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Evaluation of a Modified Pulsed-Field Gel Electrophoresis Approach for the Identification of *Leptospira* Serovars

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Abstract. The genus *Leptospira* is composed of spirochetes that are morphologically indistinguishable and cannot be differentiated by phenotypic methods. Determining the identity of an infecting serovar is valuable from both epidemiologic and public health standpoints. Pulsed-field gel electrophoresis (PFGE) of *Leptospira* has had limited use in few laboratories. In this study, we modified the existing PFGE protocol to reduce time to completion and developed a PFGE database using the restriction endonuclease *Not* I to generate PFGE profiles for the identification and evaluation of *Leptospira.* Reference strains (n = 206) from a collection maintained by the Centers for Disease Control and Prevention were studied. Eighty-nine percent of the serovars produced distinct patterns, and different strains of the same serovar showed profiles that were highly similar. Pulsed-field gel electrophoresis of *Leptospira* is a useful tool for molecular characterization of reference serovars, the investigation of potentially new species or serovars, and ultimately for the routine identification of clinical isolates.

INTRODUCTION

Leptospirosis is an acute febrile disease caused by pathogenic spirochetes of the genus *Leptospira*. The disease is maintained in nature by chronic renal infection of carrier animals and acquired by direct or indirect contact with urine or tissues from infected animals.[1] The genus is divided into serovars, each characterized by cross-agglutinin absorption testing.[2] Historically, several hundred serovars of *Leptospira* were classified into two species, *L. interrogans* and *L. biflexa*,[3] which contained pathogenic and saprophytic strains respectively. Based upon DNA-DNA hybridization data, the genus is now classified into 17 species,[4–9] several of which contain both pathogenic and non-pathogenic serovars. Identification is further complicated because several serovars are distributed between different species, apparently the result of horizontal gene transfer.[10]

The epidemiology of leptospirosis in industrialized countries has changed in recent years. The traditional patterns of occupational exposure have become infrequent, and there is an increasing trend toward recreational exposures. Moreover, climatic events associated with excess rainfall and flooding have led to large outbreaks of disease,[11] and with the increase in global travel, the outbreaks may be initially recognized as sporadic cases in distant geographic locations at the same time, away from the site of the exposure.[12] The identity of causative serovars cannot be assumed, especially when infection is acquired in tropical environments[11,13] where the ecology of leptospirosis has not been studied extensively. Serovar identity can have a significant effect on public health because potential carriers of the serovar can be targeted and geographic distribution of serovars can be more accurately assessed.

To understand the changing epidemiology of the disease, serovar identification of isolates is essential. Few laboratories possess the resources to maintain the traditional serological identification schemes[2,14] and therefore most isolates are not identified fully. Several sequence-based molecular methods have been applied to the identification of leptospires,[15,16] but these do not yet have wide application. Molecular techniques such as multiple locus variable number tandem repeat analysis[17–19] and multilocus sequence typing[20,21] have disadvantages in serovar identification[22] and have yet to be widely applied. Multiple locus variable number tandem repeat analysis[17] can identify to the serovar level, but requires primers specific for each species (except for *L. interrogans* and *L. kirschneri*), and therefore cannot be applied easily to all isolates.[17–19] The use of pulsed-field gel electrophoresis (PFGE) as a method for identification of leptospiral serovars was previously described,[23] but this method was not broadly adopted.

In this study, we describe a modified PFGE procedure using protocols similar to those extensively used for identifying foodborne enteric organisms.[24] Our objectives were to reduce the time required for serovar identification of an isolate and to evaluate the molecular patterns of a large number of reference serovars to develop a database that will facilitate the exchange of PFGE data between laboratories.

