Predominance of the ST143 and ST50 *Leptospira* clones in the urban rat populations of Peninsular Malaysia

Leptospirosis is a zoonotic and an emerging infectious disease caused by the pathogenic *Leptospira* species (Levett, 2001). Leptospirosis is transmitted directly or indirectly through urine of infected animals or environments contaminated with urine of reservoir animals (Bharti et al., 2003; Ullmann & Langoi, 2011). Serovar identification of *Leptospira* isolates constitutes an important component in understanding leptospiral epidemiology and establishment of appropriate control and preventive measures, especially in areas where the disease is prevalent (Bourhy et al., 2013). To date, more than 300 serovars that belong to 29 serogroups have been identified and described (Saito et al., 2005; Slack et al., 2010). Consequently, various publications have appeared addressing several epidemiological questions, such as the prevalent *Leptospira* species and serovars in rodent hosts and the environment (Benacer et al., 2013a, b; Mohamed-Hassan et al., 2010, 2012; Sapian et al., 2012; Benacer et al., 2013b). However, there is no study available to date from Malaysia that employs MLST to explore the genetic diversity of pathogenic *Leptospira* species. Therefore, we initiated this research by analyzing local pathogenic *Leptospira* isolates by MLST to examine their genetic relationships.

Of the 63 *Leptospira* isolates analyzed, 60 isolates were recovered from rats and we included one isolate each from dog, swine and human (Table 1). The dog isolate (D7) was isolated from urine sample of a male stray dog, aged more than 2 years and that showed some skin lesions, while the swine isolate (LS5) was from a healthy female swine from a farm in Selangor state. Isolates in this study were identified and characterized as *Leptospira* using culture, microscopic agglutination test (MAT) and PFGE techniques; in addition, the pathogenic status of the strains was determined using PCR targeting the 16S rDNA and secY genes followed by sequencing (Benacer, et al., 2013a, 2016). The human isolate was provided by the Royal Tropical Institute (KIT), Amsterdam, Netherlands, and was originally isolated from a patient in Malaysia (Alexander et al., 1957). All isolates were maintained in culture in Ellinghausen McCullough Johnson Harris (EMJH) medium.

Genomic DNA was extracted from 7 day-old culture using Wizard™ Genomic DNA Purification Kit (Promega) following the manufacturer’s instructions. The quantity of DNA was measured by Biophotometer (Eppendorf). MLST was performed according to the procedures described by Boonsilp et al. (2013). Briefly, a 25 µl PCR reaction consisted of 1× PCR buffer, 1.5 mM MgCl₂, 100 ng of DNA template. The cycling conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for pntA, sucA, pykB, tpiA, mreA, caifB and 50 °C for glmU for 30 s, and extension at 72 °C for 2 min, and lastly a final extension at 72 °C for 10 min. The PCR products were analyzed by gel electrophoresis, purified using QIAquick PCR purification kit (Qiagen) and sent to a commercial sequencing facility (First BASE Laboratories Sdn Bhd).

Sequencing data were analyzed using Seq Scanner 2 (Applied Biosystems) and BioEdit software (http://www.ebio.ncsu.edu/bioedit/bioedit.html). Both the allelic numbers and sequence types (STs) were obtained from the *Leptospira* MLST database (http://leptospira.mlst.net/). In one case (isolate ID R183) raw sequencing data for both tpiA and caifB were submitted to the database curator for assignment of new allelic numbers and ST. The eBURST algorithm (http://leptospira.mlst.net/eburst/) was used to assign STs into clonal complexes and clones were considered genetically related if they are either single-locus variants (SLVs) or double-locus variants (DLVs) to each other. Genetic diversity was measured by using the Simpson’s index of diversity as described previously (Hunter & Gaston, 1988).

MLST analysis identified seven unique STs comprising six known clones and one new clone (ST205) (Table 1). The genetic diversity of the Malaysian pathogenic *Leptospira* population was low with Simpson’s index of diversity of 0.579. The most prevalent clone was ST143 (32 isolates, 50.8%), followed by ST50 (26 isolates, 41.3%). Two clonal complexes were identified by eBURST, one consisted of ST143–ST144 and the other one, ST37–ST140 with predicted primary founder ST143 and ST37, respectively.

