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***Leptospira* as an emerging pathogen: a review of its biology, pathogenesis and host immune responses**

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Abstract

Leptospirosis, the most widespread zoonosis in the world, is an emerging public health problem, particularly in large urban centers of developing countries. Several pathogenic species of the genus *Leptospira* can cause a wide range of clinical manifestations, from a mild, flu-like illness to a severe disease form characterized by multiorgan system complications leading to death. However, the mechanisms of pathogenesis of *Leptospira* are largely unknown. This article will address the animal models of acute and chronic leptospire infections, and the recent developments in the genetic manipulation of the bacteria, which facilitate the identification of virulence factors involved in pathogenesis and the assessment of their potential values in the control and prevention of leptospirosis.

Keywords

epidemiology; leptospirosis; pathogenesis; virulence

Leptospirosis, the most widespread zoonosis, is emerging as a major public health problem. The clinical manifestations of human leptospirosis are diverse, ranging from mild, flu-like illness to a severe disease form known as Weil's syndrome. Severe disease is characterized by jaundice, acute renal and hepatic failure, pulmonary distress and hemorrhage, which can lead to death [1]. Leptospirosis is caused by spirochetes belonging to the genus *Leptospira*, which comprises both saprophytic and pathogenic species. Leptospirosis has a broad geographical distribution, occurring in both rural and urban areas of tropical, subtropical and temperate regions. The disease outbreaks in developed countries are usually associated with occupational exposure [2,3], tourism or sporting events [4–6]. Developing countries carry the major burden of the disease, with half a million cases reported yearly and a mortality rate ranging from 5 to 10% [7].

This article will focus on the recent advances in understanding leptospirosis pathogenesis; in particular, the different factors identified to have roles in virulence. The development of

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genetic tools and animal models available for further identification and characterization of the different leptospire proteins involved in pathogenesis will also be discussed.

Biology of *Leptospira*

Classification

Traditional classification specified that all saprophytic species are under *Leptospira biflexa*, while *L. interrogans* included all pathogenic species of leptospires [8,9]. The currently used genetically based classification indicates that there are at least 19 species (13 pathogenic and six saprophytic [10,11]), identified through DNA hybridization analysis [12,13]. Seven of these species: *L. interrogans*, *L. borgpetersenii*, *L. santarosai*, *L. noguchii*, *L. weilli*, *L. kirschneri* and *L. alexanderi* are the main agents of leptospirosis [14].

All recognized species of *Leptospira* are categorized into 24 serogroups and 250 serovars [15], based on the expression of surface-exposed lipopolysaccharide (LPS) [10]. The structural differences in the carbohydrate moiety of LPS determines antigenic diversity among the numerous serovar groups. Serovars containing overlapping antigenic determinants are classified into a larger serogroup. Phylogenetic analyses of 16S rRNA genes suggest that *Leptospira* species cluster into three groups designated pathogenic, saprophytic and intermediate [16,17].

Cell biology

Leptospires are thin, helically coiled, motile spirochetes usually 6–20 µm in length. The hooked ends of this bacterium give its distinctive question-mark shape. The leptospires have surface structures that share features of both Gram-positive and -negative bacteria. The double-membrane and the presence of LPS are characteristic of Gram-negative bacteria, while the close association of the cytoplasmic membrane with murein cell wall is reminiscent of Gram-positive envelope architecture [13,18–20].

Motility in leptospires is a function of the two periplasmic flagella or endoflagella, which arise from each end of the bacterium. The disruption of the flagellin gene *flaB* by a kanamycin marker in saprophytic *L. biflexa* through homologous recombination resulted in the absence of the endoflagella with the corresponding loss in bacterial movement [21–23]. On the other hand, the flagellar motor switch *fliY* mutant of the pathogenic *L. interrogans* exhibited attenuated rotative motion in both liquid and semi-solid media [24]. Additionally, guinea pigs infected with the *fliY* mutant had a higher survival rate compared with those infected with wild-type strains, suggesting that endoflagellar rotation and consequent bacterial motility might have roles in the pathogenesis of *Leptospira* infection.

