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Advances and challenges in barcoding pathogenic and environmental *Leptospira*

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SUMMARY

Leptospirosis is a zoonotic bacterial disease of global importance. A large spectrum of asymptomatic animal hosts can carry the infection and contribute to the burden of human disease. Environmental sources of human contamination also point to the importance of a hydrotelluric reservoir. Leptospirosis can be caused by as many as 15 different pathogenic or intermediate *Leptospira* species. However, classification of these bacteria remains complicated through the use of both serological and genetic classification systems that show poor correlation. With the advent of molecular techniques, DNA-based barcoding offers a conceptual framework that can be used for leptospirosis surveillance as well as source tracking. In this review, we summarize some of the current techniques, highlight significant successes and weaknesses and point to the future opportunities and challenges to successfully establish a widely applicable barcoding scheme for *Leptospira*.

Key words: *Leptospira*, epidemiology, zoonotic reservoir, barcoding, genotyping.

INTRODUCTION

Leptospirosis is a zoonotic disease with a worldwide distribution. An estimated 1·03 million human cases and almost 60 000 deaths occur annually (Costa *et al*. 2015). Human leptospirosis is caused by infection with pathogenic *Leptospira* spp. bacteria through indirect exposure to water or moist environments contaminated with the urine of infected animals, or through direct contact with infected animals or their tissues. Although it is recognized as an emerging infectious disease, leptospirosis is also considered a neglected disease that places its greatest burden on impoverished populations from developing countries and tropical regions (McBride *et al*. 2005). Human leptospirosis is often diagnosed late, due to its broad spectrum of signs and symptoms that range from a flu-like syndrome to multi-organ failure, and because the clinical presentation of leptospirosis mimics that of many other diseases, including dengue fever, chikungunya and malaria. Laboratory confirmation of a clinical suspicion of leptospirosis is therefore essential to ensure optimal patient care (Hartskeerl and Smythe, 2015; Goarant, 2016).

The genus *Leptospira* belongs to the phylum of Spirochaetes, order Spirochaetales, family Leptospiraceae (Paster *et al*. 1991). Two classification schemes are used for leptospires, one of which is based on serology with the serovar as the basic taxon, and another which uses molecular taxonomy to identify the *Leptospira* species, sometimes referred to as genomospecies (Levett, 2001). Serological classification is based on antigenic determinants related to the structural heterogeneity of the lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria. *Leptospira* have been classified serologically into 26 serogroups and over 300 serovars (both saprophytic and pathogenic) using Microscopic Agglutination Test and Cross-Agglutination Absorption Test, respectively (Cerqueira and Picardeau, 2009; Hartskeerl and Smythe, 2015).

Phylogenetically, 22 species of the *Leptospira* genus have been described so far, based on 16S rRNA phylogeny and DNA–DNA hybridization (until recently the gold standard for defining bacterial species) (Fouts *et al*. 2016). Those species are arranged into three large groups based on their pathogenicity: pathogenic species (*Leptospira interrogans*, *L. kirschneri*, *L. borgpetersenii*, *L. mayottensis*, *L. santarosai*, *L. noguchii*, *L. weilii*, *L. alexanderi*, *L. kmetyi* and *L. alstonii*), intermediate (i.e. species of unclear or low pathogenicity: *L. broomii*, *L. fainei*, *L. inadai*, *L. licerasiae*, *L wolffii*) and saprophytic species (i.e. free-living organisms found in water and soil and generally considered not to infect animal hosts: *L. biflexa*, *L. idonii*, *L. meyeri*,

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L. terpstrae, *L. vanthielli*, *L. wolbachii*, *L. yanagawae*) (Faine *et al*. 1999). Intermediate leptospires cause predominantly mild self-resolving illnesses without fatal complications, whilst pathogenic species produce disease of varying severity in both humans and animals, ranging from subclinical infections to severe disease and death. The most severe forms are frequently caused by pathogenic strains belonging to the evolutionarily related species *L. interrogans*, *L. kirschneri* and *L. noguchii* (Lehmann *et al*. 2014; Xu *et al*. 2016), although severe disease might involve any other species (Levett, 2001).

Overall, there is poor correlation between the two *Leptospira* classification schemes and serological classification cannot be used to reliably predict the species of *Leptospira* isolates. For example, a single serogroup may contain serovars from different *Leptospira* species, and similarly a single genomic species may also contain representatives from several different serogroups (Levett, 2001). Furthermore, a number of *Leptospira* serovars are found in more than one *Leptospira* species (Levett, 2015). Although serological classification is still essential to support clinical diagnostics and surveillance, the development of *Leptospira* genomics is revealing new insights into the biology and pathogenesis of *Leptospira* infection (Lehmann *et al*. 2014; Fouts *et al*. 2016). Increasingly, genetic typing approaches for leptospires are being used to further understand the epidemiology of *Leptospira* infection in a range of clinical and research settings.

In the same way that the black stripes of the 'Universal Product Code' or 'barcode' distinguish products in a supermarket, DNA barcoding was developed as a molecular identification technique in which a single short DNA sequence can be used for species identification (Hebert *et al*. 2003). DNA barcoding was originally proposed as an identification tool for animals, and used PCR amplification and sequence analysis of a ~650 bp (conserved) region of the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene as a universal target. Barcoding has been very largely used in Arthropods, notably Lepidopterans, but was also developed in many other animals, as well as (frequently with other gene targets) in plants, algae, protists, fungi and eubacteria. In prokaryotes, sequencing of the small ribosomal 16S rRNA subunit gene (*rrs*) has been regarded as a standard for bacterial species identification for some time, and is analogous to the barcoding approach used in higher species. Unique sequence types (STs) could not always be attributed to a single bacterial species though, and reversely, some bacterial species could display sequence variations within the barcode. The term 'molecular operational taxonomic unit' or mOTU was therefore created to describe unique STs when studying complex communities. Prokaryote barcoding has been most notably used to describe microbial assemblages by directly amplifying *rrs* genes from environmental DNA extracts, cloning the PCR product and sequencing as many clones as possible (Pace, 1997), an ancestor form of what became 'metabarcoding', aimed at barcoding every individual from a complex community.

DNA barcoding of a group of organisms requires a good understanding of the diversity of the genome in question, and the identification of suitable gene targets that can be used to discriminate between different species within the group. For *Leptospira* species, the genomes range from 3·9 to 4·7 megabases (MB) in size and consist of two circular chromosomes: a large chromosome CI (~3·6–4·3 MB) and a smaller chromosome CII ~350 kilo-bases (KB) in length (Picardeau, 2015). Some saprophytic species, such as *L. biflexa*, also have an extra-chromosomal element p74 of around 74 KB that has not been detected in any of the pathogenic *Leptospira* species (Picardeau *et al*. 2008; Fouts *et al*. 2016). Comparative genomic analysis suggests that pathogenic species have a common progenitor with a genome resembling that of *L. biflexa*. Many of the essential housekeeping genes are located on CI, as are most of the genes encoding virulence factors, such as *lipL32* and *ligB* (Picardeau *et al*. 2008). In saprophytic species (e.g. *L. biflexa*), some housekeeping genes are also located on the p74 extra-chromosomal replicon.

Leptospira show unusual mechanisms of gene regulation and patterns of genetic organization (Saint Girons *et al*. 1992; Bulach *et al*. 2006). In contrast to most bacteria where the rRNA genes are clustered and co-transcribed, in the *Leptospira* genome, these genes are not linked to one another and are widely scattered along the CI chromosome (Picardeau, 2015). There also appears to be substantial amount of functional gene redundancy in *Leptospira*, particularly in pathogen-specific genes notably through gene duplication (Adler *et al*. 2011; Xu *et al*. 2016), and little synteny between pathogenic *Leptospira* species despite the short evolutionary distance between them (Picardeau *et al*. 2008). Pseudogenes and insertion sequences (IS) are common features in the *Leptospira* genomes (Picardeau, 2015). Comparative genomics revealed both overall genetic similarities and significant structural differences at the genus level, confirming genomic plasticity (Xu *et al*. 2016). IS-mediated sequence disruption and large chromosomal inversion or deletion are thought to be an important mechanisms in the evolution of *Leptospira*, and the number of IS elements varies between species and serovars (Bulach *et al*. 2006). In general however, the *Leptospira* genome is considered relatively stable and *Leptospira* serovar identity can be maintained during *in vitro* cultures for more than 80 years in the absence of selective pressure (Picardeau, 2015).

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In this article, we review the use of DNA-based 'barcoding' approaches to identify and explore the diversity of pathogenic *Leptospira* from clinical and environmental samples.

UTILITY OF *LEPTOSPIRA* BARCODING

Barcoding *Leptospira* in clinical and environmental samples is essential for a better understanding of the local epidemiology of infection and can provide knowledge to inform the development of disease control strategies. Routine barcoding of infecting *Leptospira* supports surveillance of predominant strains and genotypes of *Leptospira* in local human and animal populations and may indicate of changing trends of *Leptospira* infection within populations, such as new or atypical sources of infection or the emergence of a novel genotype. Studies of circulating *Leptospira* types can prove useful to monitor the evolution of the disease on a long-term perspective within a specific area, where the disease is endemic and/or where recurrent epidemic outbreaks occur. As an example, a prospective study conducted in northern Thailand between 2000 and 2005 identified the emergence of a dominant clone of *L. interrogans* serovar Autumnalis, which was a major cause of human disease during the outbreak unfolding in the early 2000s and has since diminished (Thaipadungpanit *et al*. 2007).

