Understanding leptospirosis: application of state-of-the-art molecular typing tools with a One Health lens

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ABSTRACT
Leptospirosis is an archetypal One Health problem as described in the companion Currents in One Health article in the October 2022 issue of the Journal of the American Veterinary Medical Association by Sykes et al. A thorough understanding of leptospirosis requires a detailed analysis of the elaborate interplay among pathogenic leptospiral strains, host species, and the environment. Such an understanding is required to inform appropriate preventative measures including vaccine design, prophylaxis efforts, educational programs that help to reduce exposure to pathogenic spirochetes, as well as policy development. Because of the complex epidemiology of leptospirosis, a One Health approach as defined by the One Health Initiative Task Force is critical—an approach that calls for “the collaborative efforts of multiple disciplines working locally, nationally, and globally, to attain optimal health for people, animals and our environment.” Over the last three decades, progressive advances in cutting-edge molecular typing techniques, as well as our ability to rapidly generate and share large amounts of sequence data through establishment and growth of databases, have been central to accelerating a One Health understanding of the epidemiology of leptospirosis. Nevertheless, our dependence on serotype information because of the serovar-specific nature of current vaccines means that laborious serotyping efforts continue. With the advent of new approaches such as mRNA vaccines that are based on lipopolysaccharide immunogens, sequence- and/or proteomics-based typing methods may replace these methods.

A globally important, zoonotic bacterial disease caused by pathogenic spirochetes of the genus Leptospira, leptospirosis is estimated to affect more than one million people worldwide, accounting for over 60,000 deaths/annum, with over 70% of cases occurring in tropical regions.1,2 As discussed in the companion Currents in One Health article in the October 2022 issue of the Journal of the American Veterinary Medical Association by Sykes et al, leptospirosis is also responsible for significant morbidity and mortality in domestic animals and wildlife species, with profound impacts on food production and the human-animal bond. The purpose of this article is to illustrate how cutting-edge transdisciplinary approaches are accelerating our comprehension of this important One Health problem, with a focus on detection and identification of leptospiral strains.

Importance of a Harmonized Strain Typing Approach
Central to a One Health approach for improved understanding and prevention of leptospirosis is accurate detection and precise classification of leptospires in incidental hosts, in reservoir hosts, and in the environment. The success of a transdisciplinary approach requires harmonization of strain typing approaches with use of a common microbial sequence repository across disciplines. Typing methods should be simple to perform, affordable, and have high discriminatory power that is applicable across regions and countries, and newly identified strains should be deposited in a single, search-able, open access sequence database (according to the FAIR concept—that scientific material should be findable, accessible, interoperable, and reusable).3 Inclusion of both phenotypic and genotypic information should be promoted. The database should include quality controls (including curator verification) to ensure deposited material is validated and authentic. Scientific journals can promote database construction by requiring deposition of strain type information as part of journal policy. For example, the journal Nature requires that authors submit sequence data to a public repository within the International Nucleotide Sequence Database Collaboration (INSDC).4
Evolution of Leptospiral Strain Identification Approaches

In the past, leptospires were classified as saprophytes (Leptospira biflexa sensu lato) or pathogens (Leptospira interrogans sensu lato) based on phenotypic characteristics (ability to grow at 13 and 30 °C, growth in 8-azaguanine).\(^6\)\(^,\)\(^6\) Serotyping reagents were then used to classify pathogenic leptospires into over 200 serovars grouped into 24 antigenically related serogroups.\(^6\) However, culture of leptospires has been challenging because leptospires are fastidious, often inactivated during transport to the laboratory, and require special media and prolonged incubation times for growth. Serotyping is laborious and time consuming; reference antisera and monoclonal antibodies are fastidious, often inactivated during transport to the laboratory, and require special media and prolonged incubation times for growth. Serotyping is laborious and time consuming; reference antisera and monoclonal antibodies are required and an experienced laboratory that maintains antisera, yet worldwide the number of laboratories with this capability is limited. Manipulation of live pathogenic leptospires during typing efforts also represents a hazard to laboratory workers. In the late 1980s and early 1990s, use of DNA-DNA hybridization with recombinant radioactive probes as well as pulsed-field gel electrophoresis provided insight into the genomic heterogeneity of Leptospira interrogans sensu lato.\(^8\)\(^-\)\(^11\) with the identification of 8 pathogenic and 2 saprophytic species.\(^9\)

