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Leptospira: The Dawn of the Molecular Genetics Era for an Emerging Zoonotic Pathogen

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Abstract

Leptospirosis is a zoonotic disease which has emerged as a major cause of morbidity and mortality among impoverished populations. One centenary after the discovery of the causative spirochaetal agent, little is understood of *Leptospira* pathogenesis, which in turn has hampered the identification of new intervention strategies to address this neglected disease. However the recent availability of complete genome sequences for *Leptospira* and discovery of genetic tools to transform the pathogen has led to major insights into the biology and pathogenesis of this pathogen. We discuss the life cycle of the bacterium and the new advances that have been made and their implications for the future prevention of this disease.

Descriptions of leptospirosis-like syndromes were reported in the scripts of ancient civilisations [1], but the first modern clinical description of the leptospirosis was that of Weil in 1886 [2]. Inada *et al.*, in their landmark study from 1916, isolated leptospires, identified the organism as the causal agent of leptospirosis, and determined that rats are a reservoir for transmission to humans [3]. Leptospires were subsequently isolated from a wide range of animal reservoir species and classified into serogroups and serovars as a function of their antigenic determinants (Box 1).

Leptospirosis, a zoonotic disease **[AU: GT]** with a worldwide distribution, is now recognised as an emerging infectious disease [4]. Over the last decade, outbreaks during sporting events, adventure tourism and disasters underscore the ability of the disease to become a public health problem in non-traditional settings [4-6]. Yet leptospirosis is mostly a neglected disease which imparts its greatest burden on impoverished populations from developing countries and tropical regions [6]. Leptospirosis, in addition to being an endemic disease of subsistence farmers [1, 4, 5], has emerged as a widespread problem in urban slum populations where inadequate sanitation has produced the conditions for rat-borne transmission of the disease [7-9]. More than 500,000 cases of severe leptospirosis are reported each year, with case fatality rates exceeding 10% [10].

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Previous reviews summarized our knowledge of the epidemiology, diagnosis, and clinical features of leptospirosis [1, 4-6] as well as the genomics of *Leptospira* spp. [11]. This review will focus on the pathogenesis of leptospirosis and highlight the recent advances with respect to genetic approaches taken and the virulence factors discovered.

The question mark-shaped bacteria

The genus *Leptospira* comprises of saprophytic and pathogenic species and belongs to the phylum of spirochaetes (Box 1) [12]. Saprophytic leptospires, such as *L. biflexa*, are free-living organisms found in water and soil and unlike pathogenic *Leptospira* spp., do not infect animal hosts [1]. Leptospires are thin, highly motile, slow-growing obligate aerobes with an optimal growth temperature of 30°C and can be distinguished morphologically from other spirochaetes on the basis of their unique hook or question mark-shaped ends [13] (Figure 1A).

The genomes for two pathogenic species, *L. interrogans* and *L. borgpetersenii*, and one saprophytic species, *L. biflexa*, have been sequenced [14-17]. The majority (77-81%) of the genes in the *Leptospira* genome do not have orthologues found in the genomes of other spirochaetes, confirming that large degree by which leptospires have genetically diverged from other members of the phylum [12]. Furthermore, comparative analysis of genomes of pathogens and saprophytes [11, 17] has provided insights on the genetic determinants that may be involved in pathogenesis (Box 2).

The transmission cycle

Transmission requires continuous enzootic circulation **[AU: GT]** of the pathogen among animal reservoir, or as commonly referred, maintenance hosts (Figure 2). *Leptospira* serovars demonstrate specific host preferences with respect to their ability to produce high-grade carriage. For example, rats (genus *Rattus*) serve as reservoirs for the Icterohaemorragiae serogroup, whereas house mice (*Mus musculus*) are the reservoir for the Ballum serogroup [4, 5, 18]. Furthermore, serovars often do not cause significant disease in reservoir hosts to which they are highly adapted (Box 3).

The pathogen colonises and is shed from the renal tubules of a broad spectrum of animals (Box 3). Leptospires survive for weeks to months in moist soil and water after excretion in the urine [19]. Cell aggregation [19] and biofilm formation [20] (Figure 1B) may contribute to the survival of leptospires outside their hosts.

Disease pathogenesis

Pathogenic *Leptospira* spp. produce a systemic infection after an environmental exposure, establish persistent renal carriage and urinary shedding in reservoir animals, and cause tissue damage in multiple organs of susceptible hosts. Acute disease and chronic colonisation represent opposite poles of a wide spectrum of disease presentations (Box 3). Humans are incidental hosts in which leptospirosis causes acute disease manifestations and yet does not induce a carrier state required for transmission of the pathogen.

Dissemination in the host

Leptospires penetrate abraded skin and mucous membranes and quickly establish a systemic infection by crossing tissue barriers and haematogenous **[AU:GT]** dissemination [1]. It was believed that leptospires, like other spirochaetes, spread by transiting though intercellular junctions [21]. However, leptospires have been shown to efficiently enter host cells *in vitro* [22, 23] and rapidly translocate across polarized cell monolayers without altering the

trans-epithelial electrical resistance [24, 25]. Leptospires are not facultative intracellular organisms for they are rarely observed intracellulary in infected tissues and appear to reside transiently within host cells as they cross cell monolayers *in vitro* [25]. The process by which leptospires enter host cells is not understood: internalized leptospires have been observed in cytoplasmic [24, 25] and phagosome compartments [23] of normally non-phagocytic host cells. Nonetheless, these findings suggest that leptospires use host cell entry and rapid translocation as a mechanism to spread to target organs and evade immune killing.

Infection causes a prolonged leptospiraemia until the host mounts an effective acquired immune response which occurs one to two weeks after exposure (Figure 3A) [26]. Leptospires are isolated from the bloodstream within minutes after inoculation [1] and are detected in multiple organs by the 3rd day after infection [26-29]. Leptospires, whose burden in blood and tissues may reach up to 10^6-10^7 organisms/ml or g in patients [30, 31] and infected animals [29], are able to evade the host innate immune response during the early-phase of infection. The organism is resistant to the alternative pathway of complement activation [32, 33] and acquire Factor H and related fluid-phase regulators [34, 35], through ligands such as Leptospiral endostatin-like (LEN) proteins [36, 37]. Host C4BP binds to the surface of leptospires [38], suggesting that a similar process may confer some protection against the classical pathway of complement activation.

Persistent colonisation

The essential component of the pathogen's life cycle is its ability to produce persistent renal carriage in reservoir animals. In rats, leptospires cause a systemic infection but are cleared from all organs except the renal tubules [28, 39]. Colonised tubules are densely populated with leptospires, which aggregate together with an amorphous biofilm-like structure (Figure 1D). Rats have been shown to excrete leptospires in high concentrations (10^7 organisms/ml [39]) for periods of 9 months after experimental infection [40].

Leptospires isolated from chronically-infected rat kidneys have significantly higher amounts of lipopolysaccharide (LPS) O-antigen than those isolated from livers of hamsters with acute disease, suggesting that expression of O-antigen content may facilitate induction of carriage [39]. The renal tubule is an immunoprivileged site, a feature which may contribute to high-grade persistence of the pathogen. Moreover leptospires which are shed in the urine down-regulate the expression of proteins recognised by the humoral immune response in rats [41].