Characterization of *Leptospira* spp. detected in different animal hosts or environmental sources is now a prerequisite for epidemiological and source attribution studies as it allows human *Leptospira* infection to be traced back to the probable source of contamination. A wide variety of mammals may become infected with pathogenic *Leptospira* and act as reservoirs of infection for people and other animals through excretion of infectious bacteria in their urine (Levett, 2001). Rodents are considered to be one of the main sources of *Leptospira* infection (Adler, 2015), but livestock, other domestic animals and many wild animal species can also carry and transmit pathogenic leptospires through urinary shedding (Weekes *et al*. 1997; Gay *et al*. 2014). Notable associations between animal reservoir host species and specific *Leptospira* serovars have been reported and are considered as a hallmark of leptospirosis epidemiology. Most of the knowledge of which animal hosts carry different *Leptospira* serovars relies on data from serological typing of isolates and seroprevalence surveys acquired over several decades. However, since the advent of the molecular taxonomy, studies that have used molecular analysis to explore host–pathogen relationships have revealed new insights into trends in host specificity [e.g. in bats and small mammals in the Western Indian Ocean islands (Dietrich *et al*. 2014; Gomard *et al*. 2016)]. Publicly available databases also allow the comparison of genotypes between regions and are important to trace the movement of different *Leptospira* types from a phylogeographic perspective.

Identifying sources of *Leptospira* infection is essential to develop evidence-based infection control and prevention strategies for both human and animal infection. Detecting animal or environmental reservoirs of infection can prove particularly useful to infer transmission routes, and identify 'at-risk' groups of people and high-transmission settings (Ganoza *et al*. 2006; Viau and Boehm, 2011). Vaccinations against *Leptospira* are available but are serovar- (or at very best serogroup-) specific. Vaccine design and implementation policies require a good characterization of predominant *Leptospira* in the target human or animal population and have been used in some settings as an effective method to reduce infection in animal reservoirs of infection [e.g. vaccination of dairy cattle in New Zealand to reduce human infection (Marshall and Manktelow, 2002)].

LEPTOSPIRA BARCODING: TARGETS AND DATABASES

Identification and barcoding of pathogenic *Leptospira* species is usually performed on bacterial isolates obtained from infected human or animals. Increasingly, these approaches are also applied to DNA extracted directly from clinical or environmental samples following PCR-based pathogen detection. A variety of PCR assays have been described for use in the diagnosis of *Leptospira* infection in people and animals, and some of these can also be used for DNA-based typing of the infecting *Leptospira*. *Leptospira*-specific targets used for molecular diagnosis and typing target either conserved housekeeping genes where sequence-specific primers are used to differentiate between pathogenic and non-pathogenic species, or genes that are found only in the pathogenic species. One advantage of PCR assays designed for medical diagnosis is that they have usually been optimized for both sensitivity and specificity, assets that are useful when attempting to detect and characterize *Leptospira* in low numbers or in samples that contain high concentrations of non-*Leptospira* DNA. However, a drawback of these approaches is that diagnostic assays typically target highly conserved gene regions to ensure their sensitivity in detecting a variety of pathogenic *Leptospira*. Therefore, some of the gene targets commonly used for the diagnosis of *Leptospira* [e.g. *lip*L32 (Levett *et al*. 2005; Stoddard *et al*. 2009)] show poor discriminatory power in differentiating between *Leptospira* species when used alone. Some gene targets that have been used in the diagnosis and typing of *Leptospira* are shown in Table 1.

Amongst the housekeeping genes, the *secY* gene has been used most widely for both the diagnosis and typing of *Leptospira* infections. The *Leptospira*

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Table 1. PCR used or of potential use to generate *Leptospira* 'barcodes'

Initial purpose	Gene target	Technology	Product size (bp)	Reference
Diagnostic	16S rRNA *rrs*	Conventional nested PCR	331 290	Merien *et al*. (1992) Primers A and D detect all *Leptospira* species
Diagnostic	*lipL32*	SYBR Green I qPCR	423	Levett *et al*. (2005)
Diagnostic	*lipL32*	TaqMan qPCR	242	Stoddard *et al*. (2009)
Diagnostic	*secY*	SYBR Green I qPCR	202	Ahmed *et al*. (2009)
Diagnostic	*lfb1*	SYBR Green I qPCR	331	Merien *et al*. (2005)
Diagnostic	*flab*	Conventional PCR	793	Kawabata *et al*. (2001)
Diagnostic	*secY*	Conventional PCR	285	Gravekamp *et al*. (1993)
Typing	*gyrB*	Conventional PCR or SYBR Green I qPCR	504	Slack *et al*. (2006)
Typing	*rpoB*	Conventional PCR	600	La Scola *et al*. (2006)
Typing (MLST)	16SrRNA *rrs*	Conventional PCR	541	Ahmed *et al*. (2006)
Typing (MLST)	*secY*	Conventional PCR	549	Ahmed *et al*. (2006)
Typing (MLST)	*lipL32*	Conventional PCR	474	Ahmed *et al*. (2006)
Metagenomics	16SrRNA *rrs* regions V3–V4	Conventional PCR	~450	Klindworth *et al*. (2013)
Metagenomics	*cpn60* (*hsp60*)	Conventional PCR	~550–600	Goh *et al*. (1996); Klindworth *et al*. (2013)
Barcoding	COI-1	Conventional PCR	~550	Smith *et al*. (2012)

The PCR published for diagnostic purpose are usually optimized for sensitivity and specificity.

secY gene is a housekeeping gene located on the CI chromosome that encodes a pre-protein translocase important for the export of proteins across the cytoplasmic membrane (Durack *et al*. 2015; Haake and Levett, 2015). Conserved regions of this gene were the target of one of the earliest diagnostic PCR assays (Gravekamp *et al*. 1993) and a real-time PCR has been designed and validated for use in human clinical diagnosis (Ahmed *et al*. 2009). However, other regions of the *secY* gene demonstrate marked nucleotide polymorphism. A seminal study of 131 well-characterized *Leptospira* serovars by Victoria *et al*. (2008) demonstrated the phylogenetic value of the variable regions of the *secY* gene. Sequence analysis of these regions can discriminate between known pathogenic *Leptospira* species and strains, and have been used in many studies to identify the infecting species of *Leptospira* (examples are listed in Table 2).

An assay that is proving promising with regards to diagnosis and typing from a single assay is a SYBR Green I diagnostic qPCR developed to target *lfb1*, a putative fibronectin-binding protein present in pathogenic leptospires (Merien *et al*. 2005). Interestingly, the PCR product generated has been shown to have a sequence polymorphism of epidemiological interest, and performs well in comparison to multi-locus sequence typing (MLST) for a selection of serovars belonging to the *L. interrogans* and *L. borgpetersenii* species (Perez and Goarant, 2010). Originally described for rapid identification of infecting *Leptospira* species from human clinical cases in New Caledonia, this approach has also revealed novel insights into the epidemiology and diversity of *Leptospira* infection in rodents in Madagascar (Moseley *et al*. in preparation) and livestock in Tanzania (K. Allan, unpublished data).

Another target that has been used to discriminate between *Leptospira* species is the *flaB* gene. This gene target, which encodes a flagellum protein, has been used successfully to identify other bacterial species including *Campylobacter* (Harrington *et al*. 2003) and *Borrelia* (Lin *et al*. 2004). Restriction fragment length polymorphism of the PCR product of this gene has been successfully used to discriminate between *Leptospira* species in the laboratory (Kawabata *et al*. 2001) and was recently used in field settings to study the molecular epidemiology of canine leptospirosis in Japan (Koizumi *et al*. 2013) as well as to study the phylogenetic diversity in fruit-bat kidney specimens from Congo, revealing unique genotypes (Ogawa *et al*. 2015).

With regards to *Leptospira* barcoding, it is worth considering the so-called 'universal' highly conserved genes used in large population or metagenomics studies of bacteria. In particular, the ribosomal 16S rRNA gene (*rrs*) has a recognized phylogenetic value for bacterial species classification, and was the first DNA sequence available for most *Leptospira* species as well as being used as a target for many diagnostic PCR assays (Merien *et al*. 1992; Smythe *et al*. 2002). Of note, among the published diagnostic PCRs, these two latter ones targeting *rrs* are currently the only ones which can detect pathogen as well as intermediate species, in spite of a single-nucleotide polymorphism in the sequence of intermediates matching the LeptoF primer (Smythe *et al*. 2002) or by using a combination of the forward primer A and the reverse primer D (Merien *et al*. 1992). The 16S rRNA variable regions 3 and 4 sequence frequently used in

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Table 2. Examples of successful typing of *Leptospira* from single short DNA sequences and corresponding discriminatory capacity

