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Analysis of Multilocus Sequence Typing for Identification of *Leptospira* Isolates in Brazil[▽]

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A collection of 101 *Leptospira* isolates was tested by multilocus sequence typing (MLST) and by traditional serotyping. MLST divided the isolates into 4 sequence types (STs), while serotyping classified them into 6 serogroups. Two isolates failed to generate products for some genes by MLST. MLST was less discriminatory than serotyping for uncommonly occurring isolates from humans in Brazil.

Leptospirosis, caused by pathogenic leptospires, is one of the most widespread zoonotic diseases known. The genus *Leptospira* is divided into 20 species with more than 200 pathogenic serovars organized into 24 serogroups on the basis of antigenic relatedness (2, 9, 13, 30). The disease occurs in wild and domesticated animals, both of which can be a source of human infection. Exposures that occur during flooding events are the main risk factors of human leptospirosis in Brazil (1, 3, 14, 22). In São Paulo State, Brazil, 13,620 cases have been reported in the last 20 years (ftp://ftp.cve.saude.sp.gov.br/doc_tec/zoo/lepto09_perfil.pdf). However, information about circulating isolates of *Leptospira* spp. in Brazil is limited. Identification of isolates to the serovar level is essential for understanding the epidemiology of the disease in both humans and animals.

Since certain serovars are often associated with specific mammalian hosts and with the symptoms and severity of the disease, the identification of the serovar usually permits the prediction of sources of infection, thereby enabling control of the spread of the disease (17). Monitoring the serovars and genetic profiles of strains collected over time and from different regions is also important to better understand the current circulating *Leptospira* population worldwide. Isolate discrimination can be performed by a microagglutination test (MAT) at the serogroup level and by a cross-agglutinin absorption test (CAAT) at the serovar level (5). However, performing those methods is tedious, as live cultures of collection strains must be maintained for use as antigens and rabbit hyperimmune sera are required. Although identification by serotyping is valuable, molecular methods with higher reproducibility and discriminatory power may be more useful in epidemiological investigations.

New molecular methods such as multilocus sequence typing (MLST) have been recently developed and applied to the study of many bacterial species (4, 6, 7, 15, 18). MLST is a simple PCR-based technique that makes use of automated DNA sequencers to assign and characterize the alleles present in different target genes. The method allows one to generate sequence data on a low- to high-throughput scale that is unambiguous and suitable for epidemiological and population studies. The selected loci are generally housekeeping genes, which evolve very slowly over an evolutionary time scale (8).

Our goal was to evaluate the discriminatory power of MLST compared to serotyping using a set of Brazilian human isolates. A total of 101 *Leptospira* clinical strains (95 from blood, 5 from cerebrospinal fluid, and 1 from urine) isolated from humans in São Paulo, Brazil, between 1986 and 2009 were analyzed by MLST and serotyped by MAT. The isolates used in this study are part of the collection of the Leptospirosis Laboratory, Instituto Adolfo Lutz, São Paulo, Brazil. This study was approved by Instituto Adolfo Lutz ethical committees.

MLST was performed according to the method of Thaipadungpanit et al. (28) with the following housekeeping genes: *mreA*, *pfkB*, *pntA*, *sucA*, *tpiA*, *fadD*,and *glmU*. Briefly, PCR amplification was carried out using chromosomal DNA and an initial denaturing step at 94°C for 5 min, followed by 30 cycles of 94°C for 10 s, 52°C (*mreA*, *pfkB*, *pntA*, *sucA*, and *tpiA*), or 50°C (*fadD* and *glmU*) for 15 s, 72°C for 50 s, and then 72°C for 7 min. PCR products were purified using ExoSAP-IT (USB Corp.), and sequencing reactions were carried out in each direction using the primers that were used for the initial PCR amplification, a BigDye Terminator v. 3.1 cycle sequencing kit (ABI), and an ABI Prism 3130xl Genetic Analyzer automated DNA sequencer. The sequences were analyzed using *DNASTAR* Lasergene 8 software and submitted to the international public database (http://www.mlst.net/) to generate an allelic profile and to assign the sequence type (ST). Pulsed-field gel electrophoresis (PFGE) and 16S rRNA sequencing were performed as previously described (11, 19) to verify the identity of isolates that failed to amplify all seven housekeeping genes.