Disease manifestations and determinants

Infection does not produce disease until 5-14 days (incubation period, 2-30 days) after environmental exposure (Figure 3A) [1]. In humans, leptospirosis causes a febrile illness which in its early-phase, often cannot be differentiated from other causes of acute fever. In most patients, illness resolves after the 1st week of symptoms. Yet a subset (5-15%) of patients progress to develop severe late-phase manifestations [6]. Unlike bacterial infections such as gram-negative sepsis, leptospirosis does not cause a fulminating disease manifestations shortly after the onset of illness, which may relate to the low endotoxic potency of *Leptospira* LPS [1]. Severe late-phase manifestations occurs four to six days after onset of illness (Figure 3A) but may vary depending on the infecting inoculum dose and other disease determinants. Weil's disease is the classic presentation of severe leptospirosis and characterized by jaundice, acute renal failure and bleeding. In addition, there is increasing awareness of a new emerging severe disease form, leptospirosis-associated pulmonary haemorrhage syndrome (LPHS) (Box 4) for which the case fatality rate is >50% [6].

Development of leptospirosis and disease progression are influenced by the virulence characteristics of the strain, host susceptibility factors and infecting inoculum size during environmental exposure. Specific *Leptospira* species and serovars are more frequently found to cause severe disease in humans [42, 43]. Thaipadungpanit *et al.* found that a single circulating clone caused a large and sustained nationwide epidemic in Thailand [44]. Clonal transmission of strains has been described in other outbreaks and settings of endemic transmission [45, 46] and may reflect localized clusters of transmission [45]. However the magnitude and duration of the epidemic in Thailand suggests that predominant clones may indeed possess specific factors which contribute to their overall biological success. The advent of high-throughput whole-genome sequencing provides an opportunity to determine whether such factors exist by screening isolate genomes for genetic polymorphisms associated with clinical and transmission-related phenotypes.

Our understanding remains limited on the acquired and innate host factors which influence infection and disease progression. An investigation of a triathlon-related outbreak identified HLA-DQ6 genotype as the first and to date only genetic susceptibility factor reported for leptospirosis [47]. The authors found a synergistic risk interaction between HLA-DQ6 and swallowing water while swimming during the triathlon event. This environmental exposure was a likely proxy for an inoculum size effect. It is well known that increasing inoculum size shortens the incubation period and decreases survival in a dose-dependent manner in experimental animals (Figure 3B) [26, 48]. The synergism between HLA-DQ6 and environmental exposures found during the triathlon outbreak constitutes the first gene-environment interaction identified for an infectious disease.

Tissue damage

The onset of disease correlates with the appearance of agglutinating antibodies and clearance of leptospires by antibody-mediated opsonisation and lysis (Figure 3A) [1]. Vascular endothelial damage is a hallmark feature of severe leptospirosis [49, 50] and causes capillary leakage, haemorrhage, and in a subset of cases, vasculitis. Leptospirosis activates the coagulation cascade [51, 52] and has been reported to cause disseminated intravascular coagulation in up to 50% of patients with severe disease manifestations [51].

Leptospiral components released after immune killing stimulate production of pro-inflammatory cytokines [53-56] and mediate inflammation and damage of end-organ tissues. The Jarisch-Herxheimer reaction, caused by the sudden release of these cytokines, is a complication of antimicrobial therapy for leptospirosis. Moreover, TNF-alpha may play a key role in disease progression since levels of this cytokine are a predictor of poor clinical outcomes [57].

The *Leptospira* LPS has been shown to activate Toll-like receptor 2 (TLR2) rather than the TLR4 pathway in human cells [58], an unusual finding that may relate to a 1-methylphosphate moiety which is not found in other bacterial lipid A [59]. In addition, leptospiral lipoproteins induce innate responses by activating the TLR2 pathway [58, 60]. As a caveat, *Leptospira* LPS activates both TLR2 and TLR4 pathways in mouse cells, indicating that there are species-specific differences with respect to TLR activation [61]. Leptospires stimulate expansion of gamma-delta T cell populations in naïve peripheral blood mononuclear cells and leptospirosis patients have increased numbers of this specific subset [54], suggesting that acquired cell-mediated responses, in addition to innate and acquired humoral responses, may promote inflammation.

Infection causes pronounced physiological disturbances in the kidney and liver, which has led to the speculation that leptospires liberate a toxin. Leptospirosis produces a peculiar hypokalaemic non-oliguric form of acute renal failure characterized by impaired tubular

sodium reabsorption [62]. *Leptospira*-derived non-esterified unsaturated fatty acids have been found to inhibit kidney Na+, K+ ATPase [63]. However, it seems more plausible that the renal manifestations are the direct result of a focal tubulointerstitial nephritis. Leptospiral outer membrane proteins, such as LipL32, activates TLR-dependent pathways which leads to induction of nuclear transcription factor kappa B, mitogen-activated kinases and cytokines and subsequently, tubular damage [60]. Furthermore, activation of these pathways may provide a possible explanation for the dysregulation of sodium transporters in infected kidneys, a finding which has shown to be associated with impaired sodium reabsorption [64, 65].

Leptospires have been reported to induce apoptosis in macrophages and hepatocytes [22, 66, 67], yet the overall contribution of apoptosis in disease pathogenesis has not been delineated. Leptospirosis elicits production of autoantibodies, such as anti-cardiolipin antibodies [68]. Several reports suggest that autoimmune mechanisms may play a role in the development of uveitis [37] and LPHS [69] during infection.

Genetic tools for *Leptospira*

The virulence mechanisms, and more generally the fundamental understanding of the biology of the causative agents of leptospirosis, remain largely unknown. Before 2000, the lack of genetic tools available for use in leptospires, in either pathogenic or saprophytic species, precluded the full characterisation of genes of interest. In the first genetic studies carried out in the 1990s, several *Leptospira* genes were isolated by the functional complementation of *E. coli* mutants. This method led to the identification of the *L. biflexa recA* gene [70], the *L. interrogans rfb* genes [71], and a number of amino acid biosynthesis genes, such as *asd* and *trpE* [72, 73].

The origins of replication from the LE1 temperate leptophage [74], a 74-kb extrachromosomal element of *L. biflexa* [17], and a genomic island that can excise from the *L. interrogans* chromosome [75] were used to generate a plasmid vector able to replicate autonomously in both *L. biflexa* and *E. coli* [76]. DNA can be introduced into *Leptospira* by electroporation [76, 77] and conjugation [78]. However, to date, there is no replicative plasmid vector available for pathogenic *Leptospira*.

Deletion of chromosomal genes, including *flaB, trpE, metY, metX, metW, hemH*, and *recA* by targeted mutagenesis was achieved in the saprophyte *L. biflexa* with a suicide plasmid [79]. Recently the first gene, *ligB*, was disrupted in the pathogenic *L. interrogans* [80] by site-directed homologous recombination.