Article	Molecular target(s)	Starting material	Finding(s)
Ganoza *et al*. (2006)	Nested 16SrRNA (Ganoza *et al*. 2006) (product cloned in *Escherichia coli*)	Surface waters	Presumptive *Leptospira* species and comparison with human isolates, unknown *Leptospira* clade
Perez and Goarant (2010)	*lfb1* (Merien *et al*. 2005), *secY* (Ahmed *et al*. 2009), MLST (Thaipadungpanit *et al*. 2007)	Human serum, deer kidneys	Identification of putative serovar (as a correlate in a specific island epidemiology)
Agampodi *et al*. (2011)	16SrRNA (Agampodi *et al*. 2011)	Human blood	Identification of *Leptospira* species (*L. interrogans* and *L. weilii*) in 6/8 samples
Perez *et al*. (2011)	*lfb1* (Merien *et al*. 2005), *lipL32* (Levett *et al*. 2005), 16SrRNA (Merien *et al*. 1992)	Rodent kidneys	Identification of putative serovar, and evidence of an unknown pathogenic *Leptospira*
Agampodi *et al*. (2013)	MLST (Thaipadungpanit *et al*. 2007)	Human serum or whole blood	Determination of partial to complete ST in 12/58 samples
Koizumi *et al*. (2013)	*flaB* (Koizumi *et al*. 2008)	Blood or urine from dogs	Identification of *Leptospira* species (*L. interrogans*)
Halliday *et al*. (2013)	*secY* (Ahmed *et al*. 2009)	Kidneys from rodents	Identification of *Leptospira* species (*L. interrogans* and *L. kirschneri*)
Agampodi *et al*. (2014)	Nested 16SrRNA (Ganoza *et al*. 2006; Agampodi *et al*. 2011)	Human serum or whole blood	Identification of *Leptospira* species (*L. kirschneri*, *L. interrogans* and *L. borgpetersenii*) in 28/32 samples
Goarant *et al*. (2014)	*lfb1* (Merien *et al*. 2005), *secY* (Ahmed *et al*. 2009), *lipL32* (Levett *et al*. 2005), 16SrRNA (Merien *et al*. 1992)	Human blood	Identification of an exotic *Leptospira* species (*L. weilii*)
Gay *et al*. (2014)	*lfb1* (Merien *et al*. 2005)	Kidney or urine from dogs, pigs, deer	Identification of putative serovar
Mayer-Scholl *et al*. (2014)	*secY* (Gravekamp *et al*. 1993)	Kidneys from small mammals	Identification of *Leptospira* species (*L. kirschneri*)
Muñoz-Zanzi *et al*. (2014)	*lipL32* (Stoddard *et al*. 2009)	Surface waters	Confirmation of pathogenic leptospires
Dietrich *et al*. (2014)	*secY* , *adk*, *lipL32*, *lipL41*, 16SrRNA (Ahmed *et al*. 2009)	Kidneys from small mammals and bats	Identification of *Leptospira* species (*L. borgpetersenii*, *L. kirschneri* and *L. mayottensis*)
Cosson *et al*. (2014)	*secY*, *adk*, 16SrRNA (Ahmed *et al*. 2006)	Kidneys from rodents	Identification of *Leptospira* species (*L. borgpetersenii*, *L. interrogans*, *L. kirschneri* and *L. weilii*) and subspecific genotypes
Ogawa *et al*. (2015)	*flaB* (Koizumi *et al*. 2008), *secY* (Gravekamp *et al*. 1993), 16SrRNA (Ogawa *et al*. 2015)	Kidneys from bats	Identification of *Leptospira* species (*L. kirschneri* and *L. borgpetersenii*) as well as novel genotypes (probable novel pathogenic species)
Pagès *et al*. (2015)	*lfb1* (Merien *et al*. 2005), *secY* (Ahmed *et al*. 2009)	Human urine	Identification of *Leptospira* species (*L. interrogans*) from *lfb1* sequence only
Verma *et al*. (2015)	MLST (Ahmed *et al*. 2006)	Kidneys from pigs	Identification of *Leptospira* species (*L. interrogans*). All genes but *adk* amplified and sequenced
Gomard *et al*. (2016)	*secY*, *adk*, 16SrRNA (Ahmed *et al*. 2006)	Pool of kidney, spleen and lung from bats	Identification of *Leptospira* species (*L. kirschneri* and *L. borgpetersenii*) as well as novel genotypes (probable novel pathogenic species)
Guernier *et al*. (2016)	MLST (Ahmed *et al*. 2006), 16SrRNA (Merien *et al*. 1992; Fenner *et al*. 2010), *secY* (Gravekamp *et al*. 1993)	Human sera, kidney or urine from mammals	Identification of *Leptospira* species (*L. interrogans*, *L. kirschneri*, *L. borgpetersenii* and *L. mayottensis*) Identification of complete or partial ST from MLST

Table 2. (*Cont.*)

Article	Molecular target(s)	Starting material	Finding(s)
Hamond *et al.* (2016)	*secY* (Ahmed *et al.* 2006)	Urine from cattle	Identification of *Leptospira* species (*L. interrogans*, *L. borgpetersenii*, *L. noguchii* and *L. santarosai*) and genotype diversity
Obiegala *et al.* (2016)	*gyrB* (Slack *et al.* 2006), MLST (Thaipadungpanit *et al.* 2007)	Kidneys from small mammals	Identification of *Leptospira* species (*L. interrogans*, *L. borgpetersenii* and *L. kirschneri*). ST for some samples
Weiss *et al.* (2016)	MLST adapted from (Boonsilp *et al.* 2013)	Human samples (serum, whole blood, buffy coat, urine)	Identification of *Leptospira* species (*L. borgpetersenii*, *L. kirschneri*, *L. santarosai* and *L. weilii*). Partial or complete ST
Ayral *et al.* (2016)	16SrRNA (Merien *et al.* 1992), multispacer sequence typing (Zilber *et al.* 2014)	Kidneys from small mammals	Identification of *Leptospira* species (*L. interrogans*, *L. borgpetersenii* and *L. kirschneri*). Putative serogroup or serovar from multispacer ST
Lagadec *et al.* (2016)	16SrRNA (Merien *et al.* 1992), MLST (Ahmed *et al.* 2006; Dietrich *et al.* 2014)	Pool of kidney, spleen and lung or urine from mammals	Identification of *Leptospira* species (*L. borgpetersenii*, *L. mayottensis*, *L. interrogans*, *L. kirschneri*)
Muller *et al.* (2016)	16SrRNA (Mgode *et al.* 2005)	Human blood	Confirmation of pathogenic *Leptospira* species
Mason *et al.* (2016)	*secY* (Ahmed *et al.* 2009)	Environmental waters	Identification of *Leptospira* species (*L. interrogans*, *L. kirschneri* and *L. weilii*) as well as putative novel species
Thibeaux *et al.* (2017)	*lfb1* (Merien *et al.* 2005)	Soils from contamination areas	Identification of a particular *Leptospira* interrogans strain and identity with the clinical strain. Evidence of novel genotypes

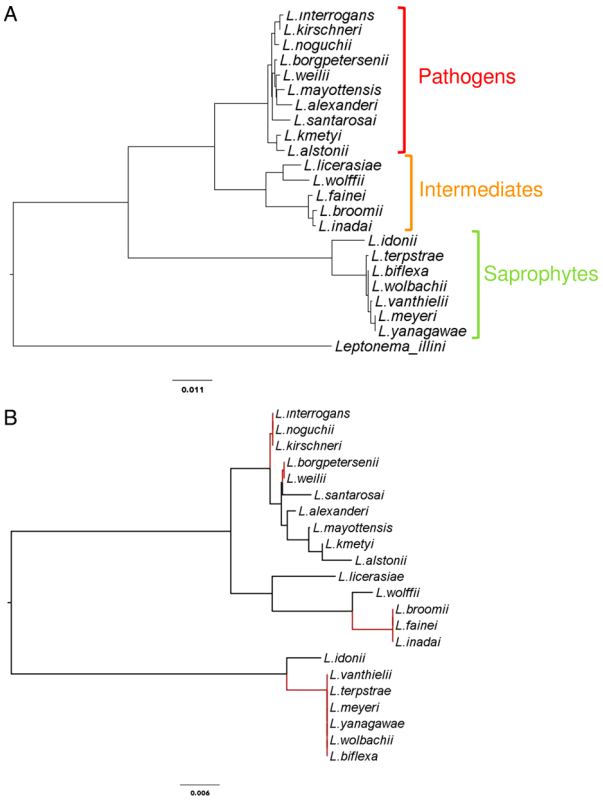


Fig. 1. Phylogeny deduced from (A) 1262 bp or (B) the variable regions V3 and V4 (440 bp) of the 16S rRNA *rrs* gene. The phylogenetic topology of the genus *Leptospira* is shown in the upper panel (A). Unique sequences of the V3 and V4 regions of the *rrs* gene, frequently used in metagenomics studies, discriminate only 12 mOTUs from the 22 *Leptospira* species currently described. The corresponding resolution losses are highlighted in red in lower panel (B). Reference sequences were retrieved from GenBank and used to establish phylogeny (neighbour joining). Scale bars are substitution rates.

metagenomics (Klindworth *et al.* 2013) has a good capacity to discriminate between the three *Leptospira* clusters, i.e. pathogens, intermediates and saprophytes. However, these regions have a low discriminatory power to differentiate between *Leptospira* species within a given clade. As an example, the 16S rRNA regions V3–V4 cannot be used to differentiate between the pathogenic species *L. interrogans*, *L. noguchii* and *L. kirschnerii*, which are classified within the same mOTU. Similarly, all saprophytic *Leptospira* species except *L. idonii* belong to a single mOTU (Fig. 1).

More recently, the RNA polymerase *β*-subunit gene *rpoB*, widely used in phylogenetic analyses of a number of bacterial genera, was proposed for *Leptospira* typing to circumvent the limitations of the 16S rRNA discrimination (La Scola *et al.* 2006). Bioinformatics studies later confirmed its high value for discrimination (Cerqueira *et al.* 2010), and its utility in epidemiological studies was demonstrated by work in Brazil and India that used this scheme

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to type *Leptospira* isolates from people and animals (Jorge *et al*. 2012; Balamurugan *et al*. 2013).

Another bacterial gene that is widely used in phylogenetic studies is the *gyrB* gene, which is reported to have a higher nucleotide divergence in *Leptospira* species than the 16S RNA *rrs* gene (Huang, 1996; Slack *et al*. 2006). This gene target has also been used for *Leptospira* diagnostic assays (Slack *et al*. 2006) and typing of isolates carried by rodents in Japan and the Philippines (Kawabata *et al*. 2006; Villanueva *et al*. 2014; Saito *et al*. 2015).

Other highly conserved genes have been proposed as targets to discriminate bacterial species by sequencing, but have rarely, if ever, been used in *Leptospira* studies. This includes *cpn60*, a chaperonin gene (also known as HSP60 in many bacteria or GroEL in *Leptospira*) proposed as a preferred universal barcode for bacteria compared with the 16S rRNA gene (Links *et al*. 2012). Such highly conserved genes might be of interest for improved discrimination between *Leptospira* species in microbial communities.