In addition to serological methods, molecular techniques such as fluorescent amplified fragment length polymorphism (FAFLP) (Vijayachari et al., 2008), pulsed-field gel electrophoresis (PFGE) (Galloway & Levett, 2008, 2010) and multiple locus variable number tandem repeat analysis (MLVA) (Majed et al., 2005; Slack et al., 2005) have been applied for the characterization of leptospiral isolates. Recently, multilocus sequence typing (MLST) schemes were developed for pathogenic *Leptospira* species (Ahmed et al., 2006; Boonsilp et al., 2013). MLST has become the gold standard in the molecular epidemiological studies of various bacterial pathogens (Maiden, 1998) and this is attributed to its portability and standardized approach to data collection in addition to being cost-effective due to the dwindling cost in DNA sequencing. The usefulness of *Leptospira* MLST was exemplified by the successful identification of a pathogenic clone, ST34 that was responsible for outbreak of human leptospirosis in Thailand (Thaipadungpanit et al., 2003).

Leptospirosis is an endemic disease in Malaysia. The number of reported cases has increased significantly since the Ministry of Health Malaysia gazetted leptospirosis as a notifiable disease in 2010. The number of reported cases has increased significantly since the Ministry of Health Malaysia gazetted leptospirosis as a notifiable disease in 2010.
<table>
<thead>
<tr>
<th>Isolate ID</th>
<th>Source</th>
<th>Sample</th>
<th>Location</th>
<th>Species and serovar when available</th>
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Table 1. Properties of *Leptospira* isolates included in this study

Predominance of *Leptospira* clones in Malaysia

http://jmm.microbiologyresearch.org
The remaining three clones appeared as singletons. The predicted primary founder of each clonal complex differed with SLVs from its subgroup clone at the *caiB* locus by a single base pair substitution.

A unidirectional correlation in which the isolates with the same ST belonged to the same serovar was apparent in our study. However, in the MLST database, there are at least 18 STs that include two or three different serovars (Boonsilp et al., 2013). The genotypes were also host-specific and therefore no evidence of clonal transmission from maintenance hosts such as rodent, swine, and dog to human was observed. Nevertheless, more data from non-hosts and humans are required especially to track the source of infection in epidemic areas and during leptospirosis outbreaks.

Our findings suggested that despite the wide geographical distribution of our isolates, the genetic diversity for the pathogenic *Leptospira* population was surprisingly low. The success of two major clones, ST143 and ST50 was responsible for the low genetic diversity because both clones constitute about 92.0% of the isolates with the same ST belonged to the same *Leptospira* species, *Leptospira interrogans* and *Leptospira borgpetersenii* as the most abundant species circulating amongst rodents in Southeast Asia (Cosson et al., 2014). However, further comparison at strain level was not possible due to the different molecular typing methods used to determine the *Leptospira* genotype. Despite successfully identifying two dominant clones in the urban rat population, their significance especially in causing human infections remained unknown. Future studies involving pathogenic *Leptospira* isolates recovered from human cases are needed to complement the present study.

This work also confirmed the results of a recent publication which proposed two *Leptospira* species, *Leptospira interrogans* and *Leptospira borgpetersenii* as the most abundant species circulating amongst rodents in Southeast Asia (Cosson et al., 2014). However, further comparison at strain level was not possible due to the different molecular typing methods used to determine the *Leptospira* genotype.

