroup Canicola, including the nine serovars analyzed, MST identified each serovar by a unique profile, except for the Jonsis and Schueffneri serovars, which exhibited the same MST combination, whereas their VNTR profiles were different.

In *L. interrogans* serogroup Australis, including six serovars, the Muenchen and Jalna serovars exhibited the same MST profile, as did the Bratislava and Australis serovars. In contrast, each serovar had a unique VNTR profile.

Finally, in *L. interrogans* serogroup Grippotyphosa, the four serovars each had a unique profile by both the MST and VNTR methods.

TABLE 3 Discrimination power of MST compared with that of the VNTR method

Typing method	No. of clusters	Size (%) of largest type	No. of unique isolates	Hunter-Gaston index
MST	26	9.1	20	0.985
VNTR	24	24.2	21	0.943

The reproducibility and repeatability of the MST method were evaluated on two serial cultures of four strains of *L. interrogans* serogroups Icterohaemorrhagiae, Australis, and Canicola and on clinical isolates; the spacer sequences obtained were identical.

The discriminatory power of MST was evaluated and compared with that of the VNTR method based on the results of the present study. MST defined 26 clusters, with the size of the largest type being the three strains of *L. interrogans* serovar Icterohaemorrhagiae. According to the Hunter-Gaston index, MST exhibited an index of discriminatory power of 0.985. With respect to the VNTR profile data, 24 clusters were identified, and the size of the largest type was eight strains of *L. interrogans* serovars Copenhageni and Icterohaemorrhagiae. Consequently, the discriminatory power of VNTR was 0.943 (Table 3).

Application to field and clinical isolates. A total of 35 field and clinical isolates belonging to *L. interrogans* serogroups Icterohaemorrhagiae and Australis based on VNTR analysis were tested by the MST method (Table 4).

Twenty-five clinical isolates were isolated from a human source. A total of 18 specimens belonged to *L. interrogans* serogroup Icterohaemorrhagiae. These isolates were divided into three MST profiles but into one VNTR profile. The unique VNTR profile corresponded to the Icterohaemorrhagiae-Copenhageni genotype, whereas the MST profiles permitted the identification of eight samples as the Copenhageni genotype strains M20 and Wijnberg, eight other samples as the Icterohaemorrhagiae genotype strain CHU Réunion, and two samples as the Icterohaemorrhagiae genotype strains RGA and Verdun. Seven clinical samples, belonging to *L. interrogans* serogroup Australis, were defined both with the VNTR and MST methods as the Australis-Bratislava genotype.

For 10 rodents' field isolates, the same VNTR profile was defined and identified as the Icterohaemorrhagiae-Copenhageni genotype, whereas three different MST profiles were obtained, with one corresponding to the Icterohaemorrhagiae genotype strain R1, one to the Icterohaemorrhagiae genotype strain CHU Réunion, and the last one to the Copenhageni genotype strains M20 and Wijnberg. The MST method was also applied on DNA extracts from the kidneys of 10 rodents, showing an MST profile identical to that of the strains isolated from the same rodents.

DISCUSSION

In this study, we investigated the MST method for the typing of *L. interrogans* strains. The selection of MST as a genotyping method is justified by its good discrimination when it has been applied to other bacteria, such as *Rickettsia conorii* (24), *Bartonella quintana*, and *Bartonella henselae* (25, 32), and by its capacity to define phylogeographical lineage, as demonstrated for Orientalis-like *Yer-*

