Utility and Limitations of Direct Multi-Locus Sequence Typing on qPCR-Positive Blood to Determine Infecting *Leptospira* Strain

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Abstract

Culture-independent molecular characterization of infecting *Leptospira* human blood specimens from a 2008 outbreak of human leptospirosis in central Sri Lanka was carried out. Of 58 quantitative real-time polymerase chain reaction-positive samples analyzed for seven multi-locus sequence typing (MLST) housekeeping genes (*mreA*, *pfkB*, *pntA*, *sucA*, *tpiA*, *fadD*, and *glmU*), interpretable data was obtained from 12 samples. Mean bacterial load was 2.2×10^5 among specimens with complete MLST profiles compared with 1.3×10^4 among specimens without complete MLST profiles; all specimens with complete profiles had at least 4.9×10^4 *Leptospira*/mL ($t = 5$, $P < 0.001$). Most (11/12) identified sequence types were ST1 (*L. interrogans* serovar Lai) and ST44 (*L. interrogans* serovar Geyaweera). MLST can be used to directly identify infecting *Leptospira* strains in blood samples obtained during acute illness without the need for culture isolation, but it shows important limitations related to bacterial load.

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Introduction

Leptospirosis is a globally emerging zoonotic disease. Epidemiological investigations of leptospirosis outbreaks are a major challenge in most settings because of varied clinical presentation, difficult diagnosis, and complex disease transmission cycle. These challenges are particularly apparent in areas where the disease is highly endemic and resources and technical capacity are lacking. Because culture isolation is insensitive and may take up to several months for a definitive result, characterization of the infecting strain, especially in outbreak investigations, is difficult. Recently, sequence-based typing techniques (including multiple-locus variable number tandem repeat analysis [MLVA], multi-locus sequence typing [MLST], etc.) have emerged as alternatives to serotyping.[1] MLST has advantages in typing *Leptospira* because of reproducibility.[2] Two MLST schemes for *Leptospira* have been published.[2,3] Importantly, MLST has typically required isolation of *Leptospira* to provide sufficient DNA for analysis. Nonetheless, because of difficulties in obtaining isolates of *Leptospira* from clinical samples (expense, expertise, problems with contamination, and intrinsically poor isolation rates), being able to identify infecting *Leptospira* in clinical samples is a high priority for clinical, epidemiological, and basic science purposes. In this study, we used a previously publishe

MLST approach to identify the infecting strain in serum and whole-blood specimens collected during a 2008 outbreak of leptospirosis in Sri Lanka.

Methods

A systematically collected sample of serum and whole blood from a 2008 outbreak of leptospirosis in Sri Lanka was used in the present study. Patient recruitment, sample collection, and processing have been published previously.[4] Of the original 401 patients, we selected samples from 58 quantitative polymerase chain reaction (qPCR) -positive patients for the present study. For MLST, DNA was extracted from 100 μL serum or whole blood using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. For analysis, we used a previously published MLST scheme based on the amplification of seven housekeeping genes (*mreA*, *pfkB*, *pntA*, *sucA*, *tpiA*, *fadD*, and *glmU*).[3] Reaction mixes were prepared using HotStar Taq master mix (Qiagen) with 5 pmol each primer and 5 μL DNA in 25 μL total volume. Amplification was carried out using DNA Engine Dyad thermal cycler (BioRad, Hercules, CA). PCR products were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA) and Sanger-sequenced in both directions using Big Dye chemistry and an ABI automated sequencer. At least four reads were obtained for each sequence. Individual sequences were assembled using Genius Pro 5.4.6 software.[5] Samples from six patients included sequences with ambiguous bases, which are potentially indicative of mixed infections, and these samples were excluded from analysis. Assembled sequences were trimmed and aligned with reference sequences downloaded from leptospira.mlst.net to assign allele numbers for all seven loci. For strain identification, allelic profiles were searched against the *Leptospira* MLST database.

Results and Discussion

Of the 58 samples tested, 12 samples provided interpretable data. Of these 12 patients, 5 patients were severe cases with myocarditis and/or renal failure, 6 patients were uncomplicated (no specific end organ involvement), and one patient lacked clinical data. Amplification and sequencing reactions were repeated to improve confidence in the underlying data. Of the seven loci, the *glmU* data were the least reliable. Three samples had complete MLST profiles (i.e., sequences from all seven loci) (Table 1), allowing confident serovar assignment. Of the specimens from which we obtained incomplete MLST profiles (and hence, tentative serovar identification), one shared two (of two) alleles with *L. interrogans* serovar Geyaweera strain Geyaweera, which is unique to Sri Lanka. All other strain types (STs) were similar to ST1, *L. interrogans* sv Lai, strains Lai and 55631. Because the MLST profiles were mostly incomplete, nodes were poorly supported after 500 bootstrap replicates (data not shown).

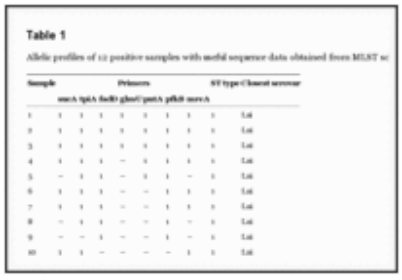
Table 1

Allelic profiles of 12 positive samples with useful sequence data obtained from MLST sc

Sample	Primers							ST type (Closest serovar
	secA	tpiA	fadD	glmU	pntA	pfkB	mreA	
1	1	1	1	1	1	1	1	Lai
2	1	1	1	1	1	1	1	Lai
3	1	1	1	1	1	1	1	Lai
4	1	1	1	–	1	1	1	Lai
5	–	1	1	–	1	1	–	Lai
6	1	1	1	–	–	1	1	Lai
7	1	1	1	–	–	1	1	Lai
8	–	1	1	–	–	1	–	Lai
9	–	–	1	–	–	1	–	Lai
10	1	1	–	–	–	1	1	Lai

Allelic profiles of 12 positive samples with useful sequence data obtained from MLST scheme

To determine whether the completeness of MLST profiles correlated with bacterial load, we compared mean leptospiremia of specimens with and without complete MLST profiles, which also included PCR-negative specimens. Mean bacterial load was 2.2×10^5 among specimens

with complete MLST profiles compared with 1.3×10^4 among specimens without, with a cutoff of 4.9×10^4 *Leptospira*/mL observed for specimens with complete profiles ($t = 5$, $P < 0.001$).

The data presented here suggest that ST1 was the predominant infecting ST among the study population during the 2008 outbreak of leptospirosis in Sri Lanka. Based on serology, ST1 seems to be *L. interrogans* serovar Lai, previously reported to be a highly virulent leptospire, and for the first time, it was reported to be present in Sri Lanka.[6] In the Sri Lanka setting, serovar Lai seems to be associated with both severe and non-severe disease, which has been reported both from China[6] and the Andaman Islands.[7] Whether the Sri Lanka outbreak of leptospirosis in 2008 was predominantly caused by ST1 is difficult to answer considering the incomplete MLST profiles and consequent poor bootstrap support. One possible explanation would be that some of the infecting serovars could be from other novel STs not currently represented in the MLST database.

The present study is the first report of using a full MLST typing scheme to directly identify infecting *Leptospira* in human clinical specimens. Although MLST was found to be relatively insensitive in this study and the poor amplification results might indicate limited homology between the primers used and target gene sequence, this method has important potential for future clinical, epidemiological, and basic science studies.

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Footnotes

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