### Acknowledgements

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#### References


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serovar javanica and leptospira interrogans
serovar bavariae as the persistent leptospira
serovars circulating in the urban rat
populations in peninsular malaysia
parasit vectors 9, 117.
bharti, a. r., nally, j. e., ricaldi, j. n.,
mattias, m. a., diaz, m. m., levett, m. a.,
levett, p. n., gilman, r. h., willig, m. r. &
other authors (2003). leptospirosis: a
zoonotic disease of global importance.
lancet infect dis 3, 757–771.
boonsilp, s., thaipadungpanit, j.,
amornchai, p., wuthiekanun, v.,
bailley, m. s., holden, m. t., zhang, c.,
jiang, x., koizumi, n. & other authors.
(2013). a single multilocus sequence typing
(mlst) scheme for seven pathogenic
leptospira species. plos negl trop dis 7,
e1954.
bourhy, p., herrmann storck, c.,
theodore, r., olive, c., nicolas, m.,
hochzed, p., lamaury, i., zini, f.,
brémont, s. & other authors (2013). serovar
diversity of pathogenic leptospira circulating in
the french west indies. plos negl trop dis 7,
e2114.
bourhy, p., collet, l., brisse, s. &
opicardeau, m. (2014). leptospira mayottensis
sp. nov., a pathogenic species of the genus
leptospira isolated from humans. int j syst evol
microbiol 64, 4061–4067.
cosson, j. f., picardeau, m., mielcarek, m.,
tatard, c., chaval, y., suputtamongkol, y.,
buchy, p., jittapalapong, s., herbreteau, v. &
other authors (2014). epidemiology of
leptospira transmitted by rodents in southeast
asia. plos negl trop dis 8, e2902.
evaluation of a modified pulsed-field gel
electrophoresis approach for the identification
of leptospira serovars. am j trop med hyg 78,
628–632.
galloway, r. l. & levett, p. n. (2010).
application and validation of pfge for serovar
identification of leptospira clinical isolates.
ploS negl trop dis 4, e824.
hunter, p. r. & gaston, m. a. (1988).
numerical index of the discriminatory ability of
typing systems: an application of simpson’s
index of diversity. j clin microbiol 26, 2465–
2466.
kanagavel, m., princess margreat, a. a.,
arunkumar, m., prabhakaran, s. g.,
shannughapriya, s. &
natarajaseenivasan, k. (2016). multilocus
sequence typing (mlst) of leptospiral strains
isolated from two geographic locations of
tamil nadu, india. infect genet evol 37, 123–
128.
microbiol rev 14, 296–326.
maiden, m. c. (2006). multilocus sequence
typing of bacteria. annu rev microbiol 60, 561–
588.
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.
mohamed-hassan, s. n., bahaman, a. r.,
mutalib, a. r. & khairani-bejo, s. (2010).
serological prevalence of leptospiral infection
in wild rats at the national service training
centres in kelantan and terengganu. trop
biomed 27, 30–32.
mohamed-hassan, s. n., bahaman, a. r.,
mutalib, a. r. & khairani-bejo, s. (2012).
prevalence of pathogenic leptospires in rats
from selected locations in peninsular malaysia.
jaAnimSc 6, 12–25.
saito, m., villanueva, s. y., kawamura, y.,
ila, k.-i., tomida, j., kanemaru, t.,
kohno, e., miyahara, s., umeda, a. &
other authors (2013). leptospira idonii sp. nov.,
isolated from environmental water. int j syst
evol microbiol 63, 2457–2462.
sapian, m., khair, m. t., how, s. h.,
rajalingam, r., sahhir, k., norazah, a.,
outbreak of melioidosis and leptospirosis co-
infection following a rescue operation. med j
malaysia 67, 293–297.
slack, a. t., dohnt, m. f., symonds, m. l. &
smythe, l. d. (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.
thaipadungpanit, j., wuthiekanun, v.,
chierakul, w., Smythe, l. d.,
petkanchanapong, w., limpaiboon, r.,
apiwanaporn, a., slack, a. t.,
suputtamongkol, y. & other authors (2007).
A dominant clone of Leptospira interrogans
associated with an outbreak of human
leptospirosis in Thailand. plos negl trop dis 1,
e56.
ullmann, l. s. & langoni, h. (2011).
interactions between environment, wild animals
and human leptospirosis. j venem Anim toxins
incl trop Dis 17, 119–129.
vijayachari, p., ahmed, n., sugunan, a. p.,
ghousunisssa, s., rao, k. r.,
hasnain, s. e. & sehgal, s. c. (2004).
Use of fluorescent amplified fragment length
polymorphism for molecular epidemiology of
leptospirosis in India. j clin microbiol 42,
3575–3580.