A system for random mutagenesis using the *Himar1 mariner* transposon has been developed in both saprophytic and pathogenic *Leptospira* strains [77, 81, 82]. In *L. biflexa* an extensive library of mutants can be generated that can be screened for phenotypes affecting diverse aspects of metabolism and physiology, such as amino-acid biosynthesis and iron acquisition systems [82, 83]. However pathogenic leptospires remain much less easily transformable with *Himar1*[77]. At the end of three years of transformation experiments performed simultaneously in two different laboratories, we obtained about 1000 random mutants with characterised transposon insertion points in *L. interrogans* (Table 1) [81]. In total, 721 of the mutations identified affected the protein coding regions of 551 different genes. The challenge at the moment is to improve existing methods and to identify more readily transformable pathogenic strains for further genetic studies in *L. interrogans*. If successful this approach should make it possible to generate a library for the high-throughput screening of mutants for specific processes known to be involved in pathogenesis.

Animal models of virulence

Guinea pigs and hamsters are the standard experimental model for acute leptospirosis [1]. Infection with low inocula (<100 leptospires) produces similar disease kinetics (Figure 3B) and severe manifestations as observed in humans (Figure 3B) [48]. Mice and gerbils have been used to study the genetics of the immune response to leptospirosis [61, 84, 85] and as models for vaccine-mediated immunity (Table 2) [86]. However mice are relatively resistant to infection and require high inocula (up to 10^8 organisms) to produce disease, a situation which may not parallel what occurs during naturally-occurring exposures. Furthermore mice, when administered with high inoculum doses required to induce a lethal infection, develop a more fulminant clinical course and tend to die within significantly shorter intervals (five days) than that observed in patients or hamsters infected with low-inoculum lethal challenges (Figure 3B). This finding raises concerns that this experimental animal model may not reproduce the disease dynamics and pathogenic processes observed in natural infections. Rats have been used as a model to study persistent colonization but also require high inocula [28, 39]. Like mice, it is not understood why this common reservoir in nature is relatively difficult to infect experimentally. Natural infection with leptospirosis occurs in non-human primates, which in turn have been used as models to study the disease [87], and more recently, the development of pulmonary haemorrhage syndrome [88].

Virulence factors

The virulence factor determined to date are primarily surface proteins, which are thought to mediate the interaction between the bacterium and the host tissues. Although several proteins are secreted by *Leptospira* spp., including degradative enzymes, there is no evidence for any dedicated protein secretion pathway for injection of proteins into host cells, such as the Type III and Type IV secretion machinery of Gram negative bacteria. Other virulence factors promote motility and iron acquisition, but many other factors, including proteins that mediate host-cell interactions or cause tissue damage are likely to be discovered.

The development of genetic tools and the availability of complete genome sequences of pathogenic *Leptospira* have made it possible to apply state-of-the-art approaches to determine the virulence and survival mechanisms used by these bacteria to ensure their persistence in different ecological niches.

Previous microarray studies have shown that exposure of *L. interrogans* to the osmolarity conditions found in host tissues induces a profound shift in global transcription profiles. Thus, osmolarity and temperature [89, 90] are important factors regulating the expression of proteins mediating the infection of mammalian hosts. Nineteen of the 25 most strongly salt-induced *L. interrogans* genes encode hypothetical proteins [90]. These genes may encode response regulators and environment-sensing proteins involved in survival or persistence in the environment or in the infected host.

Surface proteins

Moieties expressed on the surface of leptospires, are believed to be determinants in the pathogen's interaction with the host and ability to cause virulence. Leptospires adhere and enter *in vitro* host mammalian cells (Figure 1C), a phenotype which is observed in virulent leptospires and not in culture-attenuated or saprophytic organisms [22, 24, 25, 91]. The attachment of pathogenic leptospires to eukaryotic cells (Figure 1C) is a key step in the process of infection that may involve molecules secreted by the bacterium or present on its surface (Figure 4). Several leptospiral proteins have been shown to bind *in vitro* to several components of the extracellular matrix [36, 92-96]. Furthermore, virulent leptospires had

significantly lower numbers of protein particles on the outer membrane surface as determined by freeze-fracture electron microscopy, and expressed different protein and LPS profiles than culture-attenuated strains [97].

Like other spirochaetes, the genomes of *Leptospira* spp. possess a much higher number of lipoprotein genes than that of other bacterial genomes. Analysis of the genome sequences of *L. interrogans* led to the detection of approximately 145 putative lipoproteins [98] and several putative extracellular and outer membrane proteins [99, 100].

Consistent with the predicted ability of *Leptospira* to migrate through host tissues, its genome encodes a wide range of putative hemolysins and proteases that may facilitate this process. An analysis of the *L. interrogans* genome identified nine genes which encode putative hemolysins, including sphingomyelinase genes that are not found in the saprophyte *L. biflexa* [17] and a pore-forming protein gene [101]. Sphingomyelinase C was found to be up-regulated by increases in osmolarity to the levels found in mammalian host tissues [90]. The *L. interrogans* genome also contains a microbial collagenase, which is hypothesised to be involved in the destruction of host tissues.

Few proteins have been experimentally shown to be present on the leptospiral surface [102]. Together, about twelve proteins have been identified as outer membrane proteins and include OmpL1 [103], LipL32 [104], LigB [105], LenA [36], LenD [36], and Loa22 [106]. Our knowledge of the surface of leptospires thus remains limited and the further development and improvement of tools for accurate localisation of surface-associated determinants are required.

Loa22

The only gene to date that fulfils Koch's molecular postulates for a virulence factor gene is *loa22*. Disruption of *loa22* by *Himar1* insertion in *L. interrogans* led to a complete loss of virulence in the guinea pig model (Table 1) [106]. Loa22 is exposed on the bacterial surface [106] and recognised by sera from human leptospirosis patients [107] and its expression is up-regulated in an acute model of infection [108]. The observed Loa22 *in vitro* binding with components of the extracellular matrix is relatively weak [109]. The C-terminal of Loa22 consists of an OmpA domain, which contains a predicted peptidoglycan-binding motif. Although the non-pathogenic *L. biflexa* genome contains an orthologue of *loa22* [17], differential expression of this gene in pathogenic and non-pathogenic leptospires or pathogen-specific sialylation of Loa22 dependent on pathogen-specific sialic acid modification pathways (J. Ricaldi and J. Vinetz, personal communication) may explain why this protein, post-translationally modified, is a critical determinant of *L. interrogans* virulence.

LipL32

LipL32 [104], also designated Hap-1 for haemolysis-associated protein [110], is surface-exposed [104] and accounts for 75% of the outer membrane proteome [111]. The lipoprotein is highly conserved among pathogenic *Leptospira* [112]; there are no orthologues of *lipL32* in the saprophyte *L. biflexa* [17]. LipL32 was long believed to be a putative virulence factor. Higher levels of LipL32 are expressed in leptospires during acute lethal infections than in leptospires cultured *in vitro* [108]. The C-terminus of LipL32 binds *in vitro* to laminin, collagen I, collagen IV collagen V and plasma fibronectin [94, 95]. The crystal structure of LipL32 was elucidated recently and it was shown to present structural homologies with proteins such as collagenase that bind to components of the extracellular matrix [113]. Yet a LipL32 mutant, obtained by *Himar1* insertion mutagenesis, was found to be as efficient as the wild-type strain in causing an acute disease and chronic colonisation in experimental

animals (Table 1) [114]. The role of this major outer membrane protein in pathogenesis remains unclear and is a matter for debate.

