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Monitoring *Leptospira* Strain Collections: The Need for Quality Control

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The purpose of this study was to perform a 16S sequence-based quality control of two *Leptospira* strain collections. 16S rRNA gene sequencing was used to verify two *Leptospira* reference collections provided by the World Health Organization and maintained at a reference laboratory for leptospirosis in Brazil. Among the 89 serovars evaluated, four conflicting strains were identified in one of the collections. Although 16S rRNA gene sequencing cannot identify *Leptospira* beyond the species level, it is suitable for the identification of contamination and quality control of leptospiral reference collections. This study highlights the importance of the availability of high-quality 16S rRNA sequences in public databases. In addition, it emphasizes the need for periodical verifications and quality control of *Leptospira* reference collections.

INTRODUCTION

Leptospirosis is a potentially serious infectious disease caused by pathogenic *Leptospira* spp. that are maintained in a broad spectrum of mammalian reservoirs.[1–3] Currently, pathogenic leptospires are classified into nine pathogenic and four intermediate species, containing more than 260 serovars, and six saprophytic species, including over 60 serovars.[4,5] The reference test for the diagnosis of leptospirosis is the microscopic agglutination test (MAT), which is based on evaluating paired serum samples and their ability to agglutinate reference serovar strains with a battery of live *Leptospira* antigens.[6]

There are several potential problems associated with the maintenance of reference collections (RC) of *Leptospira* strains. Strain contamination, with non-*Leptospira* spp. or rapid-growing saprophytic leptospires, and mislabeling or switching of strains can be problematic.[7] Non-leptospiral contamination is easily identified on microscopic examination; however, strain switching is a major concern. Furthermore, if there is no adequate quality control carried out on the reference strains, these problems may not be identified in a timely manner. This could adversely affect outbreak investigations and epidemiological studies. The greatest burden of leptospirosis is in the developing world where the reference laboratories do not have quality-control measures in place or the capacity for the long-term storage of culture collections. These two factors increase the potential for widespread contamination and switching of the *Leptospira* strains used in the diagnosis of leptospirosis.

Ideally, the strains from a reference collection should be routinely confirmed using monoclonal antibodies or reference sera;[8] however, this is an expensive option. A robust method for the molecular speciation of bacteria is 16S rRNA gene sequencing,[9] and this has been applied to *Leptospira* spp.[10,11] Morey and others[12] found that this technique was a powerful yet simple tool for the identification of *Leptospira* species in a clinical setting. It has several important advantages including rapid turnaround time, widespread availability, and relative low cost. The present study applied 16S rRNA gene sequencing to validate two independent reference collections of *Leptospira* strains. We show that this technique was able to correctly identify the *Leptospira* spp. and also correct several cross-contaminated serovars.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The MAT battery strains were provided by the World Health Organization (WHO) reference center at the Royal Tropical Institute (KIT, Amsterdam, The Netherlands) and maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid media (Difco) at the Oswaldo Cruz Foundation (Fiocruz), Salvador, Brazil. Cultures were grown in liquid EMJH media at 30°C for up to 7 days before harvesting for DNA extraction. The strains included those from a panel (n = 29) received in 1999 (RC-99) and an expanded collection of reference strains (n = 60) received in 2004 (RC-04). RC-99 was maintained by subculturing, and no quality control had been performed on the strains. All of the reference strains in RC-04 were fully characterized by serological assays using monoclonal antibodies at KIT.

DNA extraction and 16S rRNA sequencing. Leptospiral genomic DNA was extracted using the GFX Genomic Blood DNA Purification Kit according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ). The 16S rRNA gene was amplified by the universal primers fD1 and rP2[13] and sequenced using internal primers: F2 5′-GGCGGCGC GTCTTAAACATG; F4 5′-GTGCCAGCAGCCGCGGTAA; F6 5′-AGTGAACGGGATTAGATACC; F12 5′-ACACAC GTGCTACAATGGCCG; and R3 5′-TCTTAACTGCTG CCTCCC; R11: CCTAGACATAAAGGCCATGA.[14] PCR amplification was performed using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) and the following cycling conditions: one denaturing cycle at 94°C for 2 minutes; 35 cycles of denaturing at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and elongation at 72°C for 45 seconds; and a final elongation at 72°C for 10 minutes. The amplified products were analyzed by 1% agarose gel electrophoresis. The sequencing was performed using a MegaBACE 500 DNA sequencer and the Dynamic ET-terminator technology (GE Healthcare).

