Leptospira: the dawn of the molecular genetics era for an emerging zoonotic pathogen

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Abstract | Leptospirosis is a zoonotic disease that has emerged as an important cause of morbidity and mortality among impoverished populations. One hundred years after the discovery of the causative spirochaetal agent, little is understood about *Leptospira* spp. pathogenesis, which in turn has hampered the development of new intervention strategies to address this neglected disease. However, the recent availability of complete genome sequences for *Leptospira* spp. and the discovery of genetic tools for their transformation have led to important insights into the biology of these pathogens and their pathogenesis. We discuss the life cycle of the bacterium, the recent advances in our understanding and the implications for the future prevention of leptospirosis.

Zoonotic disease

An infectious disease of animals that can be transmitted to humans, whatever its transmission mode (vector borne, directly or indirectly through a product derived from the host animal), and that causes a disease syndrome in susceptible individuals.

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Descriptions of leptospirosis-like syndromes were reported in the scripts of ancient civilizations¹, but the first modern clinical description of leptospirosis was published by Weil in 1886 (REF. 2). In a landmark study in 1916, Inada *et al.* isolated leptospires, identified the organism as the causal agent of leptospirosis and determined that rats are a reservoir for transmission to humans³. 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 with a worldwide distribution, is now recognized as an emerging infectious disease⁴. Over the past decade, outbreaks during sporting events, adventure tourism and disasters have underscored its ability to become a public health problem in non-traditional settings^{4–6}. However, leptospirosis is a neglected disease that places its greatest burden on impoverished populations from developing countries and tropical regions⁶. In addition to being an endemic disease of subsistence farmers^{1,4,5}, leptospirosis has emerged as a widespread problem in urban slums, where inadequate sanitation has produced the conditions for rat-borne transmission^{7–9}. More than 500,000 cases of severe leptospirosis are reported each year, with case fatality rates exceeding 10%¹⁰. This Review focuses on the pathogenesis of leptospirosis and then highlights the recent advances in the field with respect to the genetic approaches that have been recently developed and the virulence factors that have been discovered.

The question mark-shaped bacterium

The genus *Leptospira* belongs to the phylum Spirochaetes and comprises saprophytic and pathogenic species¹¹ (BOX 1). Saprophytic leptospires, such as *Leptospira biflexa*, are free-living organisms found in water and soil and, unlike pathogenic *Leptospira* spp., do not infect animal hosts¹. Leptospires are thin, highly motile, slow-growing obligate aerobes with an optimal growth temperature of 30 °C and can be distinguished from other spirochaetes on the basis of their unique hook or question mark-shaped ends¹² (FIG. 1a).

In addition to *L. biflexa*, the genomes of two pathogenic species, *Leptospira interrogans* and *Leptospira borgpetersenii*, have been sequenced^{13–16}. Most (77–81%) of the genes in leptospiral genomes do not have orthologues in the genomes of other spirochaetes, indicating the large degree by which leptospires have diverged from other members of the phylum¹¹. Furthermore, comparative analysis of the genomes of the pathogenic and saprophytic species^{16,17} has provided insights into the genetic determinants that may be involved in pathogenesis (BOX 2).

The transmission cycle

Transmission of leptospirosis requires continuous enzootic circulation of the pathogen among animal reservoirs or, as they are commonly called, maintenance hosts (FIG. 2). *Leptospira* serovars demonstrate specific, although not entirely exclusive, host preferences; for example, rats serve as reservoirs for the

Box 1 | Classification and molecular typing of *Leptospira* spp.