Leptospiral immunoglobulin-like proteins

A family of three high-molecular weight *Leptospira* proteins — LigA, LigB and LigC — was identified as a novel member of the bacterial immunoglobulin (Ig)-like (Big) protein superfamily [86, 105, 115]. Lig proteins are anchored to the outer membrane and have 12 to 13 tandem Big repeats domains. Like *lipL32, lig* genes are exclusively present in pathogenic *Leptospira*. Recombinant Lig proteins bind *in vitro* to host extracellular matrix proteins, including fibronectin, fibrinogen, collagen, and laminin [96, 116]. Furthermore, the repeat domain portion of the LigB molecule binds Ca^{2+} which in turn, appears to enhance its ability to adhere to fibronectin [117]. The *lig* genes are up-regulated at physiological osmolarity [90] and encode surface-exposed proteins strongly recognised by sera from human patients with leptospirosis [105, 118, 119]. Lig proteins are considered a putative virulence factor [105] since members of the bacterial Ig-like superfamily mediate pathogen-host cell interactions, such as invasion and host cell attachment, in other bacteria. However, a *ligB* mutation in *L. interrogans*, which also contains a *ligA* gene [80], does not affect the ability of the bacterium to cause acute leptospirosis or persistent renal colonisation in hamsters and rats, respectively. The presence of several other putative adhesins with potentially redundant functions, including LigA, may have obscured the detection of clear phenotypes for the *ligB* mutant.

Other potential virulence proteins

The motility of the bacterium may be of relevance to its basic biology and, despite also being common to saprophytes, may be considered a virulence factor. Freshly-isolated pathogenic leptospires have higher translational and helical motility in comparison to strains passaged *in vitro* [120]. The corkscrew motility allows these organisms to swim through gel-like medium, such as connective tissues [13]. However, it has not been determined whether loss of motility directly results in attenuation of virulence for pathogenic leptospires. *L. biflexa flaB* mutants cannot form functional endoflagella, but their cell bodies remain intact and helical [121]. The endoflagella are therefore not responsible for dictating the helical shape of the cell body in *Leptospira* spp as they do in *Borrelia burgdorferi* [122]. Proteins known to be involved in the morphogenetic system of rod-shaped bacteria, such as MreBCD and penicillin-binding proteins, are encoded by genes present in the leptospire genome. Leptospiral cell morphology may thus be determined by the cytoskeleton and maintained by the rigid murein layer.

Multiple methyl-accepting chemotaxis proteins have been identified in *Leptospira*, suggesting that chemotactic responses to various chemoattractants/repellents may occur. Unlike avirulent or saprophytic strains, *L. interrogans* displays positive chemotaxis towards haemoglobin [123].

Iron acquisition is important for virulence in many bacterial pathogens, and *Leptospira* species have been found to contain several iron uptake systems, including TonB-dependent outer membrane receptors [83]. *Leptospira* spp. possess a haem oxygenase, encoded by *hemO*, which degrades the tetrapyrrole ring of the haem molecule, releasing ferrous iron. Disruption of the *hemO* gene in *L. interrogans* decreases virulence in the hamster model of leptospirosis (Table 1) [124], suggesting that *Leptospira* uses haem as its principal source of iron during infection.

Mutations in the genes encoding the surface-associated proteins LenB and LenE, which were considered putative virulence factors [36] did not have an effect on virulence (Table

1) [80, 81]. Two attenuated mutants with disruptions in hypothetical genes may correspond to novel virulence factors in *L. interrogans* [81], but these findings need to be confirmed with complementation studies.

Immunity

The humoral response is believed to be the primary mechanism of immunity to leptospirosis [125]. LPS appears to be the major target for the protective antibody response, since passive transfer of immunity correlates with levels of agglutinating anti-LPS antibodies in patient sera [126] and anti-LPS monoclonal antibodies passively protect naïve animals from leptospirosis [127]. However, it is not known whether antibody responses against leptospiral antigens in addition to LPS also confer protection.

Recent work has contributed to the understanding that immunity to leptospirosis is not limited to the humoral response. Mice require intact TLR2 (Chassin *et al.*, submitted) and TLR4 [85] activation pathways of innate immunity in order to control a lethal infection. In contrast to immunity in hosts susceptible to acute leptospirosis, protective immunity against *L. borgpetersenii* serovar Hardjo in bovine maintenance hosts is cell-mediated. Immunisation trials in cattle found that protection against this serovar, conferred by whole *Leptospira*-based vaccines, correlated with T_H1 responses and not with agglutinating antibody titres [128-130]

Vaccines

Ido *et al.* provided the first demonstration in 1916 that immunisation with killed leptospires protects against experimental infection [131]. Since then, whole *Leptospira*-based vaccines have been routinely administered to livestock and domestic animals and used for immunization of human populations [6]. However there are major concerns with respect to their use [132]. Whole *Leptospira*-based vaccines are associated with high rates of adverse reactions and confer only short-term serovar-specific immunity [1]. Polyvalent vaccines are used to provide coverage for circulating serovar agents and need to be reformulated at significant cost when new serovars emerge [133]. Furthermore whole-*Leptospira* vaccines are not universally effective in preventing carriage, which limits their use as a transmission-blocking intervention.

Due to these limitations, efforts have focussed on developing sub-unit vaccine candidates (Table 2) and more specifically, identifying surface-associated proteins which are conserved among serovars and targets for bacteriocidal immune responses. Haake *et al.* provided the first evidence for the feasibility of this approach by demonstrating that immunisation with *E. coli* outer membrane vesicles containing recombinant LipL41 and OmpL1 partially protected against a lethal challenge of leptospires in hamsters [134]. Subsequently, LipL32 has been shown to elicit immunoprotection when administered in naked DNA [135], BCG [136], and adenovirus [137] delivery systems. Yet, overall efficacy of these formulations is low (40-75%) in experimental animals. The most promising sub-unit vaccine candidate is the Lig proteins, which have been shown to confer high-level protection (Table 2), approaching 100% in mice [86] and hamsters [138-140]. The ability of Lig proteins to elicit cross-protective immunity against the spectrum of serovar agents needs to be determined since amino acid sequence identity for this protein is 70-100% among *Leptospira* spp [141].