Sequence assembly and alignment. Sequences were assembled using the Contig Express software (Invitrogen) and submitted to basic local alignment search tool (BLAST)

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alignment (information available at www.ncbi.nlm.nih.gov/BLAST). Based on a previous study by Morey and others,[12] we determined a cut-off point of 1,000 base pairs (bp) to represent the minimum sequence length for inclusion in this study. The phylogenetic analysis was performed using the MEGA 4 software,[15] which employed 1,000 bootstrap replications and the maximum parsimony method.

Nucleotide sequence accession numbers. The nucleotide sequences from the different strains generated during this study were submitted to GenBank under the accession numbers FJ154542–FJ154600.

RESULTS

16S sequencing-based quality control of reference collections. The 16S rRNA sequences from the 29 reference strains belonging to the RC-99 collection were determined (Table 1). Based on nucleotide BLAST alignments using the Mega BLAST tool,[16] the 16S rRNA sequences were compared with those in the GenBank database. There was no evidence of contamination or serovar switching in the RC-99 strains. Thirteen of these sequences were deposited in GenBank, five of which represent previously undeposited sequences (Table 1).

The 16S rRNA sequences from 60 strains belonging to RC-04 ranged from 1150 to 1473 bp in length (Table 1). Forty-four sequences were deposited in GenBank, and of these, eight corresponded to previously undeposited sequences (Table 1). Among the sequences generated, 25 contained nucleotides (nt) 55-1423, 21 contained nt 55-1230, and 9 contained nt 94-1230. The sequences belonging to the intermediate and saprophyte strains included nt 144-1165 and 48-1217, respectively. Note that the nt coordinates are based on the *L. interrogans* serovar Icterohaemorrhagiae strain RGA 16S rRNA sequence (accession number AY631894). One sequence (*L. santarosai* serovar Rioja strain MR12) was below the cut-off point of 1 kb and was excluded from further analysis. Of the 60 *Leptospira* reference strains included in this study, 57 were confirmed either by identification through Mega BLAST (data not shown), comparison with the RC-99 16S rRNA gene sequences (Table 1), or global alignment (Figure 1).

In four cases, we found 16S rRNA sequences that apparently mismatched either their equivalent entries in GenBank or the sequences from the corresponding strains in RC-99 (Table 1). The *L. kirschneri* strain Erinaceus Auritus 670 16S sequence was 100% identical to the *L. kirschneri* 3522 C strain. However, another frozen aliquot of this strain was cultured and sequenced, and analysis of the rRNA sequence correctly identified the *L. kirschneri* Erinaceus Auritus 670 strain. Additionally, the 16S rRNA sequence of *L. weilii* Celledoni strain aligned with the *L. borgpetersenii* serovars. A second aliquot of the *L. weilii* Celledoni strain was cultured, sequenced, and confirmed as the expected strain, indicating that the strain was mislabeled during subculturing for DNA preparation. The third potential problem was the finding that the *L. noguchii* LT 796 strain showed 100% sequence identity with strain *L. noguchii* 1161 U of the same species. However, this is in accord to a previous report that mentions the renaming of *L. noguchii* LT 796 to 1161 U, confirming that the two strains are identical (Table 1).[8] The final problem identified was with the *L. santarosai* LT 117 strain, because the 16S sequence aligned with the *L. interrogans* serovars rather than with the *L. santarosai* strains. Sequencing of further aliquots of the *L. santarosai* LT 117 strain presented the same problem, suggesting that a contamination or mislabeling event occurred before the strain was stored. This was confirmed by serological characterization at KIT, and therefore, the strain was considered lost in the RC-04 collection (Table 1) and replaced by the correct strain in the KIT collection.