In vitro cultivation

Leptospires are relatively slow-growing bacteria in both liquid culture and solid medium. The optimal growth of the *Leptospira* is observed at temperatures between 28 and 30°C in medium supplemented with long-chain fatty acids, vitamins B1 and B12, and ammonium salts [1,10,13,25]. Long-chain fatty acids are the sole carbon and energy sources currently known, and are broken down through the β-oxidation pathway. The most commonly used medium is Ellinghausen–McCullough/Johnson–Harris, which contains oleic acid, bovine serum albumin and polysorbate (Tween). Contamination of the medium is prevented by autoclaving the water used for preparation, autoclaving the base medium, the addition of 5-fluorouracil and antibiotics such as nalidixic acid or rifampicin [1,10,13], and filter sterilization.

Some pathogenic strains, such as *L. interrogans*, can also survive in low-nutrient environments, such as moist soil and fresh water for long periods, with salt concentration, pH and viscosity as critical factors [26]. However, *L. borgpetersenii* does not survive outside the host, and genomic analyses indicate that the loss of critical genes necessary for survival outside the host limits its transmission through direct host-to-host contact [27]. Microscopic examination and polystyrene plate assays demonstrated that leptospires are capable of aggregating together to form a biofilm. The saprophytic strains form biofilms earlier than the pathogenic species, and among the strains tested, motility does not appear to be necessary in this process [28]. Biofilm formation is proposed to be one of the mechanisms employed by leptospires to survive in environmental niches.

Infection & disease

Leptospirosis is the most widespread zoonotic disease, infecting both human and animals. Infection by pathogenic strains of *Leptospira* commonly occurs through direct contact with infected animal urine or indirectly through contaminated water. Almost every mammal can serve as a carrier of leptospires, harboring the spirochete in the proximal renal tubules of the kidneys, leading to urinary shedding. Rats serve as the major carriers in most human leptospirosis, excreting high concentrations of leptospires (10^7 organisms per ml) months after their initial infections. Humans, on the other hand, are considered as incidental hosts, suffering from acute but sometimes fatal infections [10,19].

Leptospira enters the body via cuts or abrasions in the skin or through mucous membranes of the eyes, nose or throat. The onset of the disease in humans is variable, ranging from 1 day to 4 weeks after exposure, and in survivors, infection can last for months [9]. Leptospirosis may present a broad variety of clinical manifestations; severity depends on the *Leptospira* strain or serovar involved, inoculum size for at least some strains, as well as the age, health and immune status of the infected individual. Many of the documented cases are mild and self-limited, and may be difficult to distinguish from other infectious diseases, until an accurate diagnosis of leptospirosis is made. Other symptoms include headache, chills, nausea and vomiting, myalgia and, less commonly, skin rashes. The most severe disease form is Weil's syndrome, characterized by a multiorgan system complications, including jaundice, meningitis, pulmonary hemorrhage, hepatic and renal dysfunction, and cardiovascular collapse. Vascular injury and endothelial lesions are observed in all affected organs [10,13,19].

Endemicity & epidemiology of leptospirosis

Human leptospirosis has a wide geographic distribution, with Southeast Asia, Oceania, the Indian subcontinent, Caribbean and Latin America considered as highly endemic to the disease [29]. In these regions, climatic changes and flooding, poor levels of sanitation, and high populations of maintenance hosts (e.g., rats) are important determinants of infection. The annual incidence in these areas is 10–100 per 100,000, and this rate rises during outbreaks and in high-risk populations [30]. Some of the recently reported outbreaks in both endemic and nonendemic regions were in recreational settings. Adventure racers in Florida (USA) [6] and Borneo [31], and endurance athletes from Philippines [32] and Illinois (USA) [5] exhibited clinical manifestations of leptospirosis after joining water-sporting events.

The last decade has seen changes in the epidemiologic pattern of human leptospirosis. In regions where the disease is not common, such as Canada, continental USA and Europe, sporadic outbreaks occurred associated with water-sport activities in natural settings contaminated with pathogenic leptospires [5,6,29,33] or changing climatic patterns [29]. An increase in the incidence of leptospirosis in France and the Czech Republic was attributed to flooding events in 1997 and 2002 [29].