MULTI-LOCUS SEQUENCE TYPING OF *LEPTOSPIRA*

MLST is an unambiguous procedure for characterizing isolates of bacterial species using the sequences of internal fragments of several genes, including housekeeping genes (Maiden *et al*. 1998; Urwin and Maiden, 2003). For each gene/locus within a particular MLST scheme, a ~500 bp fragment is sequenced and analysed. The sequences present at each locus are assigned a distinct allele code and the combination of those alleles defines an allelic profile or ST for each isolate. Whilst MLST is not a barcoding method *per se* as it does not focus on a single marker, each locus considered separately can be considered as a barcode and the combination of multiple barcodes is considered one of the most robust genetic methods to identify the infecting *Leptospira* strain currently available. Three major MLST schemes exist for *Leptospira* spp. typing and *Leptospira* STs for all schemes are publically available at the PubMLST website (http://pubmlst.org/leptospira/), which also hosts molecular-typing databases for a variety of bacterial pathogens. Through this online portal, allelic profiles of *Leptospira* isolates can be easily compared with those from reference serovars compiled in the database. Presently, 13 *Leptospira* genes (including the highly discriminatory *secY* gene discussed above) from the three different MLST schemes have been included in the online database. MLST scheme #1, referred to as the 7L scheme, uses seven genes (*glmU*, *pntA*, *sucA*, *tpiA*, *pfkB*, *mreA*, *caiB*) to discriminate between the seven major *Leptospira* pathogenic species (Boonsilp *et al*. 2013). MLST scheme #3, denoted as the 6L scheme, includes three housekeeping genes (*adk*, *icdA*, *secY*), two genes encoding outer membrane proteins (*lipL32*, *lipL42*) and the 16S rRNA gene (*rrs*) that can be used for typing pathogenic and intermediate species (Ahmed *et al*. 2006). MLST scheme #2 combines the 'best' loci (*adk*, *glmU*, *icdA*, *lipL32*, *lipL41*, *mreA* and *pntA*) from schemes #1 and #3 (Varni *et al*. 2014), but to date, has been used less commonly in the literature.

Other MLST schemes have been suggested for leptospirosis research, but they are not yet supported by a website with referenced alleles or STs. As an example, *in silico* studies have proposed a reduced four-loci scheme with high discriminatory power (Cerqueira *et al*. 2010). This scheme would be usable in *L. interrogans* and *L. kirschneri* with currently validated PCRs, but would need to be further adapted for application with a wider range of pathogenic species. In the future, it is anticipated that the MLST technique will significantly contribute to gaining insights into the evolution and phylogeographic affinities of leptospires. However, the current multiplicity of available schemes leads to some confusion in the field of leptospirosis research that has limited the comparison between isolates obtained from different studies and geographic areas.

BARCODING SUCCESSES/LIMITATIONS

A major feature of leptospirosis is the great diversity of etiological agents leading to the 'leptospirosis' disease. As many as 15 different pathogenic and intermediate *Leptospira* species are infectious and have been implicated in human or animal infections (Schmid *et al*. 1986; Petersen *et al*. 2001; Levett *et al*. 2006; Matthias *et al*. 2008; Slack *et al*. 2008; Balamurugan *et al*. 2013; Tsuboi *et al*. 2017). However, as mentioned before, most diagnostic PCR only detect *Leptospira* from the pathogenic cluster and fail to detect intermediate species (Bourhy *et al*. 2011). Therefore, there is currently a strong bias towards the pathogenic cluster of *Leptospira* spp, and most frequently, only the pathogenic species might be considered with the current techniques.

An important advantage of using sequence-based genotyping of *Leptospira* is the ability for the technique to be standardized and compared between different laboratories and geographic sites. In comparison to serotyping, which is only performed at reference laboratories and requires considerable specialist expertise (Hartskeerl and Smythe, 2015), *Leptospira* barcoding techniques can be performed relatively quickly and cheaply, without the need for specialist equipment and extensive panels of reference antisera or monoclonal antibodies. The increasing availability of molecular diagnostic and sequencing facilities around the world also means that the technique can be performed in a wide variety of settings.

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Even where facilities are not available in-country, the fact that DNA-based typing does not require propagation or maintenance of live *Leptospira* cultures makes typing of *Leptospira* infecting animals and people in resource-limited settings more feasible [e.g. in Kenya and Zambia (Halliday *et al*. 2013; Ogawa *et al*. 2015)].

The ability to directly compare *Leptospira* sequences from different parts of the world has the potential to reveal new insights into the epidemiology of the infection. The application of genotyping techniques has demonstrated outbreaks of human disease caused by a single clonal complex of *L. interrogans* in Thailand (Thaipadungpanit *et al*. 2007) and revealed intriguing patterns of animal host specificity in Madagascar (Dietrich *et al*. 2014; Gomard *et al*. 2016). As yet, comparison of sequences from different geographic regions on a global scale has not been explored. However, the increasing application of standard barcoding or typing approaches has the potential for large-scale patterns of infection to be investigated.

One of the major criticisms about the use of barcoding in diversity analyses is that it relies on a single marker, and hence is often unable to discriminate between strains of a single *Leptospira* species (Dupuis *et al*. 2012; Mallo and Posada, 2016). The use of multiple barcoding loci to type *Leptospira* is a way to avoid misinterpretations because of large-scale sequence changes, such as horizontal gene transfer, which may occur between different *Leptospira* strains (Haake *et al*. 2004; Bulach *et al*. 2006; Picardeau *et al*. 2008; Victoria *et al*. 2008; Llanes *et al*. 2016). MLST (see above) is based on genotyping and establishing phylogenetic relationships between bacterial isolates using concatenated sequences derived from several loci, thus minimizing the possible biases originating from horizontally acquired DNA. MLST is considered one of the most robust and efficient methods in identifying ancestral relationships between *Leptospira* and segregating strains isolated from an outbreak, and in identifying the source(s) of human contamination (Nalam *et al*. 2010). Though not new, this method is not widely used yet for leptospirosis epidemiologic studies, mostly because it still requires bacterial isolates, in spite of ongoing efforts to type directly from non-cultured material.

DIRECT TYPING WITHOUT ISOLATION

The first objective of barcoding is to identify leptospires at the species level in patients with clinical leptospirosis.

Amongst the genes and barcoding schemes described in this paper, the 16S rRNA *rrs* gene and the translocase pre-protein *secY* have been most frequently used for typing of *Leptospira* species from patient samples. Increasingly, these typing schemes are being directly applied to biological specimens without strain isolation. In acute cases of human leptospirosis, *Leptospira* DNA can be detected and typed in whole blood, serum or urine samples. In animal carriers of infection, the kidneys or urine have been most frequently used. Similar approaches have been applied to type *Leptospira* detected in environmental surface water samples (Ganoza *et al*. 2006), a field of research that has recently gained renewed interest (Muñoz-Zanzi *et al*. 2014; Mason *et al*. 2016; Thibeaux *et al*. 2017).

Examples of successful genotyping from non-cultured clinical or environmental material are listed in Table 2.

Direct MLST typing of *Leptospira* from non-isolate clinical specimens has had limited success (Perez and Goarant, 2010; Agampodi *et al*. 2013), whatever the primers used. A revision of the MLST scheme #3 has recently been proposed, using newly designed nested primers to improve the sensitivity to make it usable directly from clinical specimens (Weiss *et al*. 2016). However, even this optimized procedure also only proved successful in a subset of clinical specimens. The success of MLST when performed directly on human or animal clinical samples has been shown to be correlated with the bacterial load in the specimen. In one study performed during an outbreak of acute human leptospirosis in Sri Lanka, Agampodi *et al* demonstrated a substantial difference in the mean bacterial load between specimens with complete MLST ($2{\cdot}2 \times 10^5$ *Leptospira* per mL) compared with specimens where the full MLST scheme could not be completed ($1{\cdot}3 \times 10^4$ *Leptospira* per mL), as measured by real-time PCR (Agampodi *et al*. 2013). A relatively high threshold of $4{\cdot}9 \times 10^4$ *Leptospira* per mL was observed for specimens with complete MLST profiles.

In addition to bacterial load, failure of sequence-based typing has also been reported in association with divergent or novel *Leptospira* types, particularly in areas that have been relatively poorly characterized for circulating *Leptospira* diversity. Standard-typing approaches may fail to amplify target gene sequences due to sequence polymorphism of local serovars at primer binding sites. In a study of *Leptospira* infection in small mammals in Madagascar (Dietrich *et al*. 2014), modification of standard *secY* primer sequences with inclusion of degenerate bases to account for sequence polymorphisms greatly improved the efficiency of single locus *secY* typing on *Leptospira* in non-isolate samples. Similarly, modified primers also facilitated direct typing of *Leptospira* in clinical samples from livestock in Tanzania (Allan, 2016) or from rodents in New Caledonia (Perez *et al*. 2011) where standard-typing assays had failed.

The ability to perform sequence-based typing of *Leptospira* spp. in the absence of bacterial isolation

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has helped to improve the utility of these techniques in a clinical setting, but risks neglecting the role of some strains or indeed species in the local epidemiology of leptospirosis. This is especially true in relatively unexplored geographic regions. For example, the most recently described *Leptospira* species, *L. mayottensis* (Bourhy *et al*. 2014), was first detected in human clinical cases on the tropical island of Mayotte, Indian Ocean (Bourhy *et al*. 2012). However, qPCR detection of this species was challenging with low diagnostic sensitivity for standard qPCR diagnostic assays (Bourhy *et al*. 2011, 2012). Following culture and isolation of the infecting *Leptospira*, more extensive phenotypic and genotypic characterization was performed leading to the description of a new species, which would have been impossible without a bacterial isolation. Similarly, a major limitation mentioned above is the failure of most current PCR designs to detect *Leptospira* belonging to the intermediate cluster (Bourhy *et al*. 2011). Because all species in this cluster have already proven pathogenic and been isolated from clinical specimens, there is growing recognition that these species will need to be considered and new PCR designs will be needed to more extensively address the contribution of these species to animal and human leptospirosis (Tsuboi *et al*. 2017). These two cases point to the current limitations of approaches only based on PCR with specific primers. Considering that the biodiversity of *Leptospira* is insufficiently recognized, they show the need for continued efforts of *Leptospira* isolation.

WHAT'S NEXT?