DISCUSSION

Leptospiral 16S rRNA gene sequencing has long been used as a typing method for molecular characterization of isolates, certification of bacterial panels, and taxonomic applications.[10–12,17,18] DNA sequencing has several advantages over other typing methods, because it is relatively cheap and available, does not require complex reagents such as type-specific sera or purified DNA, and is not a time-demanding or laborious technique. Among some sequences, only partial coverage of the query sequence with the existing GenBank databases was observed. We also found several undefined bases in some sequences (both query and subject) that precluded the finding of 100% identity and consequently, impacted negatively on the identification of the reference strains. Although efforts have been made by several groups to deposit full-length (~1,500 bp), high-quality 16S rRNA sequences, there is still more work with respect to creating a complete set of sequences available in databases, such as GenBank, so that this method can be easily applied and interpreted. Indeed, as previously noted by Victoria and others,[19] *Leptospira* speciation errors can occur in reference collections, raising the possibility that some of the 16S rRNA sequences available in public databases may not be valid.

In this study, the sequencing of the 16S rRNA gene was used for quality control of reference strains from RC-99 and RC-04 (Table 1). The results were mainly concordant, although some discrepancies were observed in RC-04. Four strains of the 89 evaluated presented as contaminated cultures, and three were identified as existing strains from RC-04, probably caused by switching or mislabeling of strains during subculturing. Although 16S sequencing is a useful technique, it can only discriminate *Leptospira* strains to the species level because of the highly conserved nature of the 16S rRNA genes. Before departure from KIT, the RC-04 panel was fully characterized using monoclonal antibodies, and it did not reveal the existence of any problems. Immediately on arrival, the RC-04 collection was quality controlled by the 16S sequencing, which showed the existence of possible switching or mislabeling of strains. Although the 16S rRNA gene sequencing presents limitations for the identification beyond the species level, we believe that a retyping of the strains confirmed as problems would provide similar results. The approach used for quality control seemed to be satisfactory for monitoring *Leptospira* strain collections and highlighted the importance of serological verifications. The ramifications of switching strains in *Leptospira* reference collections can be serious. During outbreak investigations, the serogroup is identified by the MAT and the panel of strains maintained at the local reference laboratory. Furthermore, few reference laboratories have the necessary facilities to maintain frozen stocks of their strain collections. Rather, the panels are maintained for years by repeated subculturing, significantly increasing the chances of strain switching or mislabeling. In a proficiency trial of the MAT, it was found that diagnostic laboratories often reported erroneous results.[7] The serogroup was