The outbreaks in highly endemic regions are normally associated with heavy rainfall flushing the leptospires in soil into bodies of water. In the Asia-Pacific region, leptospirosis is considered a waterborne disease; recent outbreaks in Indonesia (2003), India (2005), Sri Lanka (2008) and the Philippines (2009) occurred after major urban flooding, dispersing leptospires in contaminated waters [30,34]. In these resource-poor regions, lack of proper sanitation and climatic conditions are major contributors to human leptospirosis. The disease is also seen in rural areas, with agricultural activities such as farming and animal husbandry as potential risk factors. Geographically distant countries, including Brazil [35] and Israel [36], have reported a change in epidemiology of leptospirosis from a sporadic rural problem to an important urban disease. This is also the case for many developing countries, with increasing urban population and lack of proper sanitation, heavy rainfall and subsequent flooding lead to an increase in rodent-borne transmission of leptospirosis. With the re-emergence of the disease in nonendemic regions due to recreational exposures and tourism, and its becoming a major urban health problem in highly endemic areas, leptospirosis is now viewed as an emerging global disease.

***Leptospira* pathogenicity**

The mechanisms of leptospiral pathogenesis are poorly understood; however, recently, significant progress has been made. The whole-genome sequences of the pathogenic *L. interrogans* serovars Lai and Copenhageni, and *L. borgpetersenii* serovar Hardjo, as well as two strains of the free-living *L. biflexa* are currently available [27,37–39]. The leptospiral genome is made up of approximately 3.9–4.6 Mbp, depending on the strain and species, and is composed of two circular chromosomes. A third circular replicon, designated as p74, is found in *L. biflexa*, but not in pathogenic strains; however, 13 housekeeping genes found in p74 are present in chromosome I of the pathogenic strains. p74 has been implicated in the survival of *L. biflexa*, because mutations in the housekeeping genes affected the viability of this saprophytic strain [38]. The *L. borgpetersenii* genome is 700 kb smaller than that of *L. interrogans*, which is indicative of genome reduction [27]. Some genes necessary for environment sensing as well as metabolite transport and utilization are lost in this strain, limiting *L. borgpetersenii* survival outside of host animals. *L. interrogans*, on the other hand, can persist in aquatic environments until it encounters a mammalian host. A better understanding of the pathogenic pathways and the virulence factors involved is currently under investigation with the availability of genome sequences, genetic tools and animal models that can be utilized for different studies.

Animal models for leptospirosis

The use of animal models is indispensable in understanding the biology, transmission, host colonization and pathogenesis of *Leptospira*. Of the several animal models established for leptospirosis, hamsters are commonly used owing to their high susceptibility to leptospire infection, with acute leptospirosis exhibiting clinical features that mimics that of severe human infection [40]. This model has been useful in establishing strain infectivity and virulence, examining organ-specific damage caused by leptospirosis, and in screening potential vaccines and therapeutic efficiencies of a variety of drugs [40,41]. Leptospire-inoculated guinea pigs also exhibit fulminant leptospirosis, making them another appropriate model for severe disease [42].

The number of *Leptospira* necessary to initiate infection in small animals varies extensively. A leptospire load of 10^3 – 10^8 is usually introduced intraperitoneally to hamsters to induce lethal infection [43,44]. However, the median lethal dose (LD_{50}) is dependent on the serovar and strain used, and for most strains, the number of *in vitro* passages. Challenge experiments of 10^8 live *L. interrogans* serovar Icterohaemorrhagiae strain Verdun led to 50% mortality [45], but the same number of leptospires were found to be fatal in hamsters using *L.*

interrogans serovar Copenhageni strain Fiocruz L1-130, *L. interrogans* serovar Canicola strain Kito, *L. noguchii* serovar Australis strain Hook and serovar Autumnalis strain Bonito [44]. These strains are highly virulent in hamsters, with an LD₅₀ ranging from three to 100 leptospire.

Experimental leptospire inoculation is primarily performed through intraperitoneal injection [40]. Although this method allows reproducible amounts of leptospire to be introduced into an animal model, it does not reflect the natural transmission of the pathogen. Challenge methods by infecting the animals through skin [46] or mucous membranes of the eye [47] have been employed to mimic natural entry of leptospire into hosts.