The availability of multiple genome sequences provides an opportunity to apply high-throughput strategies for identifying novel vaccine candidates [107]. The ultimate goal for vaccine development will be to identify a candidate which protects against the spectrum of *Leptospira* agents. The *L. interrogans* and *borgpetersenii* genomes share 2708 ORFs, of which 656 are not present in the *L. biflexa* genome [16, 17] (Box 2). Strategies to refine the

number of target candidates include sequencing of a wider representation of pathogenic *Leptospira* genomes and bioinformatic analysis and selection of ORFs which are highly conserved among these genomes and encode outer membrane proteins [100]. Yet the major barrier in pursuing this strategy is the lack of *in vitro* correlates for immunity against leptospirosis. High throughput screening in experimental animals may not be feasible given the expected number of candidate antigens. A priority for vaccine development will be to prospectively determine whether infection with leptospirosis protects against subsequent re-infection in high-risk populations and identify the mechanisms of immunity which may be involved. Until epidemiologically-validated immune correlates are identified, discovery of vaccine candidates will likely continue to rely on the search for new virulence factors and outer membrane proteins.

Conclusions and future directions

There has been impressive recent progress in our knowledge of the basic aspects of the biology and pathogenesis of *Leptospira* spp., although modern molecular genetics was not applied to pathogenic leptospires until 2005, with the generation of the first mutants in *L. interrogans* [77]. Further studies need to explain why it is so difficult to introduce DNA into pathogenic leptospires by methods commonly used for other bacteria. More efficient methods are needed to test the role of putative virulence factors. The presence of prophage-like loci in the genome of pathogenic *Leptospira* [75, 142] suggest that transduction may occur and phages could be used as tools for gene transfer. Despite the large evolutionary distance between the pathogenic and non-pathogenic species, *Leptospira* spp. share a core of approximately 2000 genes [17]. *L. biflexa* could be used as a model bacterium to identify the precise functions of these common genes to gain an insight into the general biology of *Leptospira* spp.

Nevertheless, the discovery of genetic tools to transform leptospires has circumvented a major barrier to elucidating pathogen-related determinants of virulence and has led to the identification of Loa22 as the first virulence factor in *Leptospira* [106]. LipL32 and Lig proteins were long-standing hypothesized virulence factors. Yet knockout mutagenesis of the genes which encode these factors did not result in attenuation of virulence, suggesting that there may be a high degree of redundancy in function among virulence factors and that classical knockout approaches may not be useful in identifying such factors. There is therefore a real need to use convergent genomic, proteomics and metabolomic approaches to systematically identify molecular phenotypes and link these phenotypes with the pathogen's ability to cause disease in humans and animals. Our next hurdle is also to learn more about leptospiral gene regulation and the interactions among proteins. Microarrays represent a valuable tool to identify regulatory networks or pleiotropic effects of a mutation. The use of genetically distinct (or engineered) laboratory rodents together with micro-arrays or proteomic studies should permit to better delineate the mechanisms leading to chronic renal shedding. Ecological and metagenomics studies of soils will possibly provide information on the environmental persistence of leptospires which remains poorly understood.

Both host and microbiological factors probably contribute to the severity of leptospiral infection. Further studies should, for example, determine if the increasingly recognized syndrome of pulmonary hemorrhage is rather due to the emergence of a *Leptospira* clone with strain-specific factors or to innate or acquired host susceptibility factors. Elucidation of the molecular mechanisms of pathogenesis will contribute to the development of novel strategies for the treatment and prevention of leptospirosis which are urgently needed to address the large disease burden attributable to this emerging infectious disease in impoverished populations.

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Box 1: Classification and molecular typing

The genus *Leptospira* belongs to the phylum of spirochaetes [12]. The subgroup of saprophytes (*L. biflexa, L. wolbachii, L. kmetyi, L. meyeri, L. vanthielii, L. terpstrae*, and *L. yanagawae*) form the deepest branch within the genus, while another subgroup includes the pathogenic species with 8 species (*L. interrogans, L. kirschneri, L. borgpetersenii, L. santarosai, L. noguchii, L. weilii, L. alexanderi*, and *L. alstonii*). Another evolutionary branch comprises the so-called “intermediate” group (*L. inadai, L. broomii, L. fainei, L. wolffii, L. licerasiae*), which contains species of unclear pathogenicity [4, 5]. *Leptospira* spp. are also serologically classified into serovars, of which there are more than two hundred pathogenic serovars, on the basis of structural heterogeneity in the carbohydrate component of the lipopolysaccharide (LPS) [4, 5]. Serotyping of leptospires is important for clinical or epidemiological investigations, since identification of serovars and serogroups provides clues on the host reservoirs involved in transmission. However, serotyping is performed in few reference laboratories worldwide. Furthermore, several studies have shown that the system of serogroups was not related to molecular classifications [4], suggesting that genes determining serotypes may be laterally transferred into different species. Consequently, the classification system based on genetic similarities is being used in conjunction with classical antigenic classification. Recently, the releases of genome sequences allowed the introduction of several approaches to genotype *Leptospira* spp, which include multilocus variable-number tandem-repeat (VNTR) analysis [145] and multilocus sequence typing (MLST) [44, 146], a typing method that is based on the partial sequences of housekeeping genes and may evolve as a standard genotyping method as it has for other bacterial species.

Box 2: Genomes of *Leptospira* spp

A major advance in the understanding of *Leptospira* and its pathogenesis has been the recent sequencing of the genomes of two pathogenic species, *L. interrogans* and *L. borgpetersenii*, and the saprophytic species *L. biflexa* [14-17]. Overall, the genomes have a G + C content of between 35% and 41% and possess two circular chromosomes of approximately 4 Mb and 300 kb in size. The presence of a 74-kb replicon has also been identified in *L. biflexa* [17], which can possess a fourth circular replicon, the 74-kb leptospiral bacteriophage LE1 [74, 147]. A comparative analysis of *Leptospira* genomes provides clues with respect to the genetic determinants responsible for the different lifestyles of the spirochaetes [11]. Comparison of the proteins across the genomes has revealed a common backbone of 2052 proteins for this genus [17]. The *L. interrogans* and *L. borgpetersenii* genomes contain approximately 3400 and 2800 predicted coding regions (excluding transposases and pseudogenes), of which 656 of which are pathogen-specific and not found in the saprophyte *L. biflexa*. The functions of most (59%) of these genes are unknown, suggesting the presence of pathogenic mechanisms unique to *Leptospira*. The saprophyte *L. biflexa*, which survives exclusively in the external environment, has many more genes encoding environmental sensing and metabolic proteins than pathogenic leptospires [17]. Although *L. interrogans* and *L. borgpetersenii* share 2708 genes between them, there are 627 and 265 genes from *L. interrogans* and *L. borgpetersenii*, respectively which are not shared with the other pathogenic species. *L. interrogans* have retained more genes from its free-living ancestor, most of which relate to survival in the external environment [17]. *L. borgpetersenii* has a smaller genome (3.9 vs 4.6 Mb) and a much larger proportion of transposase genes or pseudogenes (20 vs 2%) than *L. interrogans*. Together these findings indicate that *L. borgspetersenii* is undergoing a process of genome reduction and specialization in the bacterium [16]. Gene loss appears to have impaired the ability of *L. borgpetersenii* to survive in the external environment, and therefore rely on direct contact between host animals (i.e., cows), rather than indirect environmental exposures, as its principle mode of transmission.