TABLE 4 MST application to clinical isolates

L. interrogans serogroup	*L. interrogans* serovar	Strain	Source	Origin	No. of genotypes by spacer			Serovar deduced (strain[s])
					MST1	MST3	MST9	
Icterohaemorrhagiae	Icterohaemorrhagiae	200700457	Human	Guadeloupe	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200704250	Human	France (Nancy)	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	200704251	Human	France (Nancy)	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	200800857	Human	French Polynesia	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200801909	Human	Guadeloupe	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200803290	Human	France (Vesoul)	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	200803792	Human	French Polynesia	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200804216	Human	Guadeloupe	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200900101	Human	French Polynesia	4	6	3	Copenhageni (Wiinberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200901382	Human	Guadeloupe	4	8	3	Icterohaemorrhagiae (RGA/Verdun)
Icterohaemorrhagiae	Icterohaemorrhagiae	200901383	Human	Guadeloupe	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	200903008	Human	French Polynesia	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	201000456	Human	Guadeloupe	4	8	3	Icterohaemorrhagiae (RGA/Verdun)
Icterohaemorrhagiae	Icterohaemorrhagiae	201000458	Human	Guadeloupe	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	201000929	Human	French Polynesia	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae	201000930	Human	French Polynesia	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	201100517	Human	Guadeloupe	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae	200201190	Human	Guadeloupe	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Australis	Australis	200701474	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	200701475	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	200703202	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	200703203	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	201001460	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	201000439	Human	French Polynesia	6	3	3	Australis/Bratislava
Australis	Australis	201100860	Human	French Polynesia	6	3	3	Australis/Bratislava
Icterohaemorrhagiae	Icterohaemorrhagiae	200210109	*Rattus*	Guadeloupe	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0505	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijinberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0834	*Rattus norvegicus*	France (Lyon)	4	7	3	Icterohaemorrhagiae (R1)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0854	*Rattus norvegicus*	France (Lyon)	4	10	3	Icterohaemorrhagiae (CHU Réunion)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0867	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0869	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0872	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0873	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0874	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)
Icterohaemorrhagiae	Icterohaemorrhagiae/Copenhageni	LYO0884	*Rattus norvegicus*	France (Lyon)	4	6	3	Copenhageni (Wijnberg/M20)

sinia pestis (33) and *Mycobacterium tuberculosis* (26). An MST database was created from spacer sequences obtained for *Leptospira* sp. strains belonging to the Icterohaemorrhagiae, Australis, Grippotyphosa, and Canicola serogroups (Table 2), which are highly prevalent in France, with the seroprevalence of the Icterohaemorrhagiae serogroup in rodents being as high as 45.6% (12, 27–30).

The choice of spacer was based on the same MLST scheme (21). In the present study, 200 loci were identified in the genomes, of which 16 spacers were selected according to the criteria defined below. We then combined three spacers for discriminating strains within the same serovar, enabling the identification of 26 genotypes among the 33 strains of *L. interrogans*.

The spacers were selected according to the following criteria. The sequence size of ≤400 bp permitted the evolution of the MST PCR method toward real-time PCR. The 70 to 99% sequence similarity between the two *L. interrogans* reference complete genomes allowed the selection of homologous spacers belonging to the same *Leptospira* species. The spacer variability of ≥5 bp permitted the selection of highly discriminatory regions. Moreover, the MST spacer selection was based on the discriminatory power of *L. interrogans* serogroup Icterohaemorrhagiae, which is the serogroup that presents ambiguous identification between the Icterohaemorrhagiae and Copenhageni serovars when analyzed by other typing method, such as PFGE and VNTR (17, 18).

Each sequence's spacer presented mutation and/or insertion or deletion genetic events. Moreover, the MST1 and MST3 spacers have deletion events and one variable region. For the MST1 spacer, the variable region permits the identification of five different genotypes in *L. interrogans* serogroups Australis, Canicola, and Grippotyphosa. For the MST3 spacer, the variable region allows the discrimination of strains belonging to *L. interrogans* serovars Icterohaemorrhagiae and Copenhageni. For the MST9 spacer, the mutation and insertion genetic events identified permit discrimination among the four serogroups when used in combination with MST1 and MST3.

In the present study, among the 33 strains studied, a total of 26 MST profiles were defined, whereas 24 genotypes were defined by the VNTR method.

A total of 14 strains of *L. interrogans* serogroup Icterohaemorrhagiae were analyzed, with four strains belonging to the Icterohaemorrhagiae serovar and four to the Copenhageni serovar. The

MST method permits the strains of the Copenhageni and Icterohaemorrhagiae serovars to be differentiated into five distinct profiles, in contrast to the unique profile by VNTR analysis (18). These two serovars are particularly difficult to discriminate when using other typing methods, such as PFGE (34), VNTR (35), and MLST (36). This MST capacity is particularly interesting for the epidemiologic surveillance of the *L. interrogans* species carried in rodents, such as *Rattus norvegicus*, since they are considered the main environmental spreader of zoonotic *Leptospira* species. Indeed, rodents play an important role for the circulation of the Icterohaemorrhagiae serogroup, with high *Leptospira* sp. loads in their renal tubules (4, 27, 37, 38). Identification of the *Leptospira* sp. genotypes in the rodent population is important for assessing the risk for human or domestic animal exposure (37, 39), as well as for a better evaluation of their role in the transmission of leptospirosis in humans. With regard to *L. interrogans* serogroup Grippotyphosa, the discrimination of MST was similar to that of the VNTR method. Identification can be achieved by either one or the other method.