Rats and mice are regarded as maintenance hosts, and leptospire infections are mostly asymptomatic with the bacteria subsequently cleared from all organs except the kidneys [48]. Hamsters, immunized with recombinant full-length or truncated LigA proteins, survived after challenging with lethal doses of *L. interrogans* serovars Copenhageni [43] or Pomona [49], suggesting the immunoprotective properties of this surface-exposed protein. While LigA was also shown to confer protection in immunized mice injected with *L. interrogans* serovar Manilae [50], the use of mice as animal model is not ideal due to their resistance to leptospire infection, as shown by their need for high inoculum doses to produce lethal infection [51]. One advantage of mice over hamster models is the availability of gene knockouts that allow the analysis of the roles of host genes *in vivo* [40], and genetic manipulation strategies for rats have recently been developed [52]. Although rat or mouse models are not reflective of disease progression in humans, they provide information on mechanisms of renal colonization and host resistance to severe leptospirosis [48,53]. However, the Toll-like receptor 4-deficient mouse strain C3H/HeJ was shown to be highly susceptible to lethal challenges of *L. interrogans* serovars Icterohaemorrhagiae, Manilae and Copenhageni [1,54,55], suggesting the potential of this particular strain of mice as a model for acute leptospirosis.

The use of zebrafish as a model for *Leptospira* pathogenesis has recently been described. Zebrafish embryos, commonly used for embryonic development studies because their transparency allows observations of living cells under the microscope, have also been applied to microbial pathogenesis research [56,57]. *L. interrogans*-infected embryos were found to be asymptomatic during early stages of infection. The spirochetes were phagocytosed and survived inside macrophages. These infected macrophages were observed to move away from the initial site of infection, suggesting an alternative mechanism by which leptospire dissemination may be facilitated [58]. Although studies using zebrafish may provide information on the initial events of leptospire infection, the relevance of this model to human leptospirosis has yet to be established.

The inoculation of leptospire into larger animals, such as dogs [59], has also been conducted, and dogs have proved to be possible models of reservoir hosts. Leptospire-infected monkeys mimic severe leptospirosis in humans, including pulmonary hemorrhage [60]. Due to their long service as models for human disease, dogs and nonhuman primates have been thought of as similar to humans in kidney physiology and function, and so are valuable in renal physiology studies [40], compared with small animal models. However, the expense of these models and the lack of tools to generate targeted genomic changes are problematic. The use of well-defined models is essential for understanding the various aspects of leptospire pathogenesis.

Genetic tools

The approaches available to leptospiral genetic manipulations are still in early phases in comparison with those of many other bacterial species. Previous attempts to perform

classical genetic studies on *Leptospira* have been thwarted by the absence of naturally occurring plasmids in pathogenic leptospires and the slow growth of the bacteria in both solid and liquid media. The last decade has seen the development of genetic tools to study leptospiral gene functions and the publication of leptospire whole-genome sequences [27,37–39].

The absence of plasmids in pathogenic leptospiral genomes raises the need to generate a replicative vector with a broad host range that can be utilized for genetic experimentation. With the discovery of the bacteriophage LE1 replicating as a circular plasmid in *L. biflexa* [61], an *Escherichia coli*–*L. biflexa* shuttle vector was developed, which contains LE1 origin of replication and antibiotic resistance markers [62]. This DNA can be introduced to leptospires by electroporation and replicates in saprophytic *L. biflexa*, but not in pathogenic species [19,62]. A recent demonstration of conjugation between *E. coli* and *Leptospira* spp. using the RP4 plasmid suggests an alternative method of introducing DNA into leptospires [63].

Site-directed mutagenesis through allelic exchange was successful in the inactivation of several genes, such as the flagellin (*flaB*) gene [22], *recA* [64], tryptophan biosynthetic gene (*trpE*) [65], heme biosynthetic gene (*hemH*) [66] and methionine biosynthesis gene (*metX*) [67] in saprophytic *L. biflexa* and *L. meyeri* using a nonreplicating vector. Pathogenic *L. interrogans* mutants lacking the adhesin *ligB*, the major outer surface protein and adhesin *lipL32*, or the flagellar motor switch *fliY* genes have also been described [24,68,69]. The disruption of these genes was carried out by replacing target sequences with antibiotic resistance genes (kanamycin or spectinomycin) through homologous recombination [67].