Features of the sequenced leptospires:

pathogenicity	survival in the environment	*Leptospira* spp. [a]	Genome size (bp)	CDS [b]	pseudogenes	transposases
strict pathogen	no survival	*L. borgpetersenii*	3,931,791	2,844	368	241
pathogen	survival	*L. interrogans*	4,627,366	3,379	41	26
non-pathogen	survival and multiplication	*L. biflexa*	3,956,088	3,590	33	10

[a] *L. borgpetersenii* serovar Hardjo strain L550, *L. interrogans* serovar Copenhageni strain Fiocruz, *L. biflexa* serovar Patoc strain Ames.

[b] excluding transposases and pseudogenes

Box 3: Leptospirosis in animals

Leptospirosis is considered the most geographically widespread zoonotic disease [4] because of the wide range of animals, mainly mammalian species, for which it infects. Rodents are the primary reservoir for maintaining enzootic transmission in most settings (Figure 2) [5]. This group includes not only rats and mice, but also voles, shrews, hedgehogs, and marsupials, all of which may serve as reservoirs of leptospirosis [1]. However, some rodents, including hamsters and guinea pigs in particular, are nevertheless highly susceptible to leptospirosis and can be used as animal models of human leptospirosis. Amphibians, snakes and freshwater fish have also been shown to have the potential to harbour pathogenic *Leptospira* [148]. Finally, although leptospires do not survive in seawater, leptospirosis has been reported in sea lions and seals, which were presumably infected in coastal rookeries [149]. Direct modes of transmission, including venereal, congenital and suckling exposures, play a more important role in animals than in humans (Figure 2A) [1]. Leptospirosis causes a broad spectrum of pathogenic processes in animals, for which acute disease and chronic colonization represent opposite poles. Humans are susceptible hosts in which infection causes severe acute manifestations but does not produce carriage. Infection in maintenance hosts such as rats causes an asymptomatic infection with persistent carriage [18]. Leptospirosis in other animals is a mixture of the two processes: infection causes a range of acute-to-chronic manifestations and produces a carrier state for which duration varies considerably between species [1]. In addition to being a human health problem, leptospirosis is a major veterinary disease associated with large economic costs [1]. In animals such as dogs, deer and pigs, leptospirosis causes acute manifestations, such as jaundice, renal failure and bleeding, as are observed in human disease [1, 150, 151]. Furthermore, leptospirosis causes a range of chronic manifestations in livestock, particularly cattle, pigs, sheep and goats, which are associated with reproductive losses, decreased mild production, stillbirths and abortions [152-154]. Recurrent uveitis due to leptospirosis is a major problem among horses [155].

Box 4: Pulmonary haemorrhage syndrome due to leptospirosis

Leptospirosis-associated pulmonary haemorrhage syndrome (LPHS), first described in Korea and China [156], was brought to world attention by a large outbreak of this severe disease form in Nicaragua in 1995 [157]. Subsequently LPHS has emerged as a major cause of haemorrhagic fever in developing countries [30, 158-160]. LPHS is striking for its fulminant presentation of massive pulmonary bleeding and acute lung injury and is associated with poorer clinical outcomes [6] indicating that the pathogenesis of LPHS may be different from that of Weil's disease. LPHS patients have high amounts of leptospiral DNA ($\geq 10^6$ organisms/g) in lung tissues [30]. However, scant numbers of intact leptospires are found in lung [49] The major lesion associated with LPHS is damage of the vascular endothelium [49, 50]. More recently several reports have observed linear deposition of immunoglobulin and complement along the alveolar basement membrane and in the intra-alveolar space of lung tissues [69, 161, 162], suggesting a possible underlying autoimmune process. The sudden appearance of LPHS in certain settings [160], suggests that introduction of clones with enhanced virulence may also contribute to the recent emergence of this syndrome.

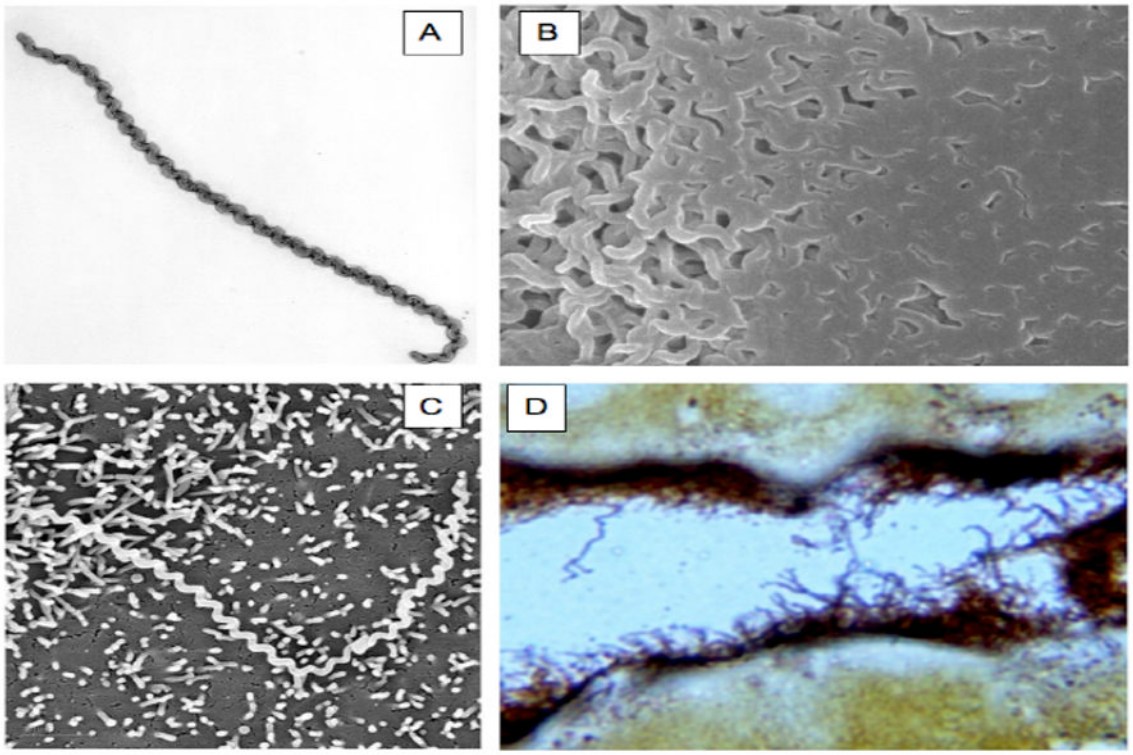

FIGURE 1. Leptospires in the environment and host

A. Leptospires are thin (cell diameter of 0.15 μm) and helical bacteria ranging from 10 to 20 μm long. Motility of leptospires is dependent on the presence of two endoflagella (or periplasmic flagella), one arising at each end of the spirochete, and extending along the cell body without overlapping in the central part of the cell.

B. Scanning electron micrograph of *L. interrogans* biofilm on a glass surface.

C. Scanning electron micrograph of *L. interrogans* adhering to polarized Mardin-Darby canine kidney cell monolayers.

D. Photomicrograph of a Warthin-Starry stained section of kidney tissue from a captured sewer rat (*Rattus norvegicus*). Leptospires are seen as silver-impregnated filamentous structures within the proximal renal tubule lumen (400x magnification).

