# Characterization of *Leptospira* isolates from patients in Hawaii using molecular and serological techniques

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## Abstract

Hawaii has the highest incidence of leptospirosis in the United States. Leptospirosis in Hawaii is a reportable disease, and isolation of the organisms from patients is used when possible to make a definitive diagnosis in conjunction with serology. However, full characterization of the isolates has not been routinely performed. Here we present results of pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and serology for 43 isolates from patients in Hawaii received at the Centers for Disease Control and Prevention (CDC) between the years 2000-2008. Isolates were collected from the blood and urine of residents of Hawaii suspected of having leptospirosis and submitted to CDC for characterization. Microscopic agglutination testing (MAT) was performed by incubating the unknown isolate with a panel of reference sera to determine serogroup. PFGE was performed using the Not I restriction enzyme to identify the serovar. MLST was done on seven housekeeping genes and sequence types (STs) were determined from the resulting allelic profiles and compared to an established internet database. Cross agglutinin absorption assay (CAAT) was completed on isolates that required further identification or confirmation of new serovar status by absorbing sera with antigens and performing subsequent titrations. MAT and PFGE grouped isolates into four clades: 43% unknown serovar (serogroup Australis), 40% serovar Icterohaemorrhagiae (serogroup Icterohaemorrhagiae), 10% serovar Ballum (serogroup Ballum), and 7% unknown serovars (serogroup Bataviae). CAAT identified the unknown member from serogroup Australis as a new serovar, closely related to Lora. However, MLST identified this isolate as ST51, the same ST as reference serovar Australis, strain Ballico. The unknown isolate from serogroup Bataviae reacted with reference sera Waimea. Formally, Waimea has not been recognized yet as a new serovar. MLST is not applicable on the species of this isolate. PFGE, MLST, MAT and CAAT grouped isolates in a similar way but offer different information in terms of discriminatory ability and genetic relatedness. Hawaii yielded a diverse collection of isolates, many of which are unique. This study highlights the value of a multifaceted approach to characterize patient isolates of *Leptospira* in Hawaii.

## Introduction

Leptospirosis is endemic in Hawaii. Although the illness ceased to be a reportable disease nationally in 1995, it remains reportable in Hawaii due to the high incidence rate.[1] Laboratories in Hawaii continued to collect patient cultures; however the isolates had not been characterized due to a lack of resources. New molecular methods as well as classical serological methods are available to characterize *Leptospira* isolates. In this study, we used a multifaceted approach to characterizing isolates using pulsed-field gel electrophoresis (PFGE), multi locus sequence typing (MLST), microscopic agglutination test (MAT) and cross agglutinin absorption test (CAAT).

## Methods

**Bacterial isolates –** Forty-three isolates were obtained in EMJH media from the blood or urine of patients in Hawaii with leptospirosis between the years 2000-2008. The cultures were sent to the Centers for Disease Control and Prevention (CDC) for characterization.

**PFGE –** Lysed DNA was embedded into agarose plugs and digested using the Not I restriction enzyme. Digests were separated on a gel in a pulsed field electrophoresis chamber and banding patterns were analyzed and compared to a reference database for serovar identification.[2]

**MLST –** Seven housekeeping genes were amplified and sequenced to generate an allelic profile and was compared to reference allele sequence types.[3]

**MAT –** A panel of *Leptospira* reference antisera produced at CDC representing 23 serogroups was serially diluted and used in reactions with the clinical isolates. Resulting agglutination titers were read using darkfield microscopy, and isolates were identified to the serogroup level.[4]

**CAAT –** Cross agglutinin absorption tests were carried out using live reference strains for absorption that were serologically related to the unknown strain and absorbed overnight. The absorbed sera were then tested using MAT. If the resulting titration using absorbed sera with the unknown strain gave a titer that was less than 10% of the homologous titer, the unknown strain was considered to belong to the same serovar as the reference strain.[4] Unknown strains that could not be identified by cross agglutinin tests were designated for inoculation into rabbits to produce hyperimmune antisera.

## Results

- PFGE was performed on all 43 isolates. Of these, MLST, MAT and CAAT were performed on 9, 39 and 16 isolates respectively
- All four methods grouped isolates similarly (Figure 1)