Libraries of transposon insertion mutants have been generated in different strains of *Leptospira* using the *Himar1 mariner* element [70,71]. The screening of *L. biflexa* mutants allowed the identification of *fecA* and *feoB* genes, as well as characterization of their role in iron acquisition [72]. This random transposon mutagenesis system resulted in the generation of 926 mutants in *L. interrogans* serovars Manilae (617), Lai (250), Pomona (17), Copenhageni (nine), Canicola (four), and serogroup Canicola strain Kito (32) [71], which include the *lipL32* [69] and *loa22* [73] mutants. Both these genes encode surface-exposed proteins that are regarded as putative virulence factors (discussed further in the following section). In the transposon library for *L. interrogans* there are 712 mutants in the protein-coding regions of 551 unique genes [19,71]. Most of these mutants await phenotypic characterization.

Pathogenesis & virulence factors

The molecular mechanisms of the pathogenesis of leptospirosis remain somewhat unclear at this time. Several candidate virulence factors have been identified that might contribute to the pathogenesis of *Leptospira* infection and disease, including LPS (which is thought of as a general virulence factor of Gram-negative bacteria), hemolysins, outer membrane proteins (OMPs) and other surface proteins, as well as adhesion molecules.

The ability of hemolysins to lyse erythrocytes and other cell membranes makes them potential virulence factors, as demonstrated in a number of other bacterial pathogens. Several putative leptospiral hemolysins have been identified with the completion of *Leptospira* genome sequencing, and work is currently underway to identify their functions. Orthologs of hemolysin proteins Tly, recognized virulence factors in the spirochete *Brachyspira hyodysenteriae* [74], are found in *L. interrogans*. Characterization of the surface-exposed TlyB and TlyC demonstrated that these leptospiral proteins did not exhibit hemolysin properties, but TlyC was found to bind extracellular matrix (ECM) components [75]. Purified sphingomyelinase C from *L. interrogans* serovar Pomona caused the lysis of

sheep erythrocytes [76]. The sphingomyelinase C gene (*sphA*) was also found in another pathogenic leptospire, *L. borgpetersenii* serovar Hardjo, and the expressed protein exhibited sphingomyelinase activities [77]. Hemolysin-encoding genes found in *L. interrogans* serovar Lai include an *sphA* homolog, *sphH*, coding a pore-forming protein without sphingomyelinase or phospholipase activities [78,79], and *sph2*, whose protein product induces endothelial cell and erythrocyte membrane damage [80]. SphH and Sph2 are both expressed during human *Leptospira* infection [81] and demonstrated cytotoxic properties [78,82]. Another group refuted the hemolytic properties of both SphH and Sph2 [81]; however, the disparity in their results may be due to different folding or other properties of the recombinant proteins used for the assays. The direct role of sphingomyelinases in pathogenesis is still unclear; the absence of sphingomyelinase genes in saprophytic leptospires [27] could suggest possible functions in virulence [10], or simply in survival in the mammalian host environment, in which certain key nutrients (e.g., iron) are limiting.

The adhesion of leptospires to host tissue components is thought of as an initial and necessary step for infection and pathogenesis. Attachment to host cells and ECM components is likely to be necessary for the ability of leptospires to penetrate, disseminate and persist in mammalian host tissues. Like other microbial pathogens, leptospires produce microbial surface components recognizing adhesive matrix molecules that might mediate colonization of host [83,84]. It has been demonstrated that *L. interrogans* binds to a variety of cell lines, including fibroblasts, monocytes/macrophages, endothelial cells and kidney epithelial cells grown *in vitro* [85]. Although it is well-established that ECM components play a role in the interaction of the pathogen with host molecules, recent data showed that pathogenic *Leptospira* bind host cells more efficiently [85]. The past decade saw identification of both host cell and ECM substrates and *Leptospira* adhesion molecules involved in this interaction.

Currently, *in silico* analysis and experimental techniques (e.g., Triton X-114 fractionation, surface immunofluorescence, surface biotinylation and membrane affinity tests) can be employed to identify leptospiral surface-exposed proteins that might have potential roles in leptospire adhesion and pathogenesis [86]. In combination, these approaches were successful in characterizing newly identified OmpL36, OmpL37, OmpL47 and OmpL54, but the functions of these proteins remain unknown.