For *L. interrogans* serogroup Canicola, the same numbers of profiles were obtained by both the MST and VNTR methods. The MST profile was the same for *L. interrogans* serovars Jonsis and Schueffneri of the Canicola serogroup, but the VNTR profiles were different. In addition, the VNTR profiles of the Canicola and Portlandvere serovars were identical, but their MST profiles were not. A joint application of the MST and VNTR methods enables a total identification of the serovars of the Canicola serogroup. In veterinary applications, Canicola serogroup surveillance is important for the protection of dogs. These animals are vaccinated against the strains of the Canicola and Icterohaemorrhagiae serogroups (40). The regular serovar identification of these serogroups is important to define more precisely the prevalence of infecting serovars and its use for the development of the vaccines (41).

For *L. interrogans* serogroup Australis, the VNTR discrimination was superior to that of MST. However, *L. interrogans* serovars Bratislava and Australis are differentiated by only one copy for one of the VNTR loci. This could lead to ambiguous results in size determination by gel electrophoresis (19). The prevalence of the Australis serogroup increased in several countries (42, 43), and this epidemiologic surveillance allowed the evolution of a future vaccine for dogs to be defined with the addition of an Australis serovar.

With respect to the studied reference strain, the MST discriminatory power (0.98) was superior to that of VNTR (0.94). This difference is explained by the fact that MST permitted the identification of strains belonging to the same serovar as the strain belonging to *L. interrogans* serovar Icterohaemorrhagiae, which leads to a better discrimination than VNTR (0.94 compared to 0.68).

Because nucleotides sequences were found identical between, for example, the Verdun strain, which was isolated in 1918 during the first World War, and recent clinical isolates of *L. interrogans* serovar Icterohaemorrhagiae, we can hypothesize that the MST sequence profiles remained identical and stable in a long period of time.

MST is a simple method based on PCR and sequencing. Therefore, because it is based on the nucleotide sequence, MST provides a robust and nonambiguous result. It requires an additional sequencing step; however, the sequencing has become a routine method of DNA analysis as the cost of sequencing has decreased. The MST allowed obtaining exportable data, which represents a great resource for other laboratories in the world through the use of nucleotide sequences deposited in GenBank. In comparison, VNTR is a rapid method based on PCR and electrophoretic migration. Thus, the VNTR analysis depends on the electrophoresis gel components and resolution limits and the locus tandem-repeat number (19, 44). These factors may affect the VNTR interpretation, which can occasionally be ambiguous at nearby high tandem-repeat numbers. This ambiguity of the serovar identification is problematic for epidemiologic applications if the research level is at the serovar or even the strain level.

For field and clinical isolates, MST and VNTR permitted the serovar identification for all isolates. The MST-PCR sensitivity was similar to that of VNTR. Moreover, the results were consistent using both the MST and VNTR methods. MST was used on isolates belonging to *L. interrogans* serogroup Icterohaemorrhagiae to test the strain identification ability. From one up to five samples were tested in geographically distinct trapping sites. Thus, MST permits the precise identification of the *Leptospira* sp. strains present in the rodent populations in each area. A study on a larger rodent population will be undertaken to validate the notion of geotyping (36). The MST method may become a new genotyping method for the epidemiological surveillance of *L. interrogans* and the geographical study of the strain's repartition of the Icterohaemorrhagiae serogroup.

In conclusion, the development of MST for the *L. interrogans* species offered a new genotyping method with a high discriminatory power. MST represents an evolved genotyping method for the identification of *L. interrogans* serogroup Icterohaemorrhagiae because the Icterohaemorrhagiae and Copenhageni serovars were clearly identified and because this method also permitted identification at the strain level. This capacity is interesting for epidemiological applications. The MST method enabled serovar identification similar to that by the VNTR method for the other serogroup, suggesting an enlargement of the MST method for supplementary serogroups and other species, such as the other pathogenic species *L. kirschneri* and *L. borgpetersenii*.

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A.-L.Z. performed the experiments, analyzed the data, and wrote the manuscript. Z.D. conceived the study, performed the experiments, analyzed the data, and wrote the manuscript. A.K. and P.D. provided the isolates and obtained the funding. A.K. analyzed the data. M.P. provided the isolates used in this study, supplied several DNA samples, and analyzed the results. M.A. coordinated this study and wrote the manuscript. F.A. provided the rodent clinical specimens. All authors read and approved the final manuscript.

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