**Figure 1. PFGE patterns, MAT, CAAT and MLST results of selected isolates from humans in Hawaii**

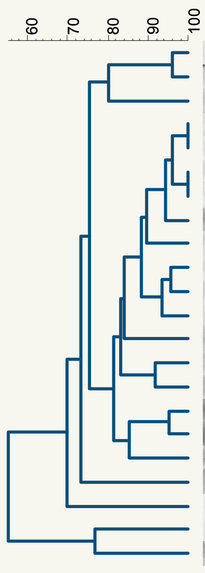

| PFGE Not I | ID | Serogroup (MAT) | Serovar (CAAT) | ST Type (MLST) |
|---|---|---|---|---|
| | 2002000626 | Australis | New | 51 |
| | 2002000627 | Australis | New | 51 |
| | 2002000621 | Australis | New[a] | 51 |
| | 2002000624 | Australis | New | 51 |
| | 2004018171 | Australis | New | 51 |
| | 2002000625 | Australis | New | 51 |
| | Reference strain | Australis | Australis | 51 |
| | 2007008424 | Icterohaemorrhagiae | Icterohaemorrhagiae | 17 |
| | 2008012813 | Icterohaemorrhagiae | Icterohaemorrhagiae | 17 |
| | Reference strain | Icterohaemorrhagiae | Icterohaemorrhagiae | 17 |
| | 2002000634 | Icterohaemorrhagiae | Icterohaemorrhagiae | 17 |
| | Reference strain | Ballum | Ballum S102 | NA |
| | 2002000620 | Ballum | Not done | NA |
| | Reference strain | Ballum | Ballum Mus 127 | NA |
| | 2007001578 | Bataviae | Waimea[b] | NA |
| | 2001034031 | Bataviae | Not done | NA |
| | 2004000358 | Bataviae | Not done | NA |

[a] Confirmed by Royal Tropical Institute (KIT) as a new serovar, closely related to Lora

[b] Confirmed by Royal Tropical Institute (KIT)

- Isolates identified as serogroup Icterohaemorrhagiae by MAT were serovar Icterohaemorrhagiae/Copenhageni by PFGE, MLST and one-way CAAT
- Isolates identified as serogroup Australis by MAT represent a potential new serovar
  - » PFGE pattern is new, but closely related to Australis
  - » Between 0-6 band differences (100%-75% similarity) among these isolates by PFGE (Figure 2)

**Figure 2. PFGE patterns, MAT, CAAT and MLST results for isolates from patients in Hawaii that represent a potential new serovar**




| PFGE Not I | ID | Serogroup (MAT) | Serovar (CAAT) | ST Type (MLST) |
|---|---|---|---|---|
| | 2002000623 | Australis | New | ND |
| | 2002000633 | Australis | New | ND |
| | 2002000630 | Australis | New | ND |
| | 2003000735 | Australis | New | ND |
| | 2003000972 | Australis | New | ND |
| | 2000010763 | Not done | New | ND |
| | Lepto0292 | Australis | New | ND |
| | 2002000621 | Australis | New* | 51 |
| | 2000010762 | Not done | New | ND |
| | 2002000624 | Australis | New | 51 |
| | 2004018171 | Australis | New | 51 |
| | 2005005624 | Australis | New | 51 |
| | 2002000629 | Australis | New | ND |
| | 2002000626 | Australis | New | 51 |
| | 2002000627 | Australis | New | ND |
| | 2002000625 | Australis | New | 51 |
| | 2002000631 | Australis | New | ND |
| | 2002000628 | Australis | New | ND |
| | Reference strain | Australis | Australis | 51 |
| | 2002000632 | Australis | New | ND |
| | Reference strain | Australis | Jalna | 24 |
| | Reference strain | Australis | Lora | 25 |

*Confirmed by KIT as a new serovar, closely related to Lora

  - » Two-way CAAT performed by KIT indicates a new serovar, closely related to Lora
  - » ST 51 by MLST, same ST type as reference serovar Australis
- Isolates identified as serogroup Ballum by MAT were serovar Ballum/Guangdong (Ballum 3)/Castellonis by PFGE. CAAT is in progress. Current MLST scheme is not appropriate for *L. borgpetersenii*
- Isolates identified as serogroup Bataviae represented 2 new patterns by PFGE of *L. noguchii* species
  - » Two-way CAAT performed by KIT was reactive with reference sera Waimea but needs to be formally named
  - » Current MLST scheme is not appropriate for *L. noguchii* species

## Discussion

We used a multifaceted approach involving both molecular and serological techniques to characterize isolates from patients in Hawaii collected over a period of 9 years. All 4 methods (PFGE, MLST, MAT and CAAT) grouped isolates similarly, but each method offered different information.

### PFGE

- Identified isolates to the serovar level
- Unable to distinguish between closely-related reference strains Icterohaemorrhagiae and Copenhageni, and Ballum, Guangdong (Ballum 3) and Castellonis
- Identified isolates that require further studies to determine their status as potential new serovars

### MLST

- Shows phylogenetic relationships
- Unambiguous results and internet database allows for direct comparison of isolates
- Not as discriminatory as PFGE or CAAT for some *Leptospira* isolates
  - » New PFGE pattern from Australis serogroup exhibited multiple differences in PFGE patterns but had the same ST type (Figure 2)
  - » Closely related to serovar Lora by CAAT, but ST Type matched reference serovar Australis. Lora and Australis ST Types differ at 6/7 MLST alleles

### MAT

- Least discriminatory, but the least costly method
- Requires large collections of live antigens and reference sera

### CAAT

- Gold standard for serovar identification and offers the highest discriminatory ability
- Requires an enormous amount of time, expertise, reagents and antigens to perform
- In our study, CAAT confirmed a new serovar that made up nearly half of the isolates collected from patients in Hawaii, and also identified one isolate as serovar Waimea, which has not yet been formally named

By using this multifaceted approach to characterize isolates from patients in Hawaii, we were able to gain an understanding of *Leptospira* isolates causing illness in Hawaii. We also identified a potentially new serovar (Australis serogroup) and another serovar (Waimea) unique to Hawaii.

## References

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