The rapid rise and development of next-generation sequencing (NGS) has exciting applications to the *Leptospira* field. The first *Leptospira* genome was fully reported 15 years ago (Ren *et al*. 2003), and since then, whole-genome sequencing has been made increasingly easier by the rise of NGS technologies. These massively parallel DNA sequencing methods provide high throughput genomic data faster and cheaper than first-generation sequencing.

A large-scale sequencing project entitled '*Leptospira* Genomics and Human Health' (http://gsc.jcvi.org/projects/gsc/leptospira/) led by the Craig Venter Institute and initiated in 2011 has generated whole-genome sequences for numerous strains belonging to 20 *Leptospira* species from diverse origins and geographical areas (Lehmann *et al*. 2014). The global analysis of these genomes has identified a core genome of more than 1760 genes, of which 737 are specific to *Leptospira*, 369 are specific to species with some degree of pathogenicity (pathogens and intermediates), and 416 are specific to pathogenic species (Fouts *et al*. 2016). Beside all basic knowledge that is (and will still be) gained from these wide comparative genomics studies (Xu *et al*. 2016), the identification of these genes offers opportunities to identify highly specific targets for *Leptospira* barcoding but also improve the feasibility of a universal, multi-locus molecular-typing system (Gerth and Bleidorn, 2013).

The availability of new 'single-molecule' sequencing technologies (generally referred to as third-generation sequencing) that can produce longer reads and highly accurate *de novo* assemblies of hundreds of microbial genomes has enabled greatly improved analysis of genome structure (Koren *et al*. 2013; Loman *et al*. 2015). When the price and computational challenges are overcome, these new technologies, with the prospect of readily available full genomic sequences data, may make core genome MLST (cgMLST) a new gold standard and trigger the downfall of DNA barcoding, especially for bacteria (Taylor and Harris, 2012).

The combination of DNA barcoding with NGS technologies has also facilitated the taxonomic profiling of complex communities through the simultaneous sequencing of many thousands of DNA barcodes from each sample. 'Metabarcoding' is distinguished from conventional barcoding by operating on the collective DNA rather than the isolated DNA of individual organisms (Baker *et al*. 2016). This broader community approach has been used by health scientists to investigate animals for zoonotic pathogens, allowing the taxonomic classification of all infecting ones (Razzauti *et al*. 2015), or by microbial ecologists to investigate genetic materials from environmental samples on a tremendous scale and without cloning. Because of this 'inventory' approach, the leptospirosis scientific community may gain unexpected information about *Leptospira* spp. through the active data mining of the sequence databases fed by metabarcoding studies with no specific focus on leptospirosis.

As an emerging and re-emerging infectious disease, a future challenge of *Leptospira* typing will be to continue to develop and adapt barcoding, typing and *Leptospira* classification schemes to deal with the increasing array of pathogen diversity described in the literature. New *Leptospira* species are still being discovered, a century after the first isolation of pathogenic *Leptospira* (Ido *et al*. 1917; Bourhy *et al*. 2014), and additional species may still be waiting to be discovered. In relatively poorly characterized regions of the world, such as Africa, studies that are performed often detect new or divergent *Leptospira* strains (Allan *et al*. 2015; Mgode *et al*. 2015; Allan, 2016). The challenge remains to identify the best DNA targets and amplification techniques for this purpose. These should fulfil two hardly compatible needs: (i) a need to be highly sensitive to account for the frequently very low bacterial burden of clinical specimens and (ii) a need to generate DNA products with a sequence polymorphism of epidemiological

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relevance. Additionally, these should still take into account the wide diversity of the genus *Leptospira*.

Estimating the true diversity of *Leptospira* is tied to how well the genomic diversity of the community is represented by the genomes available in sequence databases.

Whilst barcoding and multi-locus typing of infectious *Leptospira* allow genetic classification of the infecting leptospires, diagnosis and surveillance of infection as well as vaccination are still heavily dependent upon serology and the serological classification schemes. As yet, a major challenge for the leptospirosis community is to reconcile the serological and molecular *Leptospira* classification schemes. The different molecular-typing methods described in this review do not identify serovars but at best point to 'putative serovars' based on correlations determined previously. The effective marriage of serovars and genotypes might be achieved through a molecular-based serovar-typing system. The nucleotide sequence of the LPS biosynthetic operon *rfb* has therefore been pointed as a potential target for genotyping leptospires at the serovar level (Bezerra da Silva *et al*. 2011; Ahmed *et al*. 2012). Although the performance of the method did not provide optimal results yet, the approach in itself proved very useful and promising (Bezerra da Silva *et al*. 2011), and whole-genome sequences might be used in the future to infer serological features. For now, the ongoing challenge of a double non-convergent taxonomy – serovar *vs* genotype – looks set to continue. However, the exciting developments in the field of Whole Genome Sequencing may yet offer new insights into understanding the epidemiology, pathogenesis and taxonomy of this complex but important zoonotic pathogen.

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REFERENCES

Adler, B. (2015). History of leptospirosis and Leptospira. In *Leptospira and Leptospirosis* (ed. Adler, B.), vol. 387, pp. 1–9. Springer-Verlag, Heidelberg, Germany.

Adler, B., Lo, M., Seemann, T. and Murray, G. L. (2011). Pathogenesis of leptospirosis: the influence of genomics. *Veterinary Microbiology* **153**, 73–81.

Agampodi, S. B., Peacock, S. J., Thevanesam, V., Nugegoda, D. B., Smythe, L., Thaipadungpanit, J., Craig, S. B., Burns, M. A., Dohnt, M., Boonsilp, S., Senaratne, T., Kumara, A., Palihawadana, P., Perera, S. and Vinetz, J. M. (2011). Leptospirosis outbreak in Sri Lanka in 2008: lessons for assessing the Global burden of disease. *American Journal of Tropical Medicine and Hygiene* **85**, 471–478.

Agampodi, S. B., Moreno, A. C., Vinetz, J. M. and Matthias, M. A. (2013). Utility and limitations of direct multi-locus sequence typing on qPCR-positive blood to determine infecting *Leptospira* strain. *American Journal of Tropical Medicine and Hygiene* **88**, 184–185.

Agampodi, S. B., Dahanayaka, N. J., Bandaranayaka, A. K., Perera, M., Priyankara, S., Weerawansa, P., Matthias, M. A. and Vinetz, J. M. (2014). Regional differences of leptospirosis in Sri Lanka: observations from a flood-associated outbreak in 2011. *PLoS Neglected Tropical Diseases* **8**, e2626.

Ahmed, A., Engelberts, M. F., Boer, K. R., Ahmed, N. and Hartskeerl, R. A. (2009). Development and validation of a real-time PCR for detection of pathogenic *Leptospira* species in clinical materials. *PLoS ONE* **4**, e7093.

Ahmed, A., Grobusch, M. P., Klatser, P. R. and Hartskeerl, R. A. (2012). Molecular approaches in the detection and characterization of *Leptospira*. *Journal of Bacteriology and Parasitology* **3**, 1000133.

Ahmed, N., Devi, S. M., Valverde Mde, L., Vijayachari, P., Machang'u, R. S., Ellis, W. A. and Hartskeerl, R. A. (2006). Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Annals of Clinical Microbiology and Antimicrobials* **5**, 28.

Allan, K. J. (2016). Leptospirosis in northern Tanzania: investigating the role of rodents and ruminant livestock in a neglected public health problem. In *Institute of Biodiversity, Animal Health and Comparative Medicine* 298 PhD Glasgow, Scotland, UK, University of Glasgow. http://theses.gla.ac.uk/7565/

Allan, K. J., Biggs, H. M., Halliday, J. E., Kazwala, R. R., Maro, V. P., Cleaveland, S. and Crump, J. A. (2015). Epidemiology of leptospirosis in Africa: a systematic review of a neglected zoonosis and a paradigm for 'one health' in Africa. *PLoS Neglected Tropical Diseases* **9**, e0003899.

Ayral, F., Djelouadji, Z., Raton, V., Zilber, A. L., Gasqui, P., Faure, E., Baurier, F., Vourc'h, G., Kodjo, A. and Combes, B. (2016). Hedgehogs and mustelid species: major carriers of pathogenic *Leptospira*, a survey in 28 animal species in France (20122015). *PLoS ONE* **11**, e0162549.

Baker, C. C., Bittleston, L. S., Sanders, J. G. and Pierce, N. E. (2016). Dissecting host-associated communities with DNA barcodes. *Philos Trans R Soc Lond B Biol Sci*, **371**, 1702. pii: 20150328. doi: 10.1098/rstb.2015.0328.

Balamurugan, V., Gangadhar, N. L., Mohandoss, N., Thirumalesh, S. R., Dhar, M., Shome, R., Krishnamoorthy, P., Prabhudas, K. and Rahman, H. (2013). Characterization of *Leptospira* isolates from animals and humans: phylogenetic analysis identifies the prevalence of intermediate species in India. *Springerplus* **2**, 362.

Bezerra da Silva, J., Carvalho, E., Hartskeerl, R. A. and Ho, P. L. (2011). Evaluation of the use of selective PCR amplification of LPS biosynthesis genes for molecular typing of *Leptospira* at the serovar level. *Current Microbiology* **62**, 518–524.

Boonsilp, S., Thaipadungpanit, J., Amornchai, P., Wuthiekanun, V., Bailey, M. S., Holden, M. T., Zhang, C., Jiang, X., Koizumi, N., Taylor, K., Galloway, R., Hoffmaster, A. R., Craig, S., Smythe, L. D., Hartskeerl, R. A., Day, N. P., Chantratita, N., Feil, E. J., Aanensen, D. M., Spratt, B. G. and Peacock, S. J. (2013). A single multilocus sequence typing (MLST) scheme for seven pathogenic *Leptospira* species. *PLoS Neglected Tropical Diseases* **7**, e1954.

Bourhy, P., Bremont, S., Zinini, F., Giry, C. and Picardeau, M. (2011). Comparison of real-time PCR assays for the detection of pathogenic *Leptospira* spp. in blood and identification of variations in target sequences. *Journal of Clinical Microbiology* **49**, 2154–2160.