MATERIALS AND METHODS

Reference isolates. Strains belonging to the family *Leptospiraceae* were evaluated from a collection of reference strains maintained by the Centers for Disease Control and Prevention (Atlanta, GA). A total of 202 pathogenic, nonpathogenic, and intermediately pathogenic *Leptospira* serovars were studied. Eight of the 202 serovars had multiple strains associated with them. Of these 8 serovars, 19 strains were tested. Two strains each of *Leptonema* and *Turneriella* species were also evaluated (Table 1). Organisms were grown to late-log or stationary phase in either EMJH or PLM-5 liquid medium at 30°C and centrifuged at 4,000 rpm for 15 minutes to concentrate the bacteria. Cells were resuspended in a small volume of cell suspension buffer (100 mM Tris, pH 7.5; Invitrogen Life Technologies, Carlsbad, CA), and 100 mM EDTA, pH 8 (AMRESCO, Inc., Solon, OH) to an optical density of 0.500–0.950 at 610 nm using a Spectronic® 20 GENESYS™ spectrophotometer (Thermo Electron Corporation, Marietta, OH).

Preparation of agarose plugs. Ten microliters of proteinase K (AMRESCO, Inc.) at a concentration of 20 mg/mL was added to 200 μL of each cell suspension, followed by an equal

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TABLE 1
Reference strains used to generate the *Leptospira* database

Reference strains	No. in database
Pathogenic serovars	187
Nonpathogenic serovars	9
Intermediate pathogenicity (including *L. broomii*)	6
Turneriella	2
Leptonema	2
Total reference serovars	206

volume of molten plug agarose solution (1% SeaKem Gold agarose [Cambrex, Walkersville, MD], 1% sodium dodecyl sulfate [Roche, Alameda, CA] in Tris-EDTA buffer) to give a 1:1 dilution. The culture/agarose mixture was immediately dispensed into wells of a disposable plug mold (Bio-Rad Laboratories, Hercules, CA) and allowed to solidify. Agarose plugs were then immersed in 1.5 mL of lysis buffer (50 mM Tris-HCl, 50 mM EDTA, pH 8.0, 1% N-lauroylsarcosine, sodium salt [Sigma-Aldrich, St. Louis, MO]) containing 40 μL of proteinase K (20 mg/mL) and placed in a reciprocating water bath at 54°C for one hour. Plugs were subsequently transferred to a 50-mL conical tube with screen cap (Bio-Rad Laboratories) containing 10 mL of sterile water and washed in the reciprocating water bath at 50°C for 15 minutes. Washing was repeated three times using plug wash buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Restriction digestion. A slice was cut from each agarose plug and the embedded DNA was digested with 30 units of restriction endonuclease *Not* I (New England Biolabs, Ipswich, MA) in a 37°C water bath for two hours. *Salmonella* serotype Braenderup H9812 was digested with 50 units of *Xba* I (New England Biolabs) for use as a DNA size standard.[25]

Electrophoresis. Plug slices containing the digested DNA were placed in the wells of a 1% agarose gel (SeaKem Gold in 0.5× TBE [Tris-Borate-EDTA] buffer; Invitrogen Life Technologies) and subjected to electrophoresis in a CHEF Mapper XA or CHEF-DRIII (Bio-Rad Laboratories) for 18 hours at 14°C with recirculating 0.5× TBE buffer. Electrophoresis conditions were as follows: switch times of 2.16 and 35.07 seconds, angle of 120°, gradient of 6 V/cm, temperature of 14°C, and a linear ramping factor. After electrophoresis, gels were stained with ethidium bromide (1 μg/mL) (AMRESCO, Inc.) for 30 minutes, followed by three 20-minute cycles of destaining in water.

Fragment pattern analysis. Gel images were captured using a Gel Doc 2000 System (Bio-Rad Laboratories). Gel analysis, dendrograms, Dice band-based coefficients, and number of fragment differences were performed using BioNumerics version 4.0 (Applied Maths, Inc., Austin, TX). Patterns that had the same number of fragments at corresponding molecular weights were considered indistinguishable. Patterns with up to three fragment differences between them were considered closely related genetically, consistent with the criteria for PFGE bacterial strain typing.[26] Dice band-based coefficients were also considered when evaluating relatedness. Finally, all fragment patterns were evaluated visually in a dendrogram.

