Interlaboratory Agreement of Pulsed-Field Gel Electrophoresis Identification of *Leptospira* Serovars

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Abstract. Leptospirosis may be caused by > 250 *Leptospira* serovars. Serovar classification is a complex task that most laboratories cannot perform. We assessed the interlaboratory reproducibility of a pulsed-field gel electrophoresis (PFGE) identification technique developed by the Centers for Disease Control and Prevention (CDC). Blinded exchange of 93 Leptospiraceae strains occurred between San Antonio Military Medical Center (SAMMC) and the CDC. PFGE was performed and gel images were analyzed and compared with patterns present in each laboratory's database (CDC database: > 800 strain patterns; SAMMC database: > 300 strain patterns). Overall, 93.7% (74 of 79) of strains present in each receiving laboratory's database were correctly identified. Five isolates were misidentified, and two isolates did not match serovar PFGE patterns in the receiving laboratory's database. Patterns for these seven isolates were identical between laboratories; four serovars represented misidentified reference strains. The PFGE methodology studied showed excellent interlaboratory reproducibility, enabling standardization and data sharing between laboratories.

INTRODUCTION

Leptospirosis is a zoonotic disease of worldwide distribution caused by spirochetal bacteria of the family Leptospiraceae. Bacteria of the genus *Leptospira* have been further characterized into more than 250 serovars. Diagnosis of leptospirosis and identification of these organisms is currently centered on serological methods. Serovar identification and traditional classification is based on the serological methodology cross-absorption agglutination testing (CAAT). CAAT is a complex, time-consuming technique that most laboratories lack the capability to perform. The gold standard for diagnostic testing in leptospirosis is microscopic agglutination testing (MAT), a complex test that requires the maintenance of reference *Leptospira* serovars in culture. *Leptospira* are slow growing and difficult to maintain long term in culture. Strains are continuously subcultured and often exchanged, and they may become mislabeled or otherwise misidentified.¹ A reproducible, relatively easy molecular methodology to identify leptospires in clinical culture and verify reference strains is greatly needed. The Centers for Disease Control and Prevention (CDC; Atlanta, GA) has established pulsed-field gel electrophoresis (PFGE) as a reliable molecular method to identify *Leptospira* serovars.² This method has been suggested as a more convenient method to identify clinical isolates of *Leptospira*.^{1,3–5} PFGE has been used to identify *Leptospira* from culture collections and study disease outbreaks.^{5–7} Our study evaluated the interlaboratory reliability of PFGE identification of leptospiral serovars between the CDC and the San Antonio Military Medical Center (SAMMC; Fort Sam Houston, TX) leptospirosis laboratories.

METHODS

Isolates. Ninety strains of *Leptospira* and three strains of *Leptonema* were exchanged in a blinded fashion between

the leptospirosis laboratories at the SAMMC and the CDC. Thirty-six coded strains were sent to the CDC from the SAMMC; 57 strains were sent to the SAMMC from the CDC. Strains included reference, clinical, and uncharacterized isolates. Exchanged strains underwent PFGE at both the providing and receiving facilities.

PFGE. Strains were grown in Ellinghausen McCullough Johnson Harris (EMJH) medium (Becton Dickinson, Sparks, MD) for 7 days at 30°C. PFGE was performed according to the method developed at the CDC.² Not I-restricted Leptospiraceae DNA was separated using the CHEF Mapper or the CHEF-DRIII Chiller PFGE system (Bio-Rad Laboratories, Hercules, CA). *Salmonella enterica*, serovar Braenderup H9812 DNA restricted with Xba I was used as a standard.

PFGE data analysis and *Leptospira* identification. Gel images were analyzed using BioNumerics software (Applied Maths, Austin, TX). PFGE patterns of exchanged strains were compared with PFGE patterns of reference strains in each receiving laboratory's BioNumerics database for serovar identification. The CDC database contained PFGE patterns for more than 800 isolates; the SAMMC database contained PFGE patterns for more than 300 isolates.

RESULTS

Of 93 exchanged strains, PFGE reference patterns for 79 strains were available in the databases of the receiving laboratories; 74 of 79 (93.7%) strains were correctly identified in the blinded exchange. The SAMMC laboratory correctly identified 43 of 57 strains; 2 strains were misidentified, and 12 strains were not present in their database (Figure 1). The CDC laboratory correctly identified 31 of 36 strains. Three strains were misidentified, and two strains did not match serovars in their database (Figure 2). PFGE patterns of all isolates studied were identical at both participating laboratories. All misidentifications were proven to be because of incorrectly labeled PFGE patterns in one of the two laboratories from misidentified or possibly contaminated reference strains (Figure 3). Confirmation of the identity of these reference serovars was pursued by obtaining the same strains from

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FIGURE 1. PFGE patterns and serovar identification of isolates provided by the CDC and identified at the SAMMC laboratory. CDC C = clinical isolates provided by the CDC; CDC R = reference isolates provided by the CDC; REF = reference strains in the SAMMC database. *Misidentified serovars with correct names shown in the figure. **Isolates for which no reference strains were available in the receiving laboratory's database, resulting in no identification by PFGE.

FIGURE 2. PFGE patterns and serovar identification of isolates provided by the SAMMC and identified at the CDC laboratory. REF = reference strains in the CDC database; SAMMC C = clinical isolates provided by the SAMMC; SAMMC R = reference isolates provided by the SAMMC. *Misidentified serovars with correct names shown in the figure. **Isolates for which no reference strains were available in the receiving laboratory's database, resulting in no identification by PFGE.

other reference laboratories (e.g., KIT Leptospirosis Reference Center, Amsterdam, The Netherlands) and using their PFGE pattern for comparisons as well as performing multi-locus sequence typing (MLST) and 16S rRNA gene sequencing when appropriate.