Bourhy, P., Collet, L., Lernout, T., Zinini, F., Hartskeerl, R., van der Linden, H., Thiberge, J. M., Diancourt, L., Brisse, S., Giry, C., Pettinelli, F. and Picardeau, M. (2012). Human *Leptospira* isolates circulating in Mayotte (Indian Ocean) have unique serological and molecular features. *Journal of Clinical Microbiology* **50**, 307–311.

https://doi.org/10.1017/S0031182017001147 Published online by Cambridge University Press

Bourhy, P., Collet, L., Brisse, S. and Picardeau, M. (2014). *Leptospira mayottensis* sp. nov., a pathogenic *Leptospira* species isolated from humans. *International Journal of Systematic and Evolutionary Microbiology* **64**, 4061–4067.

Bulach, D. M., Zuerner, R. L., Wilson, P., Seemann, T., McGrath, A., Cullen, P. A., Davis, J., Johnson, M., Kuczek, E., Alt, D. P., Peterson-Burch, B., Coppel, R. L., Rood, J. I., Davies, J. K. and Adler, B. (2006). Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proceedings of the National Academy of Sciences of the USA* **103**, 14560–14565.

Cerqueira, G. M. and Picardeau, M. (2009). A century of *Leptospira* strain typing. *Infection, Genetics and Evolution* **9**, 760–768.

Cerqueira, G. M., McBride, A. J., Hartskeerl, R. A., Ahmed, N., Dellagostin, O. A., Eslabao, M. R. and Nascimento, A. L. (2010). Bioinformatics describes novel loci for high resolution discrimination of *Leptospira* isolates. *PLoS ONE* **5**, e15335.

Cosson, J. F., Picardeau, M., Mielcarek, M., Tatard, C., Chaval, Y., Suputtamongkol, Y., Buchy, P., Jittapalapong, S., Herbreteau, V. and Morand, S. (2014). Epidemiology of *Leptospira* transmitted by rodents in southeast Asia. *PLoS Neglected Tropical Diseases* **8**, e2902.

Costa, F., Hagan, J. E., Calcagno, J., Kane, M., Torgerson, P., Martinez-Silveira, M. S., Stein, C., Abela-Ridder, B. and Ko, A. I. (2015). Global morbidity and mortality of leptospirosis: a systematic review. *PLoS Neglected Tropical Diseases* **9**, e0003898.

Dietrich, M., Wilkinson, D. A., Soarimalala, V., Goodman, S. M., Dellagi, K. and Tortosa, P. (2014). Diversification of an emerging pathogen in a biodiversity hotspot: *Leptospira* in endemic small mammals of Madagascar. *Molecular Ecology* **23**, 2783–2796.

Dupuis, J. R., Roe, A. D. and Sperling, F. A. (2012). Multi-locus species delimitation in closely related animals and fungi: one marker is not enough. *Molecular Ecology* **21**, 4422–4436.

Durack, J., Burke, T. P. and Portnoy, D. A. (2015). A prl mutation in SecY suppresses secretion and virulence defects of *Listeria monocytogenes* secA2 mutants. *Journal of Bacteriology* **197**, 932–942.

Faine, S., Adler, B., Bolin, C. and Perolat, P. (1999). *Leptospira and Leptospirosis*, 2nd Edn, MedSci, Melbourne, Vic., Australia.

Fenner, J. S., Anjum, M. F., Randall, L. P., Pritchard, G. C., Wu, G., Errington, J., Dalley, C. G. and Woodward, M. J. (2010). Analysis of 16S rDNA sequences from pathogenic *Leptospira serovars* and use of single nucleotide polymorphisms for rapid speciation by D-HPLC. *Research in Veterinary Science* **89**, 48–57.

Fouts, D. E., Matthias, M. A., Adhikarla, H., Adler, B., Amorim-Santos, L., Berg, D. E., Bulach, D., Buschiazzo, A., Chang, Y. F., Galloway, R. L., Haake, D. A., Haft, D. H., Hartskeerl, R., Ko, A. I., Levett, P. N., Matsunaga, J., Mechaly, A. E., Monk, J. M., Nascimento, A. L., Nelson, K. E., Palsson, B., Peacock, S. J., Picardeau, M., Ricaldi, J. N., Thaipandungpanit, J., Wunder, E. A., Jr., Yang, X. F., Zhang, J. J. and Vinetz, J. M. (2016). What makes a bacterial species pathogenic?: comparative genomic analysis of the genus *Leptospira*. *PLoS Neglected Tropical Diseases* **10**, e0004403.

Ganoza, C. A., Matthias, M. A., Collins-Richards, D., Brouwer, K. C., Cunningham, C. B., Segura, E. R., Gilman, R. H., Gotuzzo, E. and Vinetz, J. M. (2006). Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic *Leptospira*. *PLoS Medicine* **3**, e308.

Gay, N., Soupé-Gilbert, M. E. and Goarant, C. (2014). Though not reservoirs, dogs might transmit *Leptospira* in New Caledonia. *International Journal of Environmental Research and Public Health* **11**, 4316–4325.

Gerth, M. and Bleidorn, C. (2013). A multilocus sequence typing (MLST) approach to diminish the problems that are associated with DNA barcoding: a reply to Stahlhut etal. (2012). *Systematics and Biodiversity* **11**, 15–17.

Goarant, C. (2016). Leptospirosis: risk factors and management challenges in developing countries. *Research and Reports in Tropical Medicine* **7**, 49–62.

Goarant, C., Colot, J., Faelchlin, E., Ponchet, M., Soupé-Gilbert, M. E., Descloux, E. and Gourinat, A. (2014). An exotic case of leptospirosis imported into an endemic area. *Travel Medicine and Infectious Disease* **12**, 198–200.

Goh, S. H., Potter, S., Wood, J. O., Hemmingsen, S. M., Reynolds, R. P. and Chow, A. W. (1996). HSP60 gene sequences as universal targets for microbial species identification: studies with coagulase-negative staphylococci. *Journal of Clinical Microbiology* **34**, 818–823.

Gomard, Y., Dietrich, M., Wieseke, N., Ramasindrazana, B., Lagadec, E., Goodman, S. M., Dellagi, K. and Tortosa, P. (2016). Malagasy bats shelter a considerable genetic diversity of pathogenic *Leptospira* suggesting notable host-specificity patterns. *FEMS Microbiology Ecology* **92**, fiw037.

Gravekamp, C., Van de Kemp, H., Franzen, M., Carrington, D., Schoone, G. J., Van Eys, G. J., Everard, C. O., Hartskeerl, R. A. and Terpstra, W. J. (1993). Detection of seven species of pathogenic leptospires by PCR using two sets of primers. *Journal of General Microbiology* **139**, 1691–1700.

Guernier, V., Lagadec, E., Cordonin, C., Le Minter, G., Gomard, Y., Pages, F., Jaffar-Bandjee, M. C., Michault, A., Tortosa, P. and Dellagi, K. (2016). Human leptospirosis on Reunion Island, Indian Ocean: are rodents the (only) ones to blame? *PLoS Neglected Tropical Diseases* **10**, e0004733.

Haake, D. A. and Levett, P. N. (2015). Leptospirosis in humans. *Current Topics in Microbiology and Immunology* **387**, 65–97.

Haake, D. A., Suchard, M. A., Kelley, M. M., Dundoo, M., Alt, D. P. and Zuerner, R. L. (2004). Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. *Journal of Bacteriology* **186**, 2818–2828.

Halliday, J. E., Knobel, D. L., Allan, K. J., de Bronsvoort, C. B. M., Handel, I., Agwanda, B., Cutler, S. J., Olack, B., Ahmed, A., Hartskeerl, R. A., Njenga, M. K., Cleaveland, S. and Breiman, R. F. (2013). Urban leptospirosis in Africa: a cross-sectional survey of *Leptospira* infection in rodents in the Kibera Urban Settlement, Nairobi, Kenya. *American Journal of Tropical Medicine and Hygiene* **89**, 1095–1102.

Hamond, C., Pestana, C. P., Medeiros, M. A. and Lilenbaum, W. (2016). Genotyping of *Leptospira* directly in urine samples of cattle demonstrates a diversity of species and strains in Brazil. *Epidemiology & Infection* **144**, 72–75.

Harrington, C. S., Moran, L., Ridley, A. M., Newell, D. G. and Madden, R. H. (2003). Inter-laboratory evaluation of three flagellin PCR/RFLP methods for typing *Campylobacter jejuni* and *C. coli*: the CAMPYNET experience. *Journal of Applied Microbiology* **95**, 1321–1333.

Hartskeerl, R. A. and Smythe, L. D. (2015). The role of leptospirosis reference laboratories. *Current Topics in Microbiology and Immunology* **387**, 273–288.

Hebert, P. D., Cywinska, A., Ball, S. L. and de Waard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings Biological Sciences* **270**, 313–321.

Huang, W. M. (1996). Bacterial diversity based on type II DNA topoisomerase genes. *Annual Review of Genetics* **30**, 79–107.

Ido, Y., Hoki, R., Ito, H. and Wani, H. (1917). The rat as a carrier of Spirochaeta Icterohaemorrhaguae, the causative agent of Weil's disease (Spirochaetosis Icterohaemorrhagica). *Journal of Experimental Medicine* **26**, 341–353.

Jorge, S., Hartleben, C. P., Seixas, F. K., Coimbra, M. A. A., Stark, C. B., Larrondo, A. G., Amaral, M. G., Albano, A. P. N., Minello, L. F., Dellagostin, O. A. and Brod, C. S. (2012). *Leptospira borgpetersenii* from free-living white-eared opossum (*Didelphis albiventris*): first isolation in Brazil. *Acta Tropica* **124**, 147–151.

Kawabata, H., Dancel, L. A., Villanueva, S. Y., Yanagihara, Y., Koizumi, N. and Watanabe, H. (2001). flaB-polymerase chain reaction (flaB-PCR) and its restriction fragment length polymorphism (RFLP) analysis are an efficient tool for detection and identification of *Leptospira* spp. *Microbiology and Immunology* **45**, 491–496.