RESULTS

PFGE required two days to complete after isolates grew in culture. The PFGE profiles were generated from 206 different serovars (187 pathogenic, 9 nonpathogenic, and 6 intermediate species from *Leptospira*, and 2 strains each of *Turneriella* and *Leptonema*), and were used to construct a database to serve as a searchable library for comparison with isolates submitted for identification (Table 1). Eighteen of the isolates were re-grown and PFGE was repeated to evaluate reproducibility. Nineteen strains of eight serovars were tested to determine the extent of strain differences within a serovar. Most (89%, 183 of 206) reference strains produced fragment patterns that were distinctive. Digestion of chromosomal DNA with *Not* I resulted in 3–24 restriction fragments ranging in size from 28 kb to 1,836 kb (supplemental data figure). The most commonly encountered serovars were readily differentiated from one another, except for serovars Icterohaemorrhagiae and Copenhageni, which are similar when characterized by both by serologic[27] and molecular[15] approaches (Figure 1). The remaining 23 (11%) reference serovars were difficult to differentiate and produced either indistinguishable PFGE patterns, patterns that differed from one another by only 1–2 fragment differences, or patterns that did not have a significant number of fragments (3–6 fragments). Among these remaining 23 serovars, there were 6 instances when 2 serovars could not be distinguished from each other on the basis of their PFGE pattern using *Not* I (Table 2). Seven additional patterns of serovars were closely related; they differed from each other by one or two fragments. Serovars Pomona, Lyme, Kaup, Aguaruna, and Valbuzzi appear twice in Table 2; each has zero band differences to one serovar and two band differences to others. Six serovars (*L. meyeri* serovars Hardjo, Ranarum, Semaranga, and Perameles, and *L. wolbachii* serovars Codice and Gent) produced patterns that did not have a significant number of fragments (3–6 fragments); therefore the discriminatory ability for these serovars was undetermined. The full dendrogram showing all patterns is available as supplemental data.

To determine the extent of differences between strains of the same serovar, we examined two or more different strains from each of eight serovars. The PFGE profiles for strains of a serovar belonging to the same species were indistinguishable or closely related (≤ 3 band differences, Dice correlations = 73.7–100%), but for strains of serovars that belong to two or more species the differences were substantial (10–20-band differences, Dice correlations = 31.6–43.3%) (Table 3). Figure 2 shows the differences between two strains of serovar Grippotyphosa belonging to different species. Consistent with the molecular classification of serovar Grippotyphosa into two species, *L. kirschneri* and *L. interrogans*, two distinct PFGE patterns were observed when two strains of serovar Grippotyphosa from these species were evaluated. Two hu-

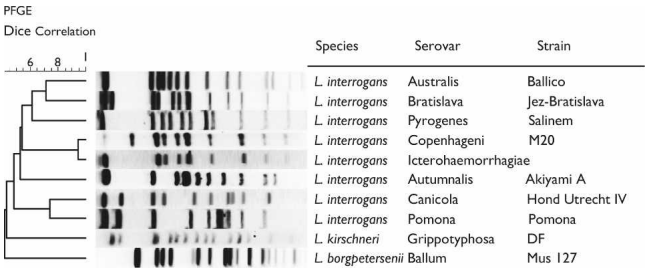

FIGURE 1. Pulsed-field gel elctrophoresis patterns of commonly encountered serovars of *Leptospira*.

TABLE 2
Leptospira serovars indistinguishable or closely-related by pulsed-field gel electrophoresis

Serovars compared	Species	No. of fragment differences	Dice coefficient, %
Bataviae (strain Van Tienen), 26-73	*L. interrogans*	0	88.9
Kennewicki/Pomona (strain 164), Guaratuba	*L. interrogans*	0	97.2
Pinchang, Sichuan	*L.* genomospecies 1	0	91.9
Lyme, Kaup	*L. inadai*	0	91.4
Aguaruna, Valbuzzi	*L. inadai*, *L. kirschneri*	0	95.0
Castellonis, Ballum (strain S102)	*L. borgpetersenii*	0	88.9
Icterohaemorrhagiae, Copenhageni	*L. interrogans*	1	94.7
Roumanica, Wolffi	*L. interrogans*	2	85.7
Panama, Pomona (strain 24K)	*L. noguchii*	2	81.8
Georgia, Szwajizak (strain Oregon)	*L. santarosai*	2	84.5
Lyme/Kaup and Aguaruna/Valbuzzi	*L. inadai*, *L. kirschneri*	2	85.3
Yunxian, Qunjisn	*L. borgpetersenii*, *L. interrogans*	2	83.3
Bratislava, Lora	*L. interrogans*	2	85.7