The advent of PCR led to additional DNA typing methods such as arbitrarily primed PCR, mapped restriction site polymorphism analysis of amplified ribosomal genes, and multilocus sequence typing (MLST).\(^6\)\(^,\)\(^12\)\(^,\)\(^13\) Use of a combination of typing methods led to recognition of leptospires with characteristics of both saprophytic and pathogenic strains (“intermediate” leptospires).\(^6\) However, these approaches were still arduous, dependent on gel electrophoresis, prone to amplification failure or contamination, and did not lend themselves to database sharing. Subsequently, 16S rRNA gene PCR amplification and sequencing were developed as simpler and more practical approaches to differentiating among pathogenic, intermediate, and saprophytic Leptospira species, despite limited discriminatory power.\(^14\) At that time, sequences from 39 strains representing 17 known Leptospira species were deposited in the National Center for Biotechnology Information (NCBI/GenBank) database, creating a foundation for subsequent comparative studies.\(^14\)

Advances in sequencing technology over the next 10 years resulted in the ability to obtain and compare whole genome sequences of 90 different leptospiral strains.\(^15\) A new perspective on leptospiral taxonomy arose, with reclassification of leptospires into 64 different genomospecies, grouped into two major clades, “Saprophytes” (organisms found in the natural environment) and “Pathogens” (which contains all species responsible for infections in animals and humans, as well as environmental species with uncertain virulence).\(^15\) Within these major clades are two subclades, P1 (formerly the pathogen group, 19 species), P2 (formerly the intermediate group, 20 species), S1, and S2 (28 species).\(^16\)

Current Approaches to Leptospiral Strain Typing

To facilitate typing based on whole genome sequence information, a core genome multilocus sequence typing (cgMLST) approach that types leptospires on the basis of 545 core genes was developed.\(^17\) Using this approach, organisms can be identified at the species, clade, clonal group (CG), and sequence type (ST) level. An open access database for deposition of cgMLST data has been created at the Institut Pasteur (https://bigsdb.pasteur.fr/leptospira/). This database currently contains 753 submissions from 58 countries, with most (35%) being L interrogans.

Given that whole genome sequencing is still not practical for routine strain typing and 16S ribosomal RNA (rRNA) gene sequencing provides limited discriminatory power, sequence information from other gene targets, such as the pppk, secY, flaB, lfb1, and glmU genes, have been used to type Leptospira species,\(^15\)\(^,\)\(^18\)\(^-\)\(^20\) as well as for PCR-based diagnostic testing.\(^21\) The use of lipL32 gene PCR sequencing has been particularly widespread, because lipL32 gene PCR is a sensitive target for diagnosis of leptospirosis and only detects pathogenic leptospires. However, by virtue of the conserved nature of the lipL32 gene sequence, this method lacks discriminatory power,\(^20\)\(^,\)\(^22\) and P2 leptospires are not detected using lipL32 gene PCR because of sequence variation.\(^7\) Three different 6- or 7-loci MLST schemes have been most widely used in the literature (Table 1).\(^20\) A simplified 3-locus (adk, lipL41, mreA) scheme for direct application to clinical specimens was recently described,\(^26\) although this still requires gel electrophoresis followed by sequencing, which is not practical in a routine clinical laboratory setting. After examination of 598 Leptospira published genomes from 20 P1 species, investigators\(^19\) in New Zealand identified the glmU locus as having sufficient discriminatory power to differentiate among serovars circulating in New Zealand. Direct application of glmU PCR sequencing to the urine of infected cattle

<table>
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<th>Scheme number</th>
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<td>adk, glmU, icdA, lipL32, lipL41, mreA, pntA</td>
<td>273</td>
<td>April 24, 2019</td>
<td>Varni et al 2014(^24)</td>
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was used to deduce infecting serovars. However, the investigators noted that their assay would only detect P1 leptospires and would not discriminate between serovars circulating in other countries.