Outer surface proteins are good candidate leptospiral adhesins because of their surface-exposed moieties. Pathogenic leptospires express a number of proteins that are at least partially surface-exposed, including LigA, LigB and LigC, which contain bacterial immunoglobulin-like domains [87,88]. Other bacterial proteins with this domain are known adhesins, such as intimin in *E. coli* [89] and invasins in *Yersinia pseudotuberculosis* [90]. Both LigA and LigB bind ECM components, such as elastin, tropoelastin, collagens I and IV, laminin, and especially fibronectin [91–93]. Fibronectin-binding is modulated by calcium [94], and this interaction is mediated by three motifs in LigB [30,92,95,96]. However, a genetic knockout of *ligB* did not affect virulence or colonization in acutely infected hamsters or chronically infected rats [68]. This suggests the presence of other proteins that are capable of similar interactions with the host, particularly LigA, which likely has overlapping or redundant functions.

A number of *L. interrogans* proteins have been shown to bind the ECM component laminin. One of the characterized laminin-binding proteins is Lsa24/LfhH or LenA, which was also shown to bind complement factor H, factor H-related protein-1, fibrinogen and fibronectin [97–100]. It is a member of the leptospiral endostatin-like protein (Len) family; other proteins belonging to this group (LenB, C, D, E, F) are also found to bind fibronectin [98]. Other leptospiral proteins identified to have laminin-binding properties include Lsa21 [101],

Lsa27 [102], Lsa63 [103] and a 36-kDa membrane protein [104]. Both Lsa27 and Lsa63 are surface-exposed and reactive with serum samples from leptospirosis patients [102,103], suggesting their possible role in host adhesion and pathogenesis, but this remains to be experimentally determined. At present, it remains unclear whether all of these proteins interact with laminin *in vivo* under physiologically relevant conditions, and this will be a key question to explore in the future.

The 32-kDa lipoprotein LipL32 is highly conserved in pathogenic species, absent from nonpathogens and expressed during human infection [105]. This major leptospiral OMP binds collagens I, IV and V, as well as laminin [105–107]. LipL32 also exhibits a calcium-dependent fibronectin binding activity [106,108]. Surprisingly and disappointingly, *lipL32* mutants constructed using transposon mutagenesis did not differ from wild-type pathogenic leptospires in morphology, growth rate or adherence to ECM, and were not attenuated in animal models [69]. Again, the question of functional redundancy will be important but challenging to address.

Loa22, the first genetically described virulence factor in *Leptospira* [73], is a lipoprotein with a peptidoglycan-binding motif similar to OmpA [109] and is upregulated during acute leptospire infection [110]. It is highly conserved among pathogenic *Leptospira*, supporting a role in pathogenesis; however, the function of Loa22 is not yet known. A *loa22* mutant obtained through transposon mutagenesis was avirulent in both the guinea pig and hamster models of leptospirosis. Virulence was restored upon complementation of the mutant [73]. The mutant is surface-exposed and recognized by sera obtained from human leptospirosis patients [111]. Together, all of these results suggest that Loa22 is a good candidate for vaccine development and for investigations into the function of the protein at the molecular level.

The exposure of *L. interrogans in vitro* to temperature and osmotic conditions mimicking the host environment resulted in changes in the expression of many genes [112–115]. In virulent strains, *ligA* and *ligB* are upregulated at physiological osmolarity (for most mammalian tissues) [91,113,116], while expression was lost when strains were culture attenuated [87]. Similarly, the expression of another putative virulence factor gene *sph2* was induced [113], while the outer surface protein gene *lipL36* was repressed [113,117] at physiologic osmolarity. However, most of the differentially expressed genes code for hypothetical proteins with unknown functions [112]. Interestingly, more surface proteins were down-regulated at physiological temperatures [113], possibly as a mechanism by which the pathogen evades the host immune system. These DNA microarray studies demonstrated the ability to pathogenic leptospires to adapt to the shift from environmental to physiological conditions, which may facilitate invasion and establishment of disease in hosts.