man isolates originally identified as *L. fainei* serovar Hurstbridge[28,29] were shown in our study to have different PFGE patterns than reference strains of serovar Hurstbridge. These isolates have been re-classified as a new species, *L. broomii*.[9]

Serovars in serogroup Pomona have been subjected to a number of re-classifications on the basis of serologic results. Monjakov and Kennewicki are no longer recognized as separate serovars, but are now classified as strains of serovar Pomona.[27] Figure 3 confirms this re-classification and the close relationship between strains of serovar Pomona, as well as showing the distinction between strains of serovar Pomona and other serovars of serogroup Pomona. One exception is a strain of serovar Pomona (24K) that appears in *L. noguchii*, unlike other strains of Pomona, which belong to *L. interrogans*. Serovar Dania strain K1 has previously been found by PFGE to resemble serovars of *L. interrogans*, different from its original classification of *L. santarosai*.[30] However, in our study, this serovar clusters in the full dendrogram with other serovars of *L. santarosai*, consistent with its original taxonomy.

Eighteen isolates were each grown and digested on at least two different occasions to evaluate reproducibility. In each instance the fragment patterns generated by the same strains were indistinguishable.

DISCUSSION

In this study, PFGE was standardized and evaluated for its potential utility to rapidly identify clinical isolates, and was used to characterize a large collection of leptospiral serovars. We modified the protocol described originally by Herrmann and others[23] to reduce the time required for completion by approximately two days and used a strain of *Salmonella* serotype Braenderup as the size marker.[25] Using this approach, we constructed a database of reference serovar patterns. Most (89%) serovars produced PFGE patterns that were unique to each serovar, but some serovars could not be distinguished. Moreover, some strains did not produce a sufficient number of bands to allow reliable characterization. In the original report describing PFGE for leptospiral characterization,[23] a second enzyme (*Sgr* AI) was used to differentiate such strains. Further development of our database to include all available serovars will require the application of this approach. However, in contrast to the previous study, we included reference strains of non-pathogenic serovars because the former distinction between pathogenic and non-pathogenic species has been replaced by a molecular classification.[6] Some of the serovars that were indistinguishable in this study were saprophytes or intermediates classified as *L.*

TABLE 3
Band differences between strains of the same serovar of *Leptospira**

Species	Serovar	Strains compared	No. of fragment differences	Dice coefficient, %
L. fainei	Hurstbridge	BUT 6^T and BKID6	0	100
L. interrogans	Icterohaemorrhagiae	RGA and Monymusk	0	88.9
L. interrogans	Pomona†	Kennewicki and 164	0	100
L. interrogans	Canicola	Ruebush and Hond Utrecht IV	1	85.7
L. interrogans	Pomona†	Kennewicki and Wickard	1	94.7
L. interrogans	Pomona†	Johnson and Monjakov	1	87.0
L. interrogans	Pomona†	Wickard and 164	1	94.7
L. interrogans	Pomona†	Kennewicki and Johnson	2	90.0
L. interrogans	Pomona†	Johnson and 164	2	90.0
L. interrogans	Pomona†	Wickard and Johnson	3	76.2
L. interrogans	Pomona†	Kennewicki and Monjakov	3	73.7
L. interrogans	Pomona†	Monjakov and 164	3	73.7
L. borpetersenii	Ballum	Mus 127 and S102	3	82.9
L. interrogans	Copenhageni	M20 and Wijnberg	3	94.7
L. interrogans, *L. kirschneri*	Grippotyphosa	Andaman and DF	10	38.5
L. interrogans, *L. noguchii*	Pomona	Pomona and 24K	12	31.6
L. santarosai, *L. interrogans*	Szwajizak	Oregon and Szwajizak	20	43.3

* Band differences between strains of the same serovar are small unless the strains are in different species.
† See Figure 3 for band differences from Pomona strain Pomona.