The genus *Leptospira* belongs to the phylum Spirochaetes¹¹. The subgroup of saprophytic species (*Leptospira biflexa*, *Leptospira wolbachii*, *Leptospira kmetyi*, *Leptospira meyeri*, *Leptospira vanthielii*, *Leptospira terpstae* and *Leptospira yanagawae*) forms the deepest branch in the genus and another subgroup includes the pathogenic species (*Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira borgpetersenii*, *Leptospira santarosai*, *Leptospira noguchii*, *Leptospira weilii*, *Leptospira alexanderi* and *Leptospira alstoni*). Another evolutionary branch comprises the so-called 'intermediate group' (*Leptospira inadai*, *Leptospira broomii*, *Leptospira fainei*, *Leptospira wolffii* and *Leptospira licerasiae*), which contains species of unclear pathogenicity^{4,5}. Pathogenic *Leptospira* spp. are classified into more than 200 serovars on the basis of structural heterogeneity in the carbohydrate component of the lipopolysaccharide^{4,5}. Serotyping of leptospires is important for clinical or epidemiological investigations, as identification of serovars and serogroups provides clues about the host reservoirs that are involved in pathogen transmission. However, serotyping is performed in only a few reference laboratories worldwide. Several studies have shown that the serogroups are not related to the groups that are formed by molecular classifications⁴, suggesting that the genes that determine the serotypes may be laterally transferred into different species. Consequently, a classification system based on genetic similarities is being used in conjunction with antigenic classification. Recently, genome sequence information allowed the introduction of several approaches to genotyping *Leptospira* spp., including multilocus variable-number tandem-repeat analysis¹⁴⁴ and multilocus sequence typing^{44,145}, a typing method that is based on the partial sequences of housekeeping genes and may evolve as a standard genotyping method for *Leptospira* spp. as it has for other bacterial species.

Icterohaemorrhagiae serogroup, whereas house mice (*Mus musculus*) are the reservoir for the Ballum serogroup^{4,5,18}. Furthermore, serovars often do not cause serious disease in reservoir hosts to which they are highly adapted.

Leptospires colonize and are shed from the renal tubules of a wide range of animals (see [Supplementary information S1](#) (box)). The bacteria survive for weeks or even months in moist soil and water after excretion in the urine¹⁹. Cell aggregation¹⁹ and biofilm formation²⁰ (FIG. 1b) may contribute to the survival of leptospires outside their hosts.

Disease pathogenesis

Pathogenic *Leptospira* spp. produce a systemic infection after 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 colonization represent the opposite poles of a wide range of disease presentations (see [Supplementary information S1](#) (box)). Humans are incidental hosts: pathogenic *Leptospira* spp. cause acute disease manifestations but do not induce a carrier state that is required for their transmission.

Dissemination in the host. Leptospires penetrate abraded skin and mucous membranes and quickly establish a systemic infection by crossing tissue barriers and by haematogenous dissemination¹. It was thought that leptospires, like other spirochaetes, spread through intercellular junctions²¹. However, they have been shown to efficiently enter host cells *in vitro*^{22,23} and to rapidly translocate across polarized cell monolayers without altering the trans-epithelial electrical

resistance^{24,25}. Leptospires are not facultative intracellular organisms; they are rarely observed within host cells but instead seem to reside only transiently within these 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 phagosomal compartments²³ of normally non-phagocytic host cells. These findings suggest that leptospires use host cell entry and rapid translocation as mechanisms to spread to target organs and evade immune killing.

Infection causes prolonged leptospiemia until the host mounts an effective acquired immune response, which occurs one to two weeks after exposure²⁶ (FIG. 3a). Leptospires can be isolated from the bloodstream within minutes after inoculation¹ and are detected in multiple organs by the third day after infection^{26–29}; they may reach 10⁶–10⁷ organisms per ml or per g in the blood and tissues of patients^{30,31} and infected animals²⁹. Leptospires evade the host innate immune response during the initial stages of infection. They are resistant to the alternative pathway of complement activation^{32,33} and acquire [complement factor H](#) and related fluid-phase regulators^{34,35} through ligands such as the leptospiral endostatin-like (Len) proteins^{36,37}. The host complement fragment C4b-binding protein alpha chain (C4BPA) binds to the surface of leptospires³⁸, suggesting that a similar process may confer some protection against the classical pathway of complement activation.

Persistent colonization. The essential component of the life cycle of pathogenic *Leptospira* spp. is their ability to give rise to persistent renal carriage in reservoir animals. In rats, leptospires cause a systemic infection but are subsequently cleared from all organs except the renal tubules^{28,39}. Colonized tubules are densely populated with leptospires, which aggregate into an amorphous, biofilm-like structure (FIG. 1d). Rats have been shown to excrete leptospires in high concentrations (10⁷ organisms per ml (REF. 39)) for 9 months after experimental infection⁴⁰.