Of five misidentified strains, four strains were misidentified because of reference strains being mislabeled at some historical point. It was determined that *L. inadai* serovar Kaup (Figure 3A), *L. santarosai* serovar Machiguengai (Figure 3B), *L. kirschneri* serovar Cynopteri (Figure 3C), and *L. meyeri* serovar Ranarum (data not shown) were mislabeled, because their PFGE patterns differed from other confirmed strains that existed in the PFGE databases. Strain SAMMC R17 was

mislabeled in the SAMMC database as *L. inadai* serovar Kaup, and the CDC identified this strain as *L. inadai* serovar Biflexa by PFGE using their confirmed database strain. Strain SAMMC R20 was mislabeled in the SAMMC database as *L. santarosai* serovar Machiguengai, and the CDC identified it as *L. interrogans* serovar Icterohaemorrhagiae by PFGE using their confirmed database strain. Strain CDC R35 was mislabeled as *L. kirschneri* serovar Cynopteri in their database, and the SAMMC identified the strain as *L. inadai* serovar Lyme. Strain CDC R30 was mislabeled as *L. meyeri* serovar Ranarum in their database, and the SAMMC identified the strain as *L. illini* serovar Illini. Despite the mislabeling, both institutions got the same PFGE pattern for all of the isolates.

FIGURE 3. Examples of misidentified serovars. Despite the mislabeling, both laboratories got the same PFGE patterns for the isolates. (A) Strain SAMMC R17 was mislabeled in the SAMMC database as *L. inadai* serovar Kaup. The CDC identified this strain as *L. inadai* serovar Biflexa using their confirmed reference strain. (B) Strain SAMMC R20 was mislabeled in the SAMMC database as *L. santarosai* serovar Machiguengai. The CDC identified the strain as *L. interrogans* serovar Icterohaemorrhagiae using their confirmed reference strain. (C) Strain CDC R35 was mislabeled as *L. kirschneri* serovar Cynopteri in the CDC database. The SAMMC identified the strain as *L. inadai* serovar Lyme using their confirmed reference strain. REF = reference strains in the SAMMC or CDC database.

The correct reference isolates were obtained from KIT (Koninklijk Instituut voor de Tropen/Royal Tropical Institute) Leptospirosis Reference Center and the National Veterinary Services Laboratories (NVSL, Ames, IA) to replace the misidentified strains in the collections and databases. The fifth misidentified strain was SAMMC C19. The SAMMC's C19 isolate was originally labeled *L. kirschneri* serovar Grippotyphosa; however, the PFGE pattern at the CDC matched *L. interrogans* serovar Canicola. The SAMMC resent isolate C19, and the CDC got a different pattern that now matched *L. kirschneri* serovar Grippotyphosa. It was determined that the isolate was mislabeled at the time of shipping to CDC.

DISCUSSION

Study of the epidemiology of leptospirosis is currently hampered by limitations in our ability to rapidly and reliably diagnose this infection and recover and identify the infecting organism. The gold standard diagnostic test, MAT, relies on the use of reference strains of the major serovars. These live antigens need to be maintained in culture. Because of the difficulty in maintaining this slow-growing organism, cultures occasionally fail to maintain viability and need to be replaced from frozen stock or obtained from new samples from other laboratories. These cultures are maintained over long periods of time and can occasionally become mislabeled or cross-contaminated. Until recently, no convenient reliable methodology has been available to monitor these cultures for purity. Identification of *Leptospira* in these reference stocks as well as those stocks recovered from clinical cultures currently requires the cumbersome serological method CAAT, a technique limited to an even smaller number of reference laboratories. Several molecular methodologies have been developed and evaluated over the past decade. These methodologies

include PFGE, DNA sequencing, and MLST.⁸⁻¹⁰ Molecular techniques have been applied to both the evaluation of the identity of reference strains and the identification of new or potentially new serovars of *Leptospira*.

Our study showed that the use of PFGE as a molecular typing method for *Leptospira* serovars is associated with excellent interlaboratory reproducibility. This high level of reproducibility has the advantage of standardization among laboratories, ensuring consistent data, and it allows for the ability of data sharing (both reference databases and individual PFGE patterns) between laboratories. The potential usefulness of this methodology to also maintain the integrity of reference serovars and the database is clearly supported by this study; 8.9% (5 of 79) of isolates exchanged in this study had been misidentified or had cultures mislabeled. Although both laboratories produced the same PFGE patterns for each isolate, errors occurred when the reference isolate used to compare unknown isolates was itself incorrect (misidentified). With the paucity of good clinical laboratory tests for leptospirosis, MAT continues to be the gold standard for diagnosis. The accuracy of this test is wholly reliant on the maintenance of *Leptospira* of clearly identified serovars. Our study confirms findings from other studies, which have examined the accuracy of serovar identification, that the integrity of reference cultures has been shown fallible.¹ PFGE of *Leptospira* seems to be a useful and accurate tool for molecular characterization and routine identification of leptospiral serovars. This tool has excellent potential to assist in the maintenance of reference culture collections as well as the investigation of the epidemiology of leptospirosis and identification of potential new serovars and species. The high interlaboratory reproducibility showed in this study shows that PFGE is an excellent tool for data sharing and allows for the comparison of isolates between laboratories.

Received December 19, 2012. Accepted for publication April 15, 2013.

Published online July 1, 2013.

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