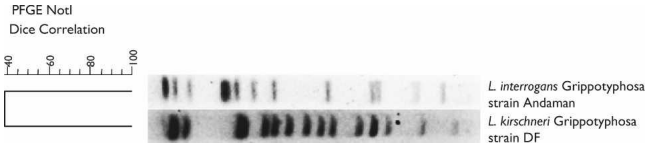

FIGURE 2. Strains of serovar Grippotyphosa of different *Leptospira* species showing different pulsed-field gel electrophoresis patterns.

meyeri, *L. wolbachii*, and *L. inadai*, and these serovars in particular will require further study using additional restriction endonucleases for improved characterization.

Repetitions of the same serovar consistently produced reproducible patterns. The PFGE patterns of different strains of the same serovar were closely related, which corresponded well to serologic classification. The exceptions were strains appearing in different species, which corresponded to the molecular classification scheme and enables identification of both species and serovar simultaneously. PFGE also has the potential to clarify taxonomic status of isolates in question because of mislabeling or contamination, such as resolving the different cultures of serovar Dania strain K1 in this and a previous study.[30]

PFGE has the capability of rapidly identifying the serovar of most clinical isolates and has the potential to replace cumbersome serologic methods of identification. The identification of clinical isolates using PFGE can associate potential reservoirs as carriers and lead to appropriate targeted prevention measures. PFGE also offers significant savings in turnaround time when compared with the definitive serologic method of serovar identification, which requires the raising of rabbit antisera against each new isolate. Moreover, PFGE can be used to rapidly identify patterns that may represent new serovars. In addition, these patterns are readily available so that future isolates can be identified as having the same pattern. In this study, two isolates reported as *L. fainei* were shown to be distinct from serovar Hurstbridge, the only described serovar of *L. fainei*[7]; this led us to the description of a new species, *L. broomii*.[9] The PFGE database is thus capable of rapidly identifying isolates that require further investigation.

Future application of this approach to identification of all *Leptospira* serovars will require expansion of the number of serovars included in the database and the use of additional endonucleases to generate banding patterns from those strains for which *Not* I was not effective.[23] Ongoing studies include validation of PFGE as a serovar identification method with CAAT using clinical isolates. In its current state, the database can be used to identify all of the serovars most commonly isolated from humans and animals and has enabled rapid detection of several clusters of isolates that are undergoing detailed serologic analysis.

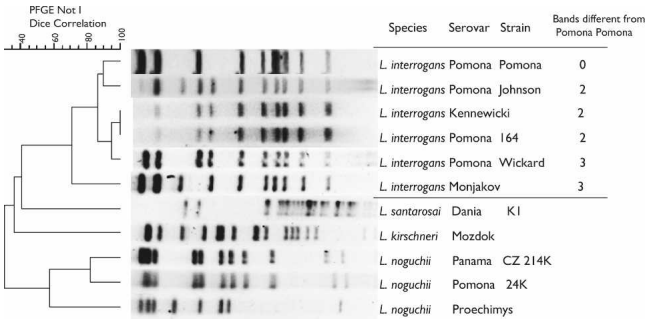

FIGURE 3. Pulsed-field gel electrophoresis (PFGE) patterns of *Leptospira* serogroup Pomona.

Improved recognition of leptospirosis acquired by travelers in tropical environments and the increased incidence of large outbreaks of leptospirosis requires rapid identification of unique leptospiral isolates that may belong to previously undescribed species and serovars. Capacity to perform PFGE is becoming increasingly widespread and standardized in public health and reference laboratories worldwide. Application of a standardized procedure, followed by submission of digital images to a centralized site for normalization and evaluation will enable analysis without shipping strains between laboratories. Moreover, the database can be shared with laboratories that use BioNumerics software, or alternatively, gel images can be submitted by e-mail for analysis using the database, which will facilitate international efforts to identify isolates from a wider variety of clinical, veterinary and environmental settings. Finally, the use of a more rapid and reliable method for serovar identification, such as PFGE, will lead to more rapid recognition of animal reservoirs and ultimately, more timely implementation of control strategies for leptospirosis.