In addition to the NCBI database, leptospiral sequence information can be deposited in and retrieved from the DNA data bank of Japan (DDBJ) (https://www.ddbj.nig.ac.jp/index-e.html), PATRIC (https://www.patricbrc.org/), and PubMLST (https://pubmlst.org/organisms/leptospira-spp/). PubMLST contains the three widely accepted MLST schemes used for typing leptospires (Table 1); using this portal, investigators can compare MLST profiles of their isolates to those of reference strains in the database. In development are PubMLST databases for a 1,565-loci cgMLST scheme as well as a 21-loci lipopolysaccharide scheme.

Despite major advances in genetic typing, the demand for serotype identification remains, because immunity provided by current leptospirosis vaccines is serotype specific, and predominant serotypes vary regionally and over time. Direct PCR sequencing only allows serogroup prediction if a matching ST has been deposited in an open access database along with with accurate serogroup characterization (i.e., based on culture and serotyping). For example, serogroups Icterohaemorrhagiae and Australis were identified in specimens from affected dogs in Italy through direct molecular detection of ST17 and ST198; these were then matched to corresponding STs with associated serogroup characterization in PubMLST. Unfortunately, serotype information is often not available in combination with sequence data. The quality of sequence information after direct application of PCR to clinical specimens can also be insufficient for typing purposes; this is especially true for MLST schemes and when divergent or novel strains are present or when bacterial loads in clinical specimens are low. In some cases, identification of new species such as *Leptospira mayotensis* and *Leptospira tipperaryensis* would not have been possible had culture (with extensive phenotyping and genome characterization) not been performed.

Although the results of serologic testing using the microscopic agglutination test (MAT) have been widely used to predict the identity of an infecting serogroup in both incidental and reservoir hosts, this approach is not reliable because of paradoxical serologic cross-reactivity and the potential for omission of the infecting serogroup from the panel. Inclusion of local serovars isolated from the kidneys and urine of domestic animals and wildlife species in Tanzania in the MAT was reported to increase leptospirosis seroprevalence in humans from 0.26% to 10.75% and in rodents from 1.9% to 16.9%. The revised panel included serovars Sokoin, Kenya, Lora, Canicola, Grippotyphosa, Hebdomedalis, Pomona, and Hardjo, while historical results used a panel that contained serovars Icterohaemorrhagiae, Canicola, Pyrogenes, Hardjo, and Grippotyphosa. Most veterinary panels include 6 to 7 serovars. In contrast, panels used in human diagnostic laboratories may include over 30 serovars. Even with the use of large panels, the serovar with the highest titer does not reliably predict the infecting serovar. Assays that include a large number of serovars are significantly more laborious to perform, report, and interpret.

**Culture Revisited**

In recent years, novel approaches have increased the speed and sensitivity of *Leptospira* culture, allowing subsequent serotyping and complete genomic characterization. The continued importance of this approach was exemplified in late 2020 when *Leptospira borgpetersenii* serovar Tarassovi, a serovar not present in bacterin vaccines for cattle, was identified for the first time in a dairy cow from a herd in Minnesota with a history of poor reproductive performance. The results of serial MAT testing using a panel of 19 serovars showed low positive titers (up to 1:200) to multiple serovars, but no reactions were identified to the reference serovar Tarassovi strain or the strain isolated from the cow. Sequence analysis of ~200 base pair secY gene sequences obtained from direct PCR sequencing yielded 100% matches with *L borgpetersenii* serovar Tarassovi and *L borgpetersenii* serovar Nyanza. Culture was pursued because of the recognition that secY sequence analysis could not be relied upon to identify serovar status. In a 2021 outbreak of leptospirosis in dogs in Santa Monica, high MAT titers to serovar Canicola in many dogs suggested Canicola as the infecting serogroup; some dogs had titers to serovar Canicola reaching 1:102,400. This was somewhat unexpected, as dogs are generally considered reservoir hosts of serogroup Canicola. Subsequent culture and serotyping did confirm the identity of the pathogen as *L interrogans* serogroup Canicola. Accordingly, the outbreak ceased in association with education of local veterinarians about the need to implement vaccination of dogs with current vaccines that contain serovars Canicola, Icterohaemorrhagiae, Grippotyphosa, and Pomona (which was not widely practiced in the region due to the perception that the risk of leptospirosis was low). It is possible the leptospiral strain in this outbreak possessed virulence factors not present in reservoir host-associated strains of serogroup Canicola or host factors (such as immunosuppression due to overcrowding in daycare centers) may have played a role.