Host immune responses to *Leptospira* species

The mechanism of resistance to leptospirosis is mediated largely by the humoral response [13,19,51]. Similar to other Gram-negative bacteria, the antibodies produced during leptospiral infection were found to be agglutinating [118]. IgM and IgG antibodies are serologically detected in patients who recovered from severe leptospirosis up to 6 years after the initial leptospire infection [119]. The antibodies produced are mainly directed against the leptospiral LPS. Monoclonal antibodies against leptospiral LPS from infected mice conferred passive protection to newborn guinea pigs against experimental infection [118]. However, anti-LPS antibody-mediated immunity is limited to homologous serovars, unlike whole leptospiral protein preparations, which demonstrated protection against challenge with both homologous and heterologous leptospire serovars [15]. Leptospiral OMPs OmpL1

and LipL41 were protective immunogens when used in combination [120], which is promising in the development of subunit vaccines with greater protective effects and fewer side-effects. Other surface-exposed proteins have potential immunoprotective properties, but testing of these has been limited to only a few. For example, immunization with conserved and variable regions of LigB, followed by *L. interrogans* serovar Pomona challenge, elicits a protective response in hamsters [121]. Identification of other protective antigens involved in immunity to leptospirosis will also facilitate early detection of *Leptospira* through development of improved diagnostic approaches.

The presence of both TLR2 and TLR4 are necessary for an effective innate immune defense against *Leptospira* [122–125]. Double-knockout mice lacking both TLR2 and TLR4 are highly susceptible to leptospire infection compared with single knockouts [122]. However, the absence of TLR4 does lead to severe leptospirosis with clinical syndromes of jaundice and pulmonary hemorrhage, as well as accumulation of leptospire in kidneys and lungs in mice infected with *L. interrogans* serovar Icterohaemorrhagiae [124]. LPS, which typically activates the TLR4 pathway in response to Gram-negative bacteria, was able to activate the TLR2 pathway in human monocytic cell lines [125]. The TLR2-deficient mice did not respond to intraperitoneal injection of leptospiral LPS, and failed to produce TNF- α and IL-6. Together, these results suggest that there may be fundamental differences in how different species react to leptospire. Further evidence supporting this hypothesis is that, while human monocytic TLR4 is not activated by LPS or heat-killed leptospire [125], TLR4-deficient mice injected intraperitoneally with *L. interrogans* serovar Icterohaemorrhagiae displayed severe leptospirosis. Nahori *et al.* demonstrated that leptospiral lipid A is highly modified compared with other Gram-negative lipid As [123]. Despite its atypical features, leptospiral lipid A can be recognized by TLR4 of murine cells. The presence of TLR4 was shown to be necessary for early production of IgM by B cells, in response to leptospire LPS [122].

The disparity in the outcomes of leptospire infection between mice and human hosts has led to studies aiming to gain a better understanding of the mechanisms of colonization and resistance to severe disease in leptospirosis. The difference in mounting an effective immune response against pathogenic leptospire between maintenance and accidental hosts might contribute to the early containment of infection. Because leptospire recognition in murine cells is mediated by both TLR4 and TLR2 receptors, while only TLR2 is activated in human cells by LPS signals [123,125], it has been suggested that leptospiral lipid A is not recognized by human TLR4, and thus fails to activate this immune pathway. The lack of leptospiral lipid A recognition in human cells, but not in murine cells, might result in failure to mount appropriate responses leading to leptospirosis susceptibility. Whether the response to a particular LPS differs between reservoir and accidental hosts for other *Leptospira* has yet to be described.

Macrophages have been shown to effectively phagocytose leptospire *in vivo* [126] and *in vitro* [13], although during infection the organisms are not cleared. Recently, Li and colleagues demonstrated that *L. interrogans* strain Lai bind and enter both murine and human macrophages with the same efficiency, but the intracellular fate of the phagocytosed pathogen differs [127]. In murine macrophages, leptospire were observed to reside in lysosomes through co-localization with late-endosomal marker LAMP-1 and were degraded by lysosomal hydrolases. By contrast, leptospire phagocytosed by human macrophages were able to escape from phagosomes into the cytosol, where they proliferated and activated apoptosis. Both the inability of TLR4 to detect leptospire and the phagosomal escape of *Leptospira* suggest possible mechanisms by which *Leptospira* are able to circumvent the human immune system. Further studies to determine the specific roles of host and other

microbial factors in the difference between disease manifestations in humans and mice are warranted.