Note: The supplemental dendrogram appears online at www.ajtmh.org.

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REFERENCES

1. Levett PN, 2004. Leptospirosis. Mandell GL, Bennett JE, Dolin R, eds. *Principles and Practice of Infectious Disease*s. Philadelphia: Elsevier, 2789–2795.
2. Dikken H, Kmety E, 1978. Serological typing methods of leptospires. Bergan T, Norris JR, eds. *Methods in Microbiology*. London: Academic Press, 259–307.
3. Johnson RC, Faine S, 1984. *Leptospira*. Krieg NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology*. Baltimore: Williams and Wilkins, 62–67.
4. Yasuda PH, Steigerwalt AG, Sulzer KR, Kaufmann AF, Rogers F, Brenner DJ, 1987. Deoxyribonucleic acid relatedness between serogroups and serovars in the family *Leptospiraceae* with proposals for seven new *Leptospira* species. *Int J Syst Bacteriol 37:* 407–415.
5. Ramadass P, Jarvis BDW, Corner RJ, Penny D, Marshall RB, 1992. Genetic characterization of pathogenic *Leptospira* species by DNA hybridization. *Int J Syst Bacteriol 42:* 215–219.
6. Brenner DJ, Kaufmann AF, Sulzer KR, Steigerwalt AG, Rogers FC, Weyant RS, 1999. Further determination of DNA relatedness between serogroups and serovars in the family Leptospiraceae with a proposal for *Leptospira alexanderi* sp. nov. and four new *Leptospira* genomospecies. *Int J Syst Bacteriol 49:* 839–858.
7. Pérolat P, Chappel RJ, Adler B, Baranton G, Bulach DM, Bill-

inghurst ML, Letocart M, Merien F, Serrano MS, 1998. *Leptospira fainei* sp. nov., isolated from pigs in Australia. *Int J Syst Bacteriol 48:* 851–858.