Successful isolation requires engagement of personnel collecting specimens in the field, as well as excellent communication among these personnel, laboratory staff, and others involved in specimen transport. The sensitivity of culture in humans and animals with suspected leptospirosis can be optimized by (1) early recognition of likely cases by clinicians based on history and clinical signs, (2) properly timed collection of correct specimens, (3) patient-side inoculation of media before administration of antimicrobials (requiring on-site storage of media), and (4) rapid specimen transport to experienced laboratory personnel that are available to inoculate media on specimen receipt. Because leptospirosis is associated with characteristic patterns of clinical and
laboratory abnormalities, use of machine learning algorithms may help to promote early recognition of leptospirosis by physicians and veterinarians; this in turn may increase the success of positive results using specific diagnostic tests. A machine learning algorithm was shown to predict leptospirosis in dogs seen at the University of California-Davis Veterinary Medical Teaching Hospital with 100% sensitivity (95% CI: 70.1 to 100%) and 90.9% specificity (95% CI: 78.8 to 96.4%). The close resemblance of human leptospirosis to the disease in dogs suggests a similar approach could be used in humans.

Submission of both urine and blood for culture is important in incidental (i.e.) hosts, because organisms are typically present in the blood for the first week of illness, and then may be shed briefly, intermittently, and in low concentrations in urine for a short period of time. Intermitten, low-level shedding reduces the clinical sensitivity of culture when applied to urine; in the case of reservoir hosts, the success of isolation can be improved by collection of multiple urine specimens over time, or use of fresh kidney tissue for culture (postmortem or by ultrasound-guided renal biopsy). Collection of serial urine specimens at weekly intervals over 23 weeks identified intermittent shedding in the cow infected with L. borgpetersenii serovar Tarassovi, with 42.6% of 47 specimens being positive with PCR and 8.7% of 46 specimens being positive with culture. In the laboratory, inoculation of several media types (e.g., HAN liquid, HAN semi-solid, Ellis semisol) followed by incubation of cultures at different temperatures improves sensitivity and has the potential to yield growth within 24 to 48 hours of incubation. Testing clinical specimens using PCR at the time of media inoculation may help to identify specimens that might require prolonged incubation. However, in addition to the problems of intermittent and low-level shedding, application of PCR to urine can lack sensitivity because urine contains inhibitors of PCR, and refrigeration of urine specimens to preserve bacteria can promote precipitation of crystals that interfere with DNA extraction.

To promote widespread access to quality reagents for culture and serotyping as well as a collection of cultured laboratory-passaged and wild-type Leptospira strains, Amsterdam UMC (University Medical Centers) has established the Leptospirosis Reference Center, a public microbial domain biological resource center (leptospira.amsterdamumc.org/leptospirosis-reference-centre). Researchers can submit published isolates to the Center, in turn promoting transdisciplinary collaborations.

**Novel Proteomics-Based Approaches to Leptospiral Strain Typing**

An alternative approach examined for rapid typing of leptospires is matrix absorption laser deionization-time of flight mass spectrometry (MALDI-TOF MS), which utilizes proteomics technology to identify bacteria. In general, initial culture is still required to yield sufficient spirochete numbers for identification. The usefulness of MALDI-TOF MS is also dependent on the development of a reference library of well-characterized strains for comparison purposes. In one study, spectra were obtained for 20 reference strains, which were grouped into 6 classes corresponding to the P1 species L. interrogans, L. borgpetersenii, L. kirschneri, L. noguchii, the P2 species L. fainei, and the S1 species L. biflexa. Spectra were uploaded to the Bruker Daltonics database, and the investigators identified peaks that had the potential to discriminate among serovars. Application of MALDI-TOF MS to the serovar Tarassovi isolate from the Minnesota dairy cow accurately identified the isolate as L. borgpetersenii. In a case report of a teenager that died of leptospirosis in Russia, manual analysis of the MALDI-TOF MS spectrum allowed prediction of the infecting serovar as Leptospira interrogans serovar Canicola, as confirmed by serotyping; however, the spectrum also closely resembled that of L. biflexa serovar Patoc. Because many commercial diagnostic laboratories now possess instruments for routine identification of bacteria using MALDI-TOF MS, combined with the availability of improved culture media, rapid identification of Leptospira spp. with strain information that could assist with surveillance efforts may be more widely achievable on a routine basis.