Conclusion & future perspective

Leptospiral infection can lead to multiorgan system complications or even death in accidental hosts such as humans, while causing only a mild chronic-to-asymptomatic infection in reservoir hosts such as rodents [10,20]. It has been proposed that the differences in host immune responses can lead to disparate disease outcomes [123]; however, the mechanisms involved are far from being established. With the availability of animal models of acute and chronic leptospirosis, and knockout mouse models, biochemical and genetic studies can be employed to gain a better understanding of host colonization and resistance to severe disease in leptospirosis. These approaches allow the identification of both host determinants and pathogen factors that are involved in disease susceptibility.

Major advancements in leptospiral genetics have been achieved, with the determination of leptospire whole genome sequences, as well as development of tools such as shuttle vectors, and site-directed and transposon mutagenesis systems. However, obstacles in genetic experimentation in pathogenic leptospires remain, such as the absence of replicative plasmids and slow growth of the bacteria. While more efficient methods of generating *L. interrogans* mutants are still under development, the use of saprophytic *L. biflexa* as a model organism allows the characterization of the functions of genes common to both free-living and pathogenic leptospires.

Recent advances leading to the generation of mutant leptospires allowed functional analysis of putative virulence factors. While this has established Loa22 as the first genetically identified virulence factor [73], disappointingly, the disruption of *lipL32* and *ligB* did not alter infectivity of mutant leptospires in the animal models that have been tested [68,69]. This may indicate that at least some of the proteins involved in pathogenesis may have overlapping functions. Additional techniques, such as generation of double knockouts, can be employed to allow simultaneous evaluation of related candidate virulence factors in the infection process of pathogenic *Leptospira*. In the future, leptospiral research can involve the application of proteomics alongside genomic methods to establish the expression pattern of putative virulence factors. The recent global proteome analysis of *L. interrogans* using mass spectrometry and 2D gel electrophoresis, showed that 65 of the 563 pathogenic leptospire proteins identified exhibited altered expression when leptospires were introduced into conditions that mimic host environment [128]. The caveat of this work, as well as the findings of Lo that leptospire protein expression in *L. interrogans* serovar Lai is altered with temperature shifts [112], is that the effect of specific environmental factors is evaluated, and thus, fails to capture accurate conditions during acute or chronic host infection. This remains technically extremely difficult. Monahan *et al.* performed proteomic analysis of *L. interrogans* serovar Copenhageni isolated from urine of chronically infected rats, and comparison with the attenuated leptospires grown *in vitro* demonstrated differences in protein profiles between the two groups [129]. Leptospires excreted by kidneys into urine are highly virulent, and may express proteins necessary for pathogenesis. Recently, using mass spectrometry techniques, Malstrom *et al.* were able to demonstrate that *L. interrogans* maintains a constant proteome concentration. Exposure of the leptospires to ciprofloxacin led to an increase in the amounts of normally unexpressed protein with a corresponding decrease in the expression of abundant proteins [130]. All of these studies suggest differential protein expression of pathogenic leptospires when outside the host, as well as during entry into, colonization of and persistence in host organisms. Further studies of the protein expression profiles of *Leptospira* as this bacterium moves from environmental

niches to different host tissues would provide information on the factors involved in the colonization process, leading to establishment and persistence of infection in hosts.

A major challenge is the application of basic research to improve diagnostics and vaccine development. The available diagnostic tests are not always serovar-specific, because crossreactivity against different serovars may occur between organisms in the same serogroup. Possible differences in pathogenic mechanisms between different strains remain to be explored. Because the initial presentation of leptospirosis may be difficult to discern from certain other infectious diseases, rapid and accurate diagnosis is essential in order to prevent the progression of the more severe form of the disease, particularly in developing countries. There are currently no widely available vaccines against leptospirosis for use in humans. Virulence factors observed to be upregulated during infection, such as OMPs with surface-exposed moieties, such as LigA and LigB, are being considered as possible targets for the development of vaccines. With the current robust leptospiral research output, in the near future we may see the development of simple and inexpensive diagnostic systems appropriate for highly endemic, resource-poor areas, as well as the application of state-of-the-art technologies to vaccine development.

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