To confirm the serogroup status, all isolates were serotyped by MAT with 23 standard group-specific rabbit antisera (5). Each isolate was assigned to the serogroup of the group serum that gave the highest titer.

The results of serotyping and MLST analysis and the dates of isolation are listed in Table 1. The serotyping performed with 23 standard antisera (group sera) revealed that 90 (89.11%) isolates belonged to serogroup Icterohaemorrhagiae, 5 (4.95%) to Canicola, 2 (1.98%) to Ballum, 2 (1.98%) to Autumnalis, 1 (0.99%) to Pomona, and 1 (0.99%) to Sejroe.

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TABLE 1. Results obtained for the 101 human isolates from Brazil analyzed by MLST and serotyping methods

Serotyping result at serogroup level	ST[a] by MLST	No. of isolates	Yr of isolation
Icterohaemorrhagiae	17[c]	9	1986
		5	1989
		7	1995
		5	1996
		10	1997
		10	1998
		4	1999
		2	2000
		10	2001
		7	2002
		5	2003
		5	2004
		5	2006
		3	2007
		3	2009
Pomona	37[d]	1	1986
Canicola	37[d]	1	1986
		1	1989
		2	2006
		1	2007
Autumnalis	27[e]	1	2004
		1	2005
Sejroe	58[f]	1	1999
Ballum	—[b]	1	1986
		1	1997

[a] The corresponding serovars are based on the results of a study by Thaipadungpanit et al. (28).
[b] MLST failed.
[c] Corresponding to serovar Copenhageni or Icterohaemorrhagiae.
[d] Corresponding to serovar Pyrogenes, Portlandvere, Canicola, Pomona, or Guaratuba.
[e] Corresponding to serovar Autumnalis.
[f] Corresponding to serovar Roumanica or Wolfii.

The ST numbers were determined on the basis of the method of Thaipadungpanit et al. (28). Four sequence types, ST 17 (90 isolates), ST 58 (1 isolate), ST 37 (6 isolates), and ST 27 (2 isolates), were identified by MLST. Two isolates identified by serotyping as belonging to serogroup Ballum failed to generate products for most genes by the MLST method. They were further confirmed to be *L. borgpetersenii* isolates by PFGE and 16S sequencing. The serogroups Pomona (1 isolate) and Canicola (5 isolates) produced the same ST by MLST. A significant finding of this study was that both methods identified Icterohaemorrhagiae as the predominant serogroup in the state of São Paulo in all years studied. The results are in agreement with the analysis of human serum samples in Brazil, which showed that the most prevalent serogroup infecting humans is Icterohaemorrhagiae (24).

Although MLST is useful for epidemiological studies of some bacterial pathogens and can be helpful in discriminating isolates during an outbreak (10, 12, 23, 26), in the present study, 89% of the isolates were serovar Icterohaemorrhagiae or Copenhageni by MLST and serogroup Icterohaemorrhagiae by MAT. MLST was able to confirm most of the isolates to the serovar level in the present study; however, there were some limitations, such as a lack of discriminatory power, when the method was used on uncommonly occurring isolates from humans in Brazil. This may limit its utility in the epidemiological studies. In addition, the method is not easily applicable in outbreak investigations because of the time and expensive equipment and reagents required. In addition, this MLST scheme (28) does not currently generate sequence data from *L. borgpetersenii* and thus could not be used to type 2 isolates in this study.

One disadvantage of the MLST assay was its inability to distinguish among some isolates at the serovar level. The inability of other techniques to genetically differentiate leptospires at the serovar level has also been previously described (16, 20, 21, 22, 25, 27, 29, 31). Our work confirms the results of another study (2) that indicated that serovars do not cluster with serogroups but cluster according to the species.

In Brazil, where this study determined that Icterohaemorrhagiae or Copenhageni was the predominant serovar nearly 90% of the time, it may be sensible and appropriate to perform MAT to identify the serogroup and infer serovar identity, since MAT is already routinely performed in reference laboratories, and since each serovar identified belonged to a separate serogroup. Therefore, this study shows that, with Brazilian isolates, the serogrouping method is easier to perform and may provide information similar to or better than that obtained on the basis of DNA sequencing using more laborious and expensive genotyping methods.

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