**Detection and Typing of Leptospirae in the Environment**

A One Health approach to understanding leptospirosis involves consideration not only of infected host species, but also the degree and distribution of environmental contamination with pathogenic leptospires, as well as other environmental factors that might promote infection of the host. Several studies have examined (1) the ability of pathogenic leptospires to persist in the environment, (2) the biodiversity of environmental leptospires, and (3) associations between environmental leptospiral DNA and the DNA of potential reservoir host species.

Different Leptospira species and strains vary in their ability to survive in the environment. Nearly a third of the 3,590 protein-coding genes present in L. biflexa are lacking in the pathogenic species L. borgpetersenii and L. interrogans. Genes common to L. borgpetersenii and L. interrogans but absent in L. biflexa are thought to be associated with virulence; pathogenic leptospires also exhibit considerable genomic rearrangements. When compared with L. borgpetersenii, L. interrogans appears to have retained genes that promote environmental survival and disease transmission through water. P2 species, many of which have been isolated from environmental samples, occupy a position closer to P1 leptospires than to saprophytes, which aligns with the observation that P2 leptospires may be more capable of causing human and animal disease than previously recognized.
In general, existing work supports persistence of P1 leptospires in moist conditions with low UV radiation for weeks to months.\textsuperscript{54-57} Although demonstration of \textit{Leptospira} viability has traditionally required culture, “viability PCR,” a novel approach that differentiates between DNA found in viable and viable cells, has also been used. Here, DNA is first treated with propidium monoazide, which removes cell-free DNA so that cell-associated DNA is available for amplification.\textsuperscript{58} Use of this approach suggested that \textit{L. interrogans} serovar Copenhageni might survive at least 16 days in soil and 28 days in spring water.\textsuperscript{59} No evidence of environmental replication was detected in this study. However, a more recent study found that the organism could replicate in waterlogged soil, but not in soil or water alone.\textsuperscript{59} This could ultimately explain why outbreaks of leptospirosis follow flooding after a lag time, typically 1 to 3 months.\textsuperscript{59-63} In addition, pathogenic \textit{Leptospira} spp. were shown to survive over a year in water under nutrient-deprived, acidic conditions,\textsuperscript{56,64} and viable spirochetes were also identified in soil (2-2°C) under a 40-cm-thick layer of snow in Sapporo, Japan, where the likelihood of contamination of the environment by infected animals was low.\textsuperscript{59} Evidence has accumulated that environmental survival of pathogenic leptospires may be promoted through biofilm formation.\textsuperscript{57,66,66} In general, bacterial biofilm consists of aggregates of bacteria embedded in a matrix composed of polysaccharides, proteins, extracellular DNA, and cell lysis products, which protects them from harsh environmental conditions and antimicrobial drugs. In an elegant study that utilized transposon mutants, biofilm formation by \textit{L. interrogans} was shown to be mediated by the signaling molecule bis-(3’,5’)-cyclic dimeric guanosine monophosphate (c-di-GMP).\textsuperscript{64} Mature biofilm was shown to contain abundant live spirochetes, extracellular DNA, and polysaccharides, but minimal protein.