8. Levett PN, Morey RE, Galloway R, Steigerwalt AG, Ellis WA, 2005. Reclassification of *Leptospira parva* Hovind-Hougen et al., 1982 as *Turneriella parv*a gen. nov., comb. nov. *Int J Syst Evol Microbiol 55:* 1497–1499.
9. Levett PN, Morey RE, Galloway RL, Steigerwalt AG, 2006. *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. *Int J Syst Evol Microbiol 56:* 671–673.
10. de la Peña-Moctezuma A, Bulach DM, Kalambaheti T, Adler B, 1999. Comparative analysis of the LPS biosynthetic loci of the genetic subtypes of serovar Hardjo: *Leptospira interrogans* subtype Hardjoprajitno and *Leptospira borgpetersenii* subtype Hardjobovis. *FEMS Microbiol Lett 177:* 319–326.
11. Trevejo RT, Rigau-Perez JG, Ashford DA, McClure EM, Jarquin-Gonzalez C, Amador JJ, de los Reyes JO, Gonzalez A, Zaki SR, Shieh WJ, McLean RG, Nasci RS, Weyant RS, Bolin CA, Bragg SL, Perkins BA, Spiegel RA, 1998. Epidemic leptospirosis associated with pulmonary hemorrhage-Nicaragua, 1995. *J Infect Dis 178:* 1457–1463.
12. Sejvar J, Bancroft E, Winthrop K, Bettinger J, Bajani M, Bragg S, Shutt K, Kaiser R, Marano N, Popovic T, Tappero J, Ashford D, Mascola L, Vugia D, Perkins B, Rosenstein N, 2003. Leptospirosis in "Eco-Challenge" athletes, Malaysian Borneo, 2000. *Emerg Infect Dis 9:* 702–707.
13. Haake DA, Dundoo M, Cader R, Kubak BM, Hartskeerl RA, Sejvar JJ, Ashford DA, 2002. Leptospirosis, water sports, and chemoprophylaxis. *Clin Infect Dis 34:* e40–e43.
14. Terpstra WJ, Korver H, Van Leeuwen J, Klatser PR, Kolk AHJ, 1985. The classification of Sejroe group serovars of *Leptospira interrogans* with monoclonal antibodies. *Zentralbl Bakteriol Mikrobiol Hygiene I Abt Orig A 259:* 498–506.
15. Majed Z, Bellenger E, Postic D, Pourcel C, Baranton G, Picardeau M, 2005. Identification of variable-number tandem-repeat loci in *Leptospira interrogans* sensu stricto. *J Clin Microbiol 43:* 539–545.
16. Vijayachari P, Ahmed N, Sugunan AP, Ghousunnissa S, Rao KR, Hasnain SE, Sehgal SC, 2004. Use of fluorescent amplified fragment length polymorphism for molecular epidemiology of leptospirosis in India. *J Clin Microbiol 42:* 3575–3580.
17. Salaun L, Merien F, Gurianova S, Baranton G, Picardeau M, 2006. Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. *J Clin Microbiol 44:* 3954–3962.
18. Slack AT, Dohnt MF, Symonds ML, Smythe LD, 2005. Development of a multiple-locus variable number of tandem repeat analysis (MLVA) for *Leptospira interrogans* and its application to *Leptospira interrogans* serovar Australis isolates from far north Queensland, Australia. *Ann Clin Microbiol Antimicrob 4:* 10.
19. Pavan ME, Cairo F, Brihuega B, Samartino L, 2008. Multiple-locus variable-number tandem repeat analysis (MLVA) of *Leptospira interrogans* serovar Pomona from Argentina reveals four new genotypes. *Comp Immunol Microbiol Infect Dis 31:* 37–45.
20. Ahmed N, Devi SM, Valverde Mde L, Vijayachari P, Machangu RS, Ellis WA, Hartskeerl RA, 2006. Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann Clin Microbiol Antimicrob 5:* 28.
21. Thaipadungpanit J, Wuthiekanun V, Chierakul W, Smythe LD, Petkanchanapong W, Limpaiboon R, Apiwatanaporn A, Slack AT, Suputtamongkol Y, White NJ, Feil EJ, Day NP, Peacock SJ, 2007. A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Negl Trop Dis 1:* e56.
22. Levett PN, 2007. Sequence-based typing of *Leptospira*: epidemiology in the genomic era. *PLoS Negl Trop Dis 1:* e120.
23. Herrmann JL, Bellenger E, Perolat P, Baranton G, Saint Girons I, 1992. Pulsed-field gel electrophoresis of *Not*I digests of leptospiral DNA: a new rapid method of serovar identification. *J Clin Microbiol 30:* 1696–1702.
24. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, Barrett TJ, 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens Dis 3:* 59–67.
25. Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kubota K, Graves L, Wrigley D, Barrett T, Ribot E, 2005. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. *J Clin Microbiol 43:* 1045–1050.
26. Tenover FC, Arbeit RD, Goering RV, Mickelsen PA, Murray BE, Persing DH, Swaminathan B, 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol 33:* 2233–2239.
27. Kmety E, Dikken H, 1993. *Classification of the Species Leptospira interrogans and History of its Serovars*. Groningen, the Netherlands: University Press Groningen.
28. Petersen AM, Boye K, Blom J, Schlichting P, Krogfelt KA, 2001. First isolation of *Leptospira fainei* serovar Hurstbridge from two human patients with Weil's syndrome. *J Med Microbiol 50:* 96–100.
29. Arzouni JP, Parola P, La Scola B, Postic D, Brouqui P, Raoult D, 2002. Human infection caused by *Leptospira fainei. Emerg Infect Dis 8:* 865–868.
30. Ciceroni L, Ciarrocchi S, Ciervo A, Petrucca A, Pinto A, Calderaro A, Viani I, Galati L, Dettori G, Chezzi C, 2002. Differentiation of leptospires of the serogroup Pomona by monoclonal antibodies, pulsed-field gel electrophoresis and arbitrarily primed polymerase chain reaction. *Res Microbiol 153:* 37–44.