Kawabata, H., Sakakibara, S., Imai, Y., Masuzawa, T., Fujita, H., Tsurumi, M., Sato, F., Takano, A., Nogami, S., Kaneda, K. and Watanabe, H. (2006). First record of *Leptospira borgpetersenii* isolation in the Amami Islands, Japan. *Microbiology and Immunology* **50**, 429–434.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. and Glockner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research* **41**, e1.

Koizumi, N., Muto, M., Yamamoto, S., Baba, Y., Kudo, M., Tamae, Y., Shimomura, K., Takatori, I., Iwakiri, A., Ishikawa, K., Soma, H. and Watanabe, H. (2008). Investigation of reservoir animals of *Leptospira* in the northern part of Miyazaki prefecture. *Japanese Journal of Infectious Diseases* **61**, 465–468.

Koizumi, N., Muto, M. M., Akachi, S., Okano, S., Yamamoto, S., Horikawa, K., Harada, S., Funatsumaru, S. and Ohnishi, M. (2013). Molecular and serological investigation of *Leptospira* and leptospirosis in dogs in Japan. *Journal of Medical Microbiology* **62**, 630–636.

Koren, S., Harhay, G. P., Smith, T. P., Bono, J. L., Harhay, D. M., McVey, S. D., Radune, D., Bergman, N. H. and Phillippy, A. M. (2013). Reducing assembly complexity of microbial genomes with single-molecule sequencing. *Genome Biology* **14**, R101.

Lagadec, E., Gomard, Y., Le Minter, G., Cordonin, C., Cardinale, E., Ramasindrazana, B., Dietrich, M., Goodman, S. M., Tortosa, P. and Dellagi, K. (2016). Identification of Tenrec ecaudatus, a wild mammal introduced to Mayotte Island, as a reservoir of the newly identified

https://doi.org/10.1017/S0031182017001147 Published online by Cambridge University Press

human pathogenic *Leptospira mayottensis*. *PLoS Neglected Tropical Diseases* **10**, e0004933.

La Scola, B., Bui, L. T., Baranton, G., Khamis, A. and Raoult, D. (2006). Partial rpoB gene sequencing for identification of *Leptospira* species. *FEMS Microbiology Letters* **263**, 142–147.

Lehmann, J. S., Matthias, M. A., Vinetz, J. M. and Fouts, D. E. (2014). Leptospiral pathogenomics. *Pathogens* **3**, 280–308.

Levett, P. N. (2001). Leptospirosis. *Clinical Microbiology Reviews* **14**, 296–326.

Levett, P. N. (2015). Systematics of leptospiraceae. *Current Topics in Microbiology and Immunology* **387**, 11–20.

Levett, P. N., Morey, R. E., Galloway, R. L., Turner, D. E., Steigerwalt, A. G. and Mayer, L. W. (2005). Detection of pathogenic leptospires by real-time quantitative PCR. *Journal of Medical Microbiology* **54**, 45–49.

Levett, P. N., Morey, R. E., Galloway, R. L. and Steigerwalt, A. G. (2006). *Leptospira broomii* sp. nov., isolated from humans with leptospirosis. *International Journal of Systematic and Evolutionary Microbiology* **56**, 671–673.

Lin, T., Oliver, J. H., Jr. and Gao, L. (2004). Molecular characterization of Borrelia isolates from ticks and mammals from the southern United States. *Journal of Parasitology* **90**, 1298–1307.

Links, M. G., Dumonceaux, T. J., Hemmingsen, S. M. and Hill, J. E. (2012). The chaperonin-60 universal target is a barcode for bacteria that enables de novo assembly of metagenomic sequence data. *PLoS ONE* **7**, e49755.

Llanes, A., Restrepo, C. M. and Rajeev, S. (2016). Whole genome sequencing allows better understanding of the evolutionary history of *Leptospira interrogans* Serovar Hardjo. *PLoS ONE* **11**, e0159387.

Loman, N. J., Quick, J. and Simpson, J. T. (2015). A complete bacterial genome assembled de novo using only nanopore sequencing data. *Nature Methods* **12**, 733–735.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. and Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences of the USA* **95**, 3140–3145.

Mallo, D. and Posada, D. (2016). Multilocus inference of species trees and DNA barcoding. *Philos Trans R Soc Lond B Biol Sci*. **371**, 1702. pii: 20150335. doi: 10.1098/rstb.2015.0335.

Marshall, R. B. and Manktelow, B. W. (2002). Fifty years of leptospirosis research in New Zealand: a perspective. *New Zealand Veterinary Journal* **50**, 61–63.

Mason, M. R., Encina, C., Sreevatsan, S. and Munoz-Zanzi, C. (2016). Distribution and diversity of pathogenic *Leptospira* species in peri-domestic surface waters from South Central Chile. *PLoS Neglected Tropical Diseases* **10**, e0004895.

Matthias, M. A., Ricaldi, J. N., Cespedes, M., Diaz, M. M., Galloway, R. L., Saito, M., Steigerwalt, A. G., Patra, K. P., Ore, C. V., Gotuzzo, E., Gilman, R. H., Levett, P. N. and Vinetz, J. M. (2008). Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the Peruvian Amazon. *PLoS Neglected Tropical Diseases* **2**, e213.

Mayer-Scholl, A., Hammerl, J. A., Schmidt, S., Ulrich, R. G., Pfeffer, M., Woll, D., Scholz, H. C., Thomas, A. and Nockler, K. (2014). *Leptospira* spp. in rodents and shrews in Germany. *International Journal of Environmental Research and Public Health* **11**, 7562–7574.

McBride, A. J., Athanazio, D. A., Reis, M. G. and Ko, A. I. (2005). Leptospirosis. *Current Opinion in Infectious Diseases* **18**, 376–386.

Merien, F., Amouriaux, P., Perolat, P., Baranton, G. and Saint Girons, I. (1992). Polymerase chain reaction for detection of *Leptospira* spp in clinical samples. *Journal of Clinical Microbiology* **30**, 2219–2224.

Merien, F., Portnoi, D., Bourhy, P., Charavay, F., Berlioz-Arthaud, A. and Baranton, G. (2005). A rapid and quantitative method for the detection of *Leptospira* species in human leptospirosis. *FEMS Microbiology Letters* **249**, 139–147.

Mgode, G., Mhamphi, G., Katakweba, A., Paemelaere, E., Willekens, N., Leirs, H., Machang'u, R. and Hartskeerl, R. (2005). PCR detection of *Leptospira* DNA in rodents and insectivores from Tanzania. *Belgian Journal of Zoology* **135**, 17–19.

Mgode, G. F., Machang'u, R. S., Mhamphi, G. G., Katakweba, A., Mulungu, L. S., Durnez, L., Leirs, H., Hartskeerl, R. A. and Belmain, S. R. (2015). *Leptospira* serovars for diagnosis of leptospirosis in humans and animals in Africa: common *Leptospira* isolates and reservoir hosts. *PLoS Neglected Tropical Diseases* **9**, e0004251.

Muller, S. K., Assenga, J. A., Matemba, L. E., Misinzo, G. and Kazwala, R. R. (2016). Human leptospirosis in Tanzania: sequencing and phylogenetic analysis confirm that pathogenic *Leptospira* species circulate among agro-pastoralists living in Katavi-Rukwa ecosystem. *BMC Infectious Diseases* **16**, 273.

Muñoz-Zanzi, C., Mason, M. R., Encina, C., Astroza, A. and Romero, A. (2014). *Leptospira* contamination in household and environmental water in rural communities in Southern Chile. *International Journal of Environmental Research and Public Health* **11**, 6666–6680.

Nalam, K., Ahmed, A., Devi, S. M., Francalacci, P., Baig, M., Sechi, L. A., Hartskeerl, R. A. and Ahmed, N. (2010). Genetic affinities within a large global collection of pathogenic Leptospira: implications for strain identification and molecular epidemiology. *PLoS ONE*. **5**(8):e12637. doi: 10.1371/journal.pone.0012637.

Obiegala, A., Woll, D., Karnath, C., Silaghi, C., Schex, S., Essbauer, S. and Pfeffer, M. (2016). Prevalence and genotype allocation of pathogenic *Leptospira* species in small mammals from various habitat types in Germany. *PLoS Neglected Tropical Diseases* **10**, e0004501.

Ogawa, H., Koizumi, N., Ohnuma, A., Mutemwa, A., Hang'ombe, B. M., Mweene, A. S., Takada, A., Sugimoto, C., Suzuki, Y., Kida, H. and Sawa, H. (2015). Molecular epidemiology of pathogenic *Leptospira* spp. in the straw-colored fruit bat (*Eidolon helvum*) migrating to Zambia from the Democratic Republic of Congo. *Infection, Genetics and Evolution* **32**, 143–147.

Pace, N. R. (1997). A molecular view of microbial diversity and the biosphere. *Science* **276**, 734–740.

Pagès, F., Kuli, B., Moiton, M. P., Goarant, C. and Jaffar-Bandjee, M. C. (2015). Leptospirosis after a stay in Madagascar. *Journal of Travel Medicine*, **22**, 136–139.

Paster, B. J., Dewhirst, F. E., Weisburg, W. G., Tordoff, L. A., Fraser, G. J., Hespell, R. B., Stanton, T. B., Zablen, L., Mandelco, L. and Woese, C. R. (1991). Phylogenetic analysis of the Spirochetes. *Journal of Bacteriology* **173**, 6101–6109.

Perez, J. and Goarant, C. (2010). Rapid *Leptospira* identification by direct sequencing of the diagnostic PCR products in New Caledonia. *BMC Microbiology* **10**, 325.

Perez, J., Brescia, F., Becam, J., Mauron, C. and Goarant, C. (2011). Rodent abundance dynamics and leptospirosis carriage in an area of hyper-endemicity in New Caledonia. *PLoS Neglected Tropical Diseases* **5**, e1361.