TABLE 1
Leptospira reference strains and isolates

Species	Serogroup	Serovar	Strain	16S sequence length (bp)		Accession number
				RC-99	RC-04	
L. biflexa	Andamana	Andamana	CH 11	1266	1420*	FJ154577
L. borgpetersenii	Ballum	Ballum	MUS 127	741	1431*	FJ154591
L. borgpetersenii	Ballum	Castellonis	Castellon 3	1318*†	1315	FJ154579
L. borgpetersenii	Javanica	Javanica	Veldrat Batavia 46		1430*	FJ154600
L. borgpetersenii	Javanica	Ceylonica	Piyasena		1342*	FJ154596
L. borgpetersenii	Javanica	Poi	Poi	1302*	1296	FJ154597
L. borgpetersenii	Mini	Mini	Sari	680	1473*	FJ154592
L. borgpetersenii	Sejroe	Hardjo	Lely 607	1391*†	1338	FJ154586
L. borgpetersenii	Sejroe	Sejroe	M 84		1320*	FJ154593
L. borgpetersenii	Tarassovi	Tarassovi	Perepelitsin	669	1366*	FJ154595
L. fainei	Hurstbridge	Hurstbridge	BUT 6		1321*	FJ154578
L. interrogans	Australis	Australis	Ballico	1431*	411	FJ154556
L. interrogans	Australis	Bratislava	Jez Bratislava	1371*		FJ154547
L. interrogans	Australis	Muenchen	Muenchen C90		1377*	FJ154565
L. interrogans	Autumnalis	Autumnalis	Akiyami A	1431*	1256	FJ154543
L. interrogans	Autumnalis	Rachmati	Rachmat	1306*†	1261	FJ154548
L. interrogans	Bataviae	Bataviae	Swart	1325	1467*	FJ154557
L. interrogans	Bataviae	Bataviae	Van Tienen		1180*	FJ154566
L. interrogans	Canicola	Canicola	Hond Utrecht IV	1442*	1265	FJ154561
L. interrogans	Djasiman	Djasiman	Djasiman	1150	1319*	FJ154550
L. interrogans	Grippotyphosa	Muelleri	RM2		1403*†	FJ154568
L. interrogans	Hebdomadis	Hebdomadis	Hebdomadis	1161	1320*	FJ154551
L. interrogans	Hebdomadis	Kremastos	Kremastos		1351*	FJ154564
L. interrogans	Icterohaemorrhagiae	Copenhageni	M 20	1432*	426	FJ154542
L. interrogans	Icterohaemorrhagiae	Copenhageni	Wijinberg		1347*	FJ154569
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Kantorowicz		1344*†	FJ154563
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	1359	1464*	FJ154549
L. interrogans	Icterohaemorrhagiae	Icterohaemorrhagiae	Verdun	1340*†	1289	FJ154555
L. interrogans	Pomona	Kennewicki	LT 1026		1273*†	FJ154571
L. interrogans	Pomona	Pomona	Pomona	1431*	869	FJ154544
L. interrogans	Pyrogenes	Manilae	LT 398		1380*†	FJ154545
L. interrogans	Pyrogenes	Pyrogenes	Salinem	1431*†	1346	FJ154552
L. interrogans	Sejroe	Hardjo	Hardjoprajitno	1322	1435*	FJ154553
L. interrogans	Sejroe	Wolffi	3705	1417*†	1103	FJ154558
L. kirschneri	Australis	Ramisi	Musa		1373*	FJ154573
L. kirschneri	Autumnalis	Erinaceiauriti	Erinaceus Auritus 670		1400‡–1405*	FJ154560
L. kirschneri	Canicola	Galtoni	LT 1014		1182*	FJ154567
L. kirschneri	Cynopteri	Cynopteri	3522 C	1280	1464*	FJ154546
L. kirschneri	Grippotyphosa	Grippotyphosa	Duyster	1091	1280*†	FJ154554
L. kirschneri	Grippotyphosa	Grippotyphosa	Mandemakers		1378*†	FJ154570
L. kirschneri	Grippotyphosa	Grippotyphosa	Moskva V		1335*	FJ154572
L. kirschneri	Hebdomadis	Kambale	Kambale		1419*	FJ154562
L. kirschneri	Pomona	Mozdok	5621		1471*	FJ154559
L. meyeri	Semaranga	Semaranga	Veldrat Semarang 173		1384*	FJ154599
L. noguchii	Australis	Nicaragua	1011		1237*	FJ154574
L. noguchii	Louisiana	Louisiana	LSU 1945	1110	1150*	FJ154587
L. noguchii	Louisiana	Orleans	LSU 2580		1332*	FJ154588
L. noguchii	Panama	Panama	CZ 214 K	1312	1452*	FJ154582
L. noguchii	Pomona	Proechimys	1161 U§	1153	1336*†	FJ154575
L. noguchii	Pomona	Proechimys	LT 796§		1122‡	NA
L. santarosai	Grippotyphosa	Canalzonae	CZ 288		1410*	FJ154584
L. santarosai	Mini	Georgia	LT 117		1292‡	NA
L. santarosai	Pomona	Tropica	CZ 299		1276*	FJ154583
L. santarosai	Pyrogenes	Alexi	HS 616		1443*	FJ154585
L. santarosai	Sejroe	Trinidad	TRVL 34056		1472*†	FJ154598
L. santarosai	Shermani	Shermani	1342 K	1170	1431*	FJ154576
L. santarosai	Tarassovi	Bakeri	LT 79		1448*	FJ154589
L. weilii	Celledoni	Celledoni	Celledoni	1431*	1377‡	FJ154580
L. weilii	Javanica	Coxi	Cox		1252*	FJ154581
L. weilii	Tarassovi	Vughia	LT 89-68		1467*	FJ154590

* Sequence deposited in GenBank.
† Previously undeposited in GenBank.
‡ Strain contaminated.
§ Identical strains. LT 796 was renamed as *L. noguchii* strain 1161 U.
NA = not applicable.

often incorrectly identified, resulting in false-negative and positive results. They concluded that contamination, mislabeling, and deterioration of the live cultures because of repeated subculturing likely contributed to these errors. Therefore, there is a need to establish an inexpensive quality-control method to identify these problems.

