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Perspective Piece

Next-Generation Sequencing Analysis of Pathogenic *Leptospira*: A Way Forward for Understanding Infectious Disease Dynamics in Low/Middle-Income, Disease-Endemic Settings

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Abstract. In the current genomic era, knowledge of diversity of *Leptospira*, the spirochetal agents of leptospirosis, is changing rapidly. Next-generation sequencing has decreased in price and increased in scale, with the potential to democratize large-scale analysis of pathogens in resource-limited, low/middle-income (LMIC) regions. Consequently, the molecular classification of *Leptospira*, a pathogen disproportionately affecting LMIC countries, has changed dramatically over the last decade. *Leptospira* classification and molecular understandings of pathogen diversity have rapidly evolved, now most precisely based on core genome analysis supplemented by new insights provided by culture-independent methods directly using body fluids such as blood and urine. In places where leptospirosis disease burden is highest, genomic technologies have not been available, and serology-based methods remain the mainstay of leptospiral classification. Understanding the epidemiology, pathogenesis, and ultimately new approaches to treating and preventing leptospirosis requires detailed knowledge of regionally circulating *Leptospira* in highly endemic settings. Next-generation sequencing–based, culture-independent typing overcomes the limitation of culture isolation of *Leptospira* from clinical samples, with promise of providing public health-actionable information applicable to leptospirosis-endemic LMIC settings.

Knowledge of pathogen diversity is an important component of infectious disease control programs. Leptospirosis, a globally widespread zoonotic disease, is caused by the most diverse bacteria genera. The genus *Leptospira*, of the phylum Spirochaetales, comprised a large number of infectious and noninfectious (saprophytic) species.[1,2] Next-generation sequencing (NGS) has the potential to rapidly obtain detailed leptospiral genomic data in a high throughput and scalable way, for example, enabling whole genome sequencing–based concatenated core genome-based generation of phylogenic trees and multi-locus sequence typing (cgMLST). Such analyses have already led to a significant change in the taxonomy of *Leptospira*, for example, recently adding 43 new species to the previously known 24 species.[1,3–5]

The serological classification of *Leptospira* was first proposed in 1954,[6] which depends on the structural heterogeneity of the carbohydrate component (O-antigen) of *Leptospira* lipopolysaccharide.[7] The proposed classification has evolved to include more than 300 serovars in 32 serogroups.[8] Serological analysis of serovars and serogroups (comprising antigenically related serovars), which does not have species-level taxonomic status, has traditionally been used to classify leptospiral isolates. The labor-intensive cross-agglutination absorption test using the microscopic agglutination test (MAT) with live *Leptospira* and corresponding rabbit antisera has traditionally been the method of choice for assigning serovars, but has become difficult to obtain from reference laboratories.[9]

The MAT is also used for the diagnosis of leptospirosis. It depends on serovar/serogroup-dependent reactions, with sera ideally obtained from acute illness and convalescence. Either a 4-fold rise in titer or a single high titer with an empirically determined cutoff based on the level of regional endemicity may be considered diagnostic in an appropriate clinical context. Microscopic agglutination test diagnostic panels comprise live *Leptospira* designed to represent known pathogenic serogroups and/or circulating serovars from a regionally optimized panel. In many countries with high incidence of leptospirosis, the panel of *Leptospira* is not optimized for the country because knowledge of circulating *leptospires* is not known. *Leptospira* isolation is fundamental to this process. *Leptospira* are fastidious and difficult to isolate and require specialized media and microscopy, and preclude culture and isolation in most endemic settings. Hence, the antigen panel used in the diagnostic process is often not regionally optimized. To improve diagnostic test sensitivity, a standard WHO/CDC MAT panel, comprising a larger number of represented serogroups, has recommended for settings where the leptospiral diversity is expected to be high but where knowledge of circulating serovars is incomplete or unknown.

The human leptospirosis literature commonly reports that the highest agglutination titer in the MAT indicates the infecting serovar; this is problematic.[10] Many case reports (only a few examples cited here)[11–13] and large hospital-[14] and community-based studies[15] have referred to the MAT titer to infer the infecting serovar. In 2003, using 151 culture-positive human leptospirosis cases, Levett demonstrated that the sensitivity of serogroup-level identification was less than 50%.[10] Another study carried out in Thailand using 101 culture-positive patients showed a 33% specificity of the infecting serovar identification.[16] Because the standard WHO/CDC MAT panel is the preferred choice in many endemic countries, the highest reacting strain in the panel may represent only the serogroup, not the infecting serovar. As Levett's work shows, even at the serogroup level, inferences of the infecting serovar from the MAT of patient sera are inaccurate. The cross-reactivity between different serogroups is high when the titer level is high. The comparison of MAT proficiency

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testing from 37 laboratories in 23 countries in 2002 also confirmed that the serogroup identification could be problematic.[17] During the proficiency test, samples from four known serogroups were sent to laboratories, and only 57% of the laboratories had identified all four serogroups correctly partly because their MAT panels did not contain a serovar within the serogroup concerned.[17] Since these observations, this issue has been discussed widely, and in general, researchers generally agree that the MAT should not be used to infer the infecting serovar. Delineating infecting serovar remains important for understanding and tracing infection source for disease control.

Although molecular methods are widely available for diagnosis, genus-level PCR does not differentiate pathogenic serovars; serovar-specific PCR remains elusive. Thus, it is important to develop efficient methods to precisely identify leptospiral serovars, thus replacing imprecise serology. Dissemination of robust molecular/genomic methods and protocols to precisely identify infecting *Leptospira* at the species and serovar levels clearly has the potential to be an important adjunct to culture isolation. Accuracy of identifying infecting *Leptospira* has clinical, public health, epidemiological, and agricultural implications for treatment, control, and prevention. Culture-independent classification at the species level was attempted with mixed results in several studies.[18–22] The usual molecular targets used for this purpose includes 16s ribosomal RNA gene, SecY, and flaB genes. Subspecies-level identification of infecting *Leptospira* was attempted mostly using different multi-locus sequence typing schemes[23–25] but remains inefficient.

Because basic knowledge on pathogen diversity is essential for disease control programs, culture isolation of *Leptospira* still should be attempted despite its inherent challenges in endemic places. Coordinated, adequately resourced programs in highly endemic countries are needed to address this public health gap. These isolates will have multiple uses including validation of culture-independent methods, including the use of NGS with high-resolution sequencing platforms to overcome the challenge of culture isolation. Classification methods such as cgMLST typing, genomic single nucleotide polymorphisms with high discriminatory power, and virulence gene content will result from this work. Recent reductions in the cost of NGS in combination with the development of efficient bioinformatic analysis pipelines have the potential to enable genomic-level *Leptospira* analysis for researchers in low/middle-income (LMIC) regions.[26] Newer technologies such as those offered by Oxford Nanopore Technologies have reduced the capital costs for equipment enough that researchers in leptospirosis-endemic regions have to access molecular testing. Researchers need to be aware of the potential for NGS-related resources to advance genomic knowledge of *Leptospira* and etiological agents of other infectious diseases in the LMIC setting.

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