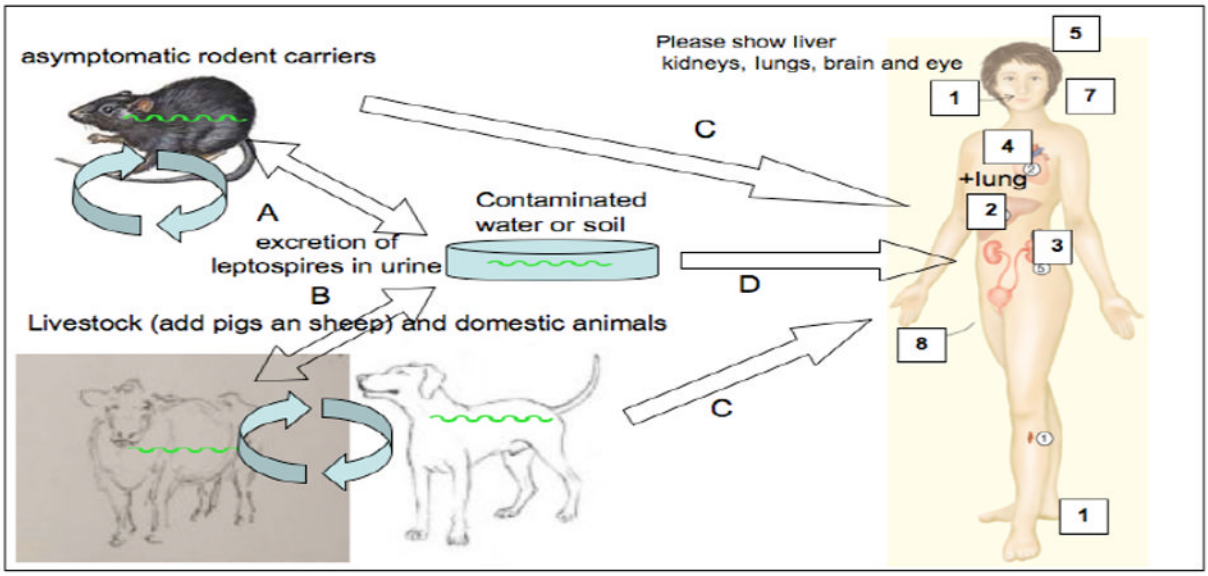

FIGURE 2. Cycle of infection

Mammalian species excrete the pathogen in their urine and serve as reservoirs for transmission. The pathogen is maintained in sylvatic and domestic environments by transmission among rodent species (-A-). In these reservoirs, infection produces chronic and persistent asymptomatic carriage in the renal tubules where *L. interrogans* forms aggregates (Figure 1D). Leptospires infect livestock and domestic animals and causes a range of disease manifestations and carrier states (Box 3). Maintenance of leptospirosis in these populations is due to continued exposure to rodent reservoirs or transmission within animal herds (-B-). Leptospirosis is transmitted to humans by direct contact with reservoir animals (-C-) or exposure to environmental surface water or soil contaminated with their urine (-D-). Leptospires penetrate abraded skin or mucous membranes (-1-), infect the bloodstream and disseminate throughout all the body tissue. Infection causes an acute febrile illness during the early “leptospiraemic” phase, which progresses during late “immune” phase to cause severe multi-system manifestations such as hepatic dysfunction and jaundice (-2-), acute renal failure (-3-), pulmonary haemorrhage syndrome (-4-), myocarditis and meningoencephilitis (-5-). Although the immune response eventually eliminates the pathogen, leptospires may persist for prolonged periods in immunoprivileged sites, such as the anterior chamber and vitreous of the eye and the renal tubules, where they can produce respectively, uveitis (-7-) months after exposure and urinary shedding weeks after resolution of the illness (-8-). Humans are an accidental host and do not efficiently shed sufficient numbers of leptospires to serve as reservoirs for transmission.

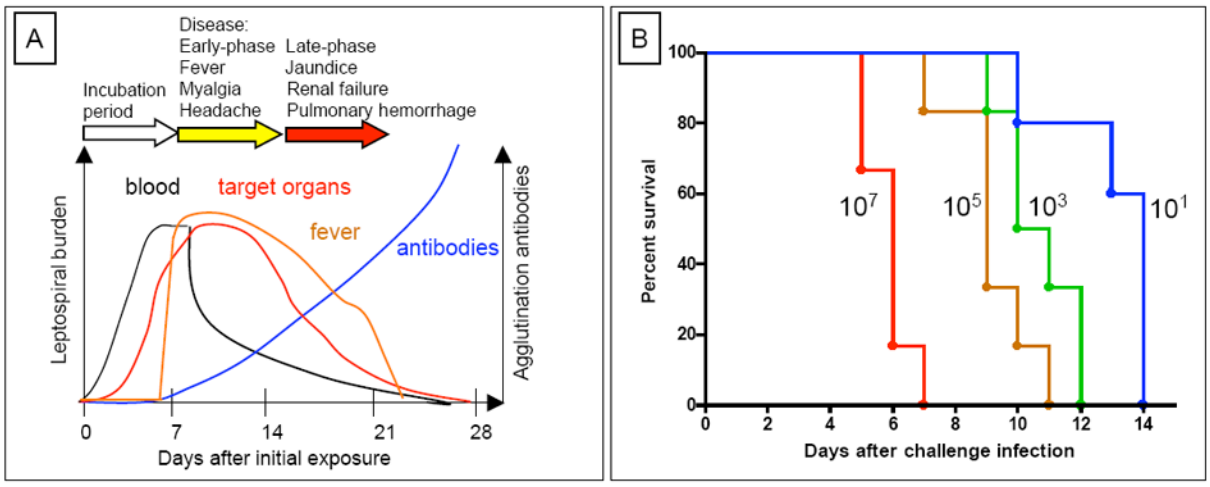

FIGURE 3. Disease kinetics of leptospirosis

A. Schematic diagram of the kinetics of leptospiral infection and disease. Infection produces a leptospiraemia (black line) within the first days after exposure, which is followed by detection of leptospires in tissues of multiple organs (red line) by the 3rd day of infection. In humans, illness (fever, brown line) develops with the appearance of agglutinating antibodies 5-14 days after exposure (blue line). Leptospires are cleared from the bloodstream and organs as serum agglutinating antibodies titres increase. Although early-phase illness (yellow arrow) is mild and resolves in the majority of infected individuals, a subset of patients progress four to six days after the onset of illness to develop severe late-phase manifestations (red arrow) during the period of immune-mediated destruction and clearance of leptospires (black line).

B. Survival curves for hamsters during experimental leptospirosis. Inoculation with increasing numbers of *L. interrogans* strain Fiocruz L1-130 (10^7 organisms, red line; 10^5, brown; 10^3, green; 10^1, blue) is associated with shortening of the incubation period and increased mortality among Golden Syrian hamsters. Infection of hamsters with low inocula produces disease manifestations which are found in patients with severe leptospirosis.