With advances in the ability to detect and identify bacteria in soil and water, great strides have been made in our understanding of the presence and distribution of pathogenic leptospires in the environment. Isolation of \textit{Leptospira} spp. from the environment has been facilitated by filtration and adding multiple antimicrobials or 5-fluorouracil to the culture medium to inhibit growth of contaminants.\textsuperscript{19,67-72} MALDI-TOF MS has also been used to rapidly identify isolates from soil.\textsuperscript{70} With the use of PCR assays that target genes such as the \textit{lipL32}, \textit{secY}, \textit{flaB}, and \textit{glmU} genes, a diverse array of pathogenic \textit{Leptospira} species have been detected widely and in abundance in soil and water samples from regions where leptospirosis is endemic.\textsuperscript{2,5,19,70,72} Because some pathogenic \textit{Leptospira} strains are not readily cultured from environmental sources, direct PCR sequencing of pathogenic leptosporal DNA from these sources has been performed using \textit{lipL32} and \textit{secY} gene PCR\textsuperscript{54,73,74} or high-throughput sequencing following environmental DNA metabarcoding.\textsuperscript{20,43,75,76} With the latter approach, environmental DNA is concentrated and subjected to multiplex or singleplex PCR; indexing PCR is used to create dual index tags for high throughput sequencing, and the resulting sequences are compared to those in the NCBI database. Assays used for environmental DNA metabarcoding have targeted broad-spectrum 16S rRNA bacterial DNA, and the \textit{Leptospira} 16S rRNA, \textit{lipL32}, \textit{flaB}, and \textit{secY} genes.\textsuperscript{43,76} Embodying a One Health approach, recent studies have used the barcoding approach to detect not only leptospiral DNA, but coincidentally the DNA of vertebrate animals (typically 12S rRNA gene sequences).\textsuperscript{43,76} Statistical analysis is then performed to correlate the presence of pathogenic leptospires with possible reservoir hosts. In the Kandy region in Sri Lanka, environmental metabarcoding was used to detect leptospiral and vertebrate DNA in 10 paddy field irrigation water samples collected from regions where leptospirosis had been reported in humans.\textsuperscript{43} Detection of the DNA of cattle (5/10 water samples), water buffalo (5/10), dogs, civet cats, sheep/goats, and Cape porcupines (each 1/10 samples) correlated strongly with detection of pathogenic leptospiral DNA. There was a high correlation between cattle DNA and \textit{L. interrogans} and \textit{L. noguchii}, and between water buffalo DNA and \textit{L. borgpetersenii}. These findings need to be interpreted with caution because of biases that might result from preferential amplification of certain gene sequences. Uniform distribution of pathogenic spirochetes as well as reservoir host DNA in environmental sources may also impact the results of such studies. It is possible that the success of host infection following exposure to organisms in the environment may depend on whether pathogenic spirochetes are free and motile, or embedded in biofilm; in this case, just finding the DNA of a pathogenic \textit{Leptospira} species in the environment may lack clinical relevance. While currently more expensive, in the future, application of in-depth whole metagenomic sequencing to capture total microbial DNA may further help to elucidate the relative importance of environmental leptospiral strains and their associations with reservoir host animals.

**Conclusion**

The ability to identify and share information on circulating leptosporal sequence types in incidental hosts, reservoir hosts, and the environment with a One Health approach is critical because it (1) allows understanding of the regional distribution of different leptosporal strains; (2) facilitates identification of potential sources of infection for incidental hosts (e.g., specific reservoir host species or environmental sources such as lakes, fountains, swamps, paddy water); (3) helps to identify strains that might be more likely to be transmitted from exposure to environmental sources than from direct exposure to infected reservoir hosts; (4) can suggest the involvement of host factors in outcome of infection (e.g., when the same leptosporal strain is detected in chronic, subclinical infections of a host species as well as in outbreaks of disease in that species); and (5) can inform the need for new vaccines (e.g., when sequence types/serogroups are detected in vaccinated animals.
with leptospirosis that are present within the vaccine). Within a particular species, as yet there have been no clear correlations made between specific sequence types and particular disease manifestations (such as recurrent uveitis in horses or leptospiral pulmonary hemorrhage syndrome), but with the increased discriminatory power of whole genome sequencing and the ability to analyze virulence factor expression, in the future such associations may be more readily identified. Ultimately, there is hope for the discovery of safe human and animal vaccines that provide broad cross-protection, such as mRNA vaccines based on one or more lipopolysaccharide immunogens such as LigB (reviewed by Barazzone et al.). With implementation of such vaccines, molecular typing tools are likely to play increasingly important roles in epidemiological studies. Regardless of the approaches used, our understanding of leptospirosis will be accelerated by uniform adoption and comprehension of new approaches by physicians, veterinarians, public health professionals, clinical microbiologists, and basic science researchers, as well as sharing of results through a unified accessible and trusted platform.

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