Petersen, A. M., Boye, K., Blom, J., Schlichting, P. and Krogfelt, K. A. (2001). First isolation of *Leptospira fainei* serovar Hurstbridge from two human patients with Weil's syndrome. *Journal of Medical Microbiology* **50**, 96–100.

Picardeau, M. (2015). Genomics, proteomics, and genetics of *Leptospira*. *Current Topics in Microbiology and Immunology* **387**, 43–63.

Picardeau, M., Bulach, D. M., Bouchier, C., Zuerner, R. L., Zidane, N., Wilson, P. J., Creno, S., Kuczek, E. S., Bommezzadri, S., Davis, J. C., McGrath, A., Johnson, M. J., Boursaux-Eude, C., Seemann, T., Rouy, Z., Coppel, R. L., Rood, J. I., Lajus, A., Davies, J. K., Medigue, C. and Adler, B. (2008). Genome sequence of the saprophyte *Leptospira* biflexa provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS ONE* **3**, e1607.

Razzauti, M., Galan, M., Bernard, M., Maman, S., Klopp, C., Charbonnel, N., Vayssier-Taussat, M., Eloit, M. and Cosson, J. F. (2015). A comparison between transcriptome sequencing and 16S metagenomics for detection of bacterial pathogens in wildlife. *PLoS Neglected Tropical Diseases* **9**, e0003929.

Ren, S., Fu, G., Jiang, X.-G., Zeng, R., Miao, Y.-G., Xu, H., Zhang, Y.-X., Xiong, H., Lu, G., Lu, L.-F., Jiang, H.-Q., Jia, J., Tu, Y.-F., Jiang, J.-X., Gu, W.-Y., Zhang, Y.-Q., Cai, Z., Sheng, H.-H., Yin, H.-F., Zhang, Y., Zhu, G.-F., Wan, M., Huang, H.-L., Qian, Z., Wang, S.-Y., Ma, W., Yao, Z.-J., Shen, Y., Qiang, B.-Q., Xia, Q.-C., Guo, X.-K., Danchin, A., Saint Girons, I., Sommerville, R. L., Wen, Y.-M., Shi, M.-H., Chen, Z., Xu, J.-G. and Zhao, G.-P. (2003). Unique physiological and pathogenic features of *Leptospira interrogans* revealed by whole-genome sequencing. *Nature* **422**, 888–893.

Saint Girons, I., Norris, S. J., Gobel, U., Meyer, J., Walker, E. M. and Zuerner, R. (1992). Genome structure of spirochetes. *Research in Microbiology* **143**, 615–621.

Saito, M., Villanueva, S. Y., Masuzawa, T., Haraguchi, Y., Ita, S., Miyahara, S., Ozuru, R., Yamaguchi, T., Yoshimura, M., Ikejiri, M., Aramaki, N., Amran, M. Y., Muslich, L. T., Iida, K. I., Yanagihara, Y., Gloriani, N. G. and Yoshida, S. I. (2015). The usefulness of semi-solid medium in the isolation of highly virulent *Leptospira* strains from wild rats in an urban area of Fukuoka, Japan. *Microbiology and Immunology* **59**, 322–330.

Schmid, G. P., Steere, A. C., Kornblatt, A. N., Kaufmann, A. F., Moss, C. W., Johnson, R. C., Hovind-Hougen, K. and Brenner, D. J. (1986). Newly recognized *Leptospira* species ('*Leptospira inadai*' serovar

https://doi.org/10.1017/S0031182017001147 Published online by Cambridge University Press

lyme) isolated from human skin. *Journal of Clinical Microbiology*, **24**, 484–486.

Slack, A. T., Symonds, M. L., Dohnt, M. F. and Smythe, L. D. (2006). Identification of pathogenic *Leptospira* species by conventional or real-time PCR and sequencing of the DNA gyrase subunit B encoding gene. *BMC Microbiology* **6**, 95.

Slack, A. T., Kalambaheti, T., Symonds, M. L., Dohnt, M. F., Galloway, R. L., Steigerwalt, A. G., Chaicumpa, W., Bunyaraksyotin, G., Craig, S., Harrower, B. J. and Smythe, L. D. (2008). *Leptospira wolffii* sp. nov., isolated from a human with suspected leptospirosis in Thailand. *International Journal of Systematic and Evolutionary Microbiology*, **58**, 2305–2308.

Smith, M. A., Bertrand, C., Crosby, K., Eveleigh, E. S., Fernandez-Triana, J., Fisher, B. L., Gibbs, J., Hajibabaei, M., Hallwachs, W., Hind, K., Hrcek, J., Huang, D. W., Janda, M., Janzen, D. H., Li, Y., Miller, S. E., Packer, L., Quicke, D., Ratnasingham, S., Rodriguez, J., Rougerie, R., Shaw, M. R., Sheffield, C., Stahlhut, J. K., Steinke, D., Whitfield, J., Wood, M. and Zhou, X. (2012). Wolbachia and DNA barcoding insects: patterns, potential, and problems. *PLoS ONE* **7**, e36514.

Smythe, L. D., Smith, I. L., Smith, G. A., Dohnt, M. F., Symonds, M. L., Barnett, L. J. and McKay, D. B. (2002). A quantitative PCR (TaqMan) assay for pathogenic *Leptospira* spp. *BMC Infectious Diseases* **2**, 13.

Stoddard, R. A., Gee, J. E., Wilkins, P. P., McCaustland, K. and Hoffmaster, A. R. (2009). Detection of pathogenic *Leptospira* spp. through TaqMan polymerase chain reaction targeting the LipL32 gene. *Diagnostic Microbiology and Infectious Diseases* **64**, 247–255.

Taylor, H. R. and Harris, W. E. (2012). An emergent science on the brink of irrelevance: a review of the past 8 years of DNA barcoding. *Molecular Ecology Resources* **12**, 377–388.

Thaipadungpanit, J., Wuthiekanun, V., Chierakul, W., Smythe, L. D., Petkanchanapong, W., Limpaiboon, R., Apiwatanaporn, A., Slack, A. T., Suputtamongkol, Y., White, N. J., Feil, E. J., Day, N. P. and Peacock, S. J. (2007). A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Neglected Tropical Diseases* **1**, e56.

Thibeaux, R., Geroult, S., Benezech, C., Chabaud, S., Soupé-Gilbert, M. E., Girault, D., Bierque, E. and Goarant, C. (2017). Seeking the environmental source of Leptospirosis reveals durable bacterial viability in river soils. *PLoS Neglected Tropical Diseases* **11**, e0005414.

Tsuboi, M., Koizumi, N., Hayakawa, K., Kanagawa, S., Ohmagari, N. and Kato, Y. (2017). Imported *Leptospira licerasiae* infection in traveler returning to Japan from Brazil. *Emerging Infectious Diseases* **23**, 548–549.

Urwin, R. and Maiden, M. C. (2003). Multi-locus sequence typing: a tool for global epidemiology. *Trends in Microbiology* **11**, 479–487.

Varni, V., Ruybal, P., Lauthier, J. J., Tomasini, N., Brihuega, B., Koval, A. and Caimi, K. (2014). Reassessment of MLST schemes for *Leptospira* spp typing worldwide. *Infection, Genetics and Evolution* **22**, 216–222.

Verma, A., Soto, E., Illanes, O., Ghosh, S. and Fuentealba, C. (2015). Detection and genotyping of *Leptospira* spp. from the kidneys of a seemingly healthy pig slaughtered for human consumption. *Journal of Infection in Developing Countries* **9**, 530–532.

Viau, E. J. and Boehm, A. B. (2011). Quantitative PCR-based detection of pathogenic *Leptospira* in Hawai'ian coastal streams. *Journal of Water and Health* **9**, 637–646.

Victoria, B., Ahmed, A., Zuerner, R. L., Ahmed, N., Bulach, D. M., Quinteiro, J. and Hartskeerl, R. A. (2008). Conservation of the *S*10-*spc*-*α* locus within otherwise highly plastic genomes provides phylogenetic insight into the genus *Leptospira*. *PLoS ONE* **3**, e2752.

Villanueva, S. Y. A. M., Saito, M., Baterna, R. A., Estrada, C. A. M., Rivera, A. K. B., Dato, M. C., Zamora, P. R. F. C., Segawa, T., Cavinta, L. L., Fukui, T., Masuzawa, T., Yanagihara, Y., Gloriani, N. G. and Yoshida, S.-I. (2014). *Leptospira*-rat-human relationship in Luzon, Philippines. *Microbes and Infection* **16**, 902–910.

Weekes, C. C., Everard, C. O. and Levett, P. N. (1997). Seroepidemiology of canine leptospirosis on the island of Barbados. *Veterinary Microbiology* **57**, 215–222.

Weiss, S., Menezes, A., Woods, K., Chanthongthip, A., Dittrich, S., Opoku-Boateng, A., Kimuli, M. and Chalker, V. (2016). An extended multilocus sequence typing (MLST) scheme for rapid direct typing of *Leptospira* from clinical samples. *PLoS Neglected Tropical Diseases* **10**, e0004996.

Xu, Y., Zhu, Y., Wang, Y., Chang, Y. F., Zhang, Y., Jiang, X., Zhuang, X., Zhu, Y., Zhang, J., Zeng, L., Yang, M., Li, S., Wang, S., Ye, Q., Xin, X., Zhao, G., Zheng, H., Guo, X. and Wang, J. (2016). Whole genome sequencing revealed host adaptation-focused genomic plasticity of pathogenic *Leptospira*. *Scientific Reports* **6**, 20020.

Zilber, A. L., Picardeau, M., Ayral, F., Artois, M., Demont, P., Kodjo, A. and Djelouadji, Z. (2014). High-resolution typing of *Leptospira interrogans* by Multispacer Sequence Typing. *Journal of Clinical Microbiology* **52**, 564–571.

https://doi.org/10.1017/S0031182017001147 Published online by Cambridge University Press