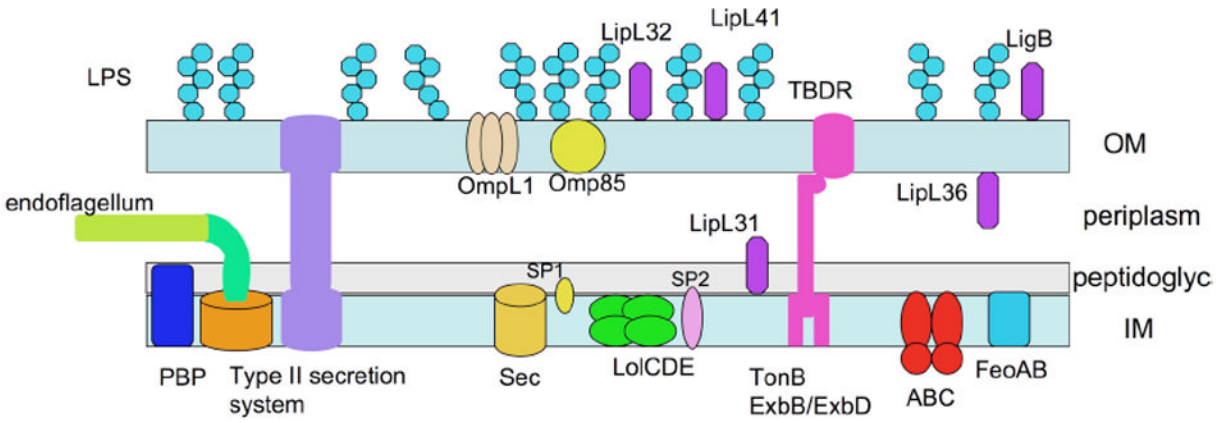

FIGURE 4. Schematic diagram of the cell wall of leptospires

Leptospira spp. possess a double-membrane structure. The peptidoglycan cell wall is associated with the inner membrane [102]. The leptospiral outer membrane is known to contain the transmembrane porin OmpL1, and lipoproteins LipL32, LipL36 (at the inner leaflet of the outer membrane), LipL41, and LigB (the surface-exposed Loa22, Len, LenD, LigA, and LigC proteins are not indicated in the schematic diagram). *Leptospira* spp. possess a lipopolysaccharide (LPS) which is composed of lipid A, a non-repeating oligosaccharide core and a distal polysaccharide (or O-antigen). Several TonB-dependent receptors (TBDR) were identified by genome analysis. Three of these TBDR were found to be involved in the transport of iron citrate (FecA-like transporter), the siderophore desferrioxamine, and hemin [83, 143]. Both transport and induction functions require energy transduction from the TonB–ExbB–ExbD complex in the inner membrane (for simplicity, only one ExbB-ExbD-TonB-TBDR system is indicated). As in other spirochaetes, the endoflagella is located in the periplasm. The inner membrane contains the FeoAB-type iron [83], penicillin-binding proteins (PBP), and the lipoprotein LipL31. Homologues of the *E. coli* export systems of outer membrane proteins (OMPs) and lipoproteins [144] were found in *Leptospira*; this includes inner membrane signal peptidases SP1 and SP2. Lipoproteins are first transported via the Sec system and bind to the ABC-transporter LolCDE. In *E. coli*, lipoproteins interact with LolA and the outer membrane receptor LolB to be inserted into the outer membrane. However, no LolA and LolB homologues are found in the *Leptospira* genomes. For OMPs, after transport via the Sec translocon, they are bound by the periplasmic chaperone Skp, then by the outer membrane protein Omp85 to be integrated into the lipid bilayer. An incomplete set of type II secretion-like genes is also present in the *Leptospira* genomes.

Table 1

Selected mutants obtained in pathogens

inactivated gene	strain [a]	method	phenotype	reference
loa22	Lai 56601	*Himar1*	attenuation of virulence [b]	Ristow *et al.* [106]
hemO	L495	*Himar1*	hemin-growth deficiency and attenuation of virulence	Murray *et al.* [124]
ligB	Fiocruz L1-130	allelic exchange	no attenuation in virulence	Croda *et al.* [80]
LipL32	L495	*Himar1*	no attenuation in virulence	Murray *et al.* [114].
lenB	L495	*Himar1*	no attenuation in virulence	Murray *et al.* [81]
uvrB	Lai 56601	*Himar1*	UV sensitivity	Murray *et al.* [81]
ligC	L495	*Himar1*	no attenuation in virulence	Murray *et al.* [81]
LA1641 [c]	L495	*Himar1*	attenuation of virulence	Murray *et al.* [81]
LA0615 [c]	L495	*Himar1*	attenuation of virulence	Murray *et al.* [81]

[a] Lai 56601: *L. interrogans* serovar Lai, Fiocruz L1-130: *L. interrogans* serovar Copenhageni, L495: *L. interrogans* serovar Manilae.

[b] Complementation of the mutant loa22 results in restoration of virulence in animal models [106].

[c] Transposon insertions were mapped onto the genome of *L. interrogans* serovar Lai strain Lai 56601

Table 2

Sub-unit vaccine candidates for leptospirosis[a]

Antigen	Adjuvant	Animal model	Inocula, serovar	Vaccine efficacy (%)[b]	Reference
LipL41/Omp	*E.coli*	hamsters	10^2	40-100 [c]	Haake *et al.* [134]
L1	OMVs		Grippotyph osa		
LipL32	Adenovirus	gerbils	10^4 Canicola	73-75 [d]	Branger *et al.*[137]
LigA/LigB	Freunds	mice	10^6 Manilae	90-100	Koizumi *et al.* [86]
LipL32	DNA	gerbils	10^7 Canicola	39 [d]	Branger *et al.* [135]
LigA	Alum	hamsters	10^8 Pomona	100 [e]	Palaniappan *et al.* [138]
LigA (C-term)	Freunds	hamsters	250 Copenhage ni	67-100	Silva *et al.* [139]
LipL32	BCG	hamsters	10^2 Copenhage ni	50 [c]	Seixas *et al.* [136]
LigA	DNA	hamsters	10^8 Pomona	100 [d]	Faisal *et al.* [163]
LigB	Alum	hamsters	10^5 Pomona	67-86	Yan *et al.* [140]
LigA (C-term)	Liposomes	hamsters	10^5 Pomona	88 [f]	Faisal *et al.* [164]

[a] Studies were included which evaluated immunisation with sub-unit vaccine candidates in protecting against mortality or survival.

[b] Vaccine efficacy against overall mortality is shown for studies which demonstrated significant protection in multiple experiments except when otherwise noted.

[c] Significant protection ($P<0.05$) against mortality in one of three experiments.

[d] Immunisation did not confer significant protection against overall mortality but was associated with a significant increase in survival rates.

[e] Immunisation did not confer significant protection against overall mortality but was associated with significant increase in survival rates when results were combined for three experiments.

[f] Significant protection against mortality in one experiment