Polymorphisms within the 16S rRNA gene sequences of pathogenic *Leptospira* spp. were reported to range from

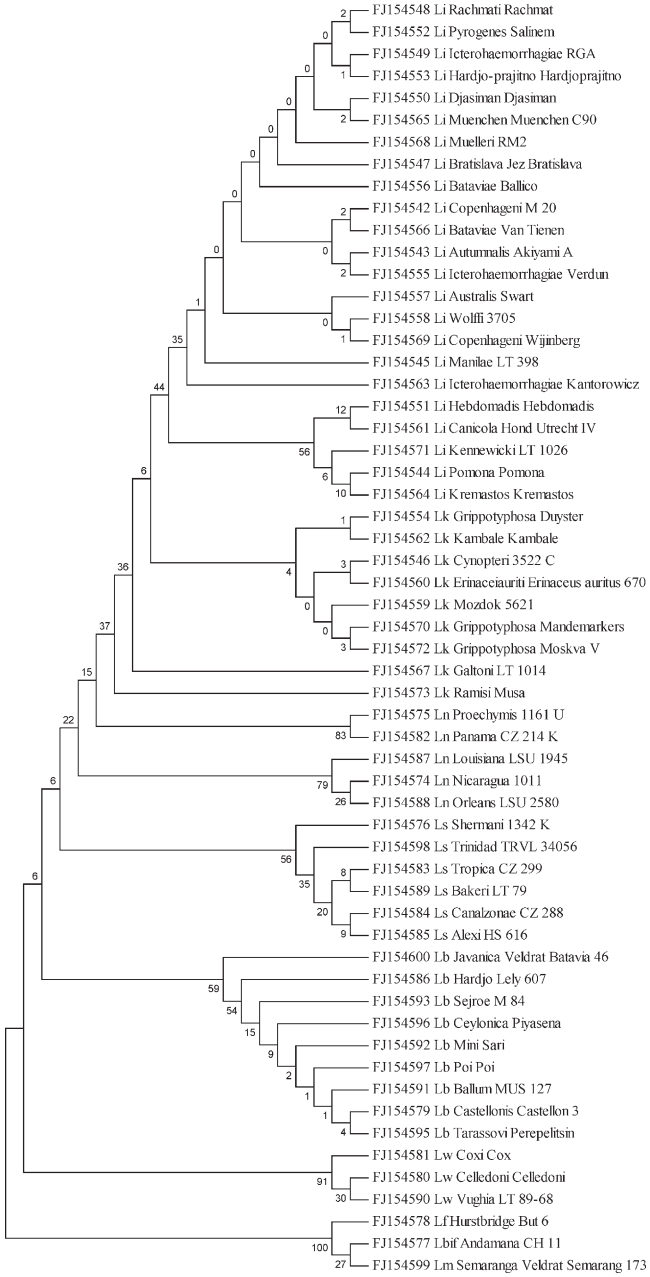

FIGURE 1. Dendrogram based on the 16S rRNA gene sequences from *Leptospira* spp. The dendrogram was built from a 1,291 bp-based alignment of nucleotide sequences by the Neighbor-joining method, using 1,000 bootstrap replications. T is indicative of type-strains. Accession numbers are presented, and they are followed by the species, serovar, and strain designations, respectively. Bootstrap values are displayed as percentages. Species names were abbreviated: Li, *L. interrogans*; Lk, *L. kirschneri*; Ln, *L. noguchii*; Ls, *L. santarosai*; Lb, *L. borgpetersenii*; Lw, *L. weilii*; Lf, *L. fainei*; Lbif, *L. biflexa*; Lm, *L. meyeri*.

1 to 19 nt among the pathogenic species.[12] In this study, we observed a similar number of mismatches (1–13 nt, data not shown). As with previous studies, although it was possible to differentiate the species, we found that it was not possible to discriminate between the serovars because of the high homology of the 16S rRNA genes.[12,20] Several groups have reported using shorter sequences (≤ 500 bp) in other genes to improve the discriminatory power over the 16S rRNA gene, including *secY*,[19] *gyrB*,[21] and *ligB*.[17] Alternative techniques such as variable-number tandem-repeat[22,23] and multi-locus sequence typing[24,25] also offer potential improvements over 16S rRNA gene sequencing. Further work is needed to evaluate these alternative strategies as applied to quality-control testing of *Leptospira* reference strains. This study has shown the need for periodical verifications and quality control in the maintenance of *Leptospira* culture collections. In addition, this study has highlighted the importance of the availability of high-quality 16S rRNA gene sequences in public databases.

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