Leptospires isolated from chronically infected rat kidneys have substantially higher amounts of lipopolysaccharide (LPS) O antigen than those isolated from the livers of hamsters with acute disease, suggesting that the expression of O antigen facilitates the induction of carriage³⁹. The renal tubule is an immunoprivileged site, a feature that may contribute to high-grade persistence of the pathogen. Moreover, those leptospires that are shed in the urine downregulate the expression of proteins that are recognized by the humoral immune response in rats⁴¹.

Disease manifestations and determinants. The incubation period for leptospirosis is 5–14 days on average, with a range of 2–30 days¹ (FIG. 3a). In humans, leptospirosis causes a febrile illness that, in its early phase, often cannot be differentiated from other acute fevers. In most patients, illness resolves after the first week of symptoms. However, a subset (5–15%) of patients develop severe late-phase manifestations⁶. Unlike bacterial infections such as Gram-negative sepsis, leptospirosis does not cause fulminating disease shortly after the onset of illness, which may

Enzootic

Pertaining to an infection that can be maintained in nature through continuous transmission among animal populations.

Haematogenous

Pertaining to anything transported by the blood.

Alternative pathway

The complement activation pathway that is stimulated by the spontaneous hydrolysis of the complement component C3 or the presence of microbial surface structures.

Classical pathway

The complement activation pathway that is stimulated by the recognition of antigen-antibody complexes on foreign-cell surfaces by the hexameric complement component C1q.

Figure 1 | Leptospire in the environment and the host. **a** | Leptospire are thin, helical bacteria with a diameter of 0.15 μm and a length ranging from 10 to 20 μm . The motility of leptospires is dependent on the presence of two endoflagella (or periplasmic flagella), one arising at each end of the spirochete, that extend along the cell body without overlapping in the central part of the cell. **b** | A scanning electron micrograph of a *Leptospira interrogans* biofilm on a glass surface. **c** | A scanning electron micrograph of *L. interrogans* adhered to polarized Mardin-Darby canine kidney cell monolayers. **d** | A photomicrograph of a Warthin-Starry-stained section of kidney tissue from a captured sewer rat (*Rattus norvegicus*). Leptospire are seen as silver-impregnated, filamentous structures in the proximal renal tubule lumen ($\times 400$ magnification).

relate to the low endotoxic potency of leptospiral LPS¹. Severe late-phase manifestations usually occur 4 to 6 days after the onset of illness (FIG. 3a) but can vary depending on the infecting inoculum dose and other disease determinants. Weil's disease is the classic presentation of severe leptospirosis and is characterized by jaundice, acute renal failure and bleeding. In addition, there is increasing awareness of an emerging severe disease form, leptospirosis-associated pulmonary haemorrhage syndrome (LPHS) (BOX 3), for which the case fatality rate is more than 50%⁶.

The development of leptospirosis and disease progression are influenced by host susceptibility factors, the dose of the infecting inoculum and the virulence characteristics of the infecting strain. Certain *Leptospira* species and serovars are more frequently found to cause severe disease in humans than others^{42,43}. A single circulating clone caused a large and sustained nationwide epidemic in Thailand⁴⁴. Clonal transmission of strains has been described in other outbreaks and in settings of endemic transmission^{45,46} and may reflect localized transmission clusters⁴⁵. However, the magnitude and duration of the epidemic in Thailand suggest that predominant clones may indeed possess specific factors that 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 that are associated with clinical and transmission-related phenotypes.

Our understanding of the acquired and innate host factors that influence infection and disease progression remains limited. An investigation of a triathlon-related outbreak identified the human leukocyte antigen serotype HLA-DQ6 as the first and to date only genetic susceptibility factor for leptospirosis⁴⁷. 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 the inoculum size shortens the incubation period and decreases survival in a dose-dependent manner in experimental animals^{26,48} (FIG. 3b). The synergism between HLA-DQ6 and environmental exposure found during the triathlon outbreak constitutes the first gene-environment interaction to be identified for an infectious disease.

Tissue damage. The onset of disease correlates with the appearance of agglutinating antibodies and the clearance of leptospires by antibody-mediated opsonization and lysis¹ (FIG. 3a). 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 manifestations⁵¹.

Leptospiral components that are released after immune killing stimulate the production of pro-inflammatory cytokines^{53–56} and mediate inflammation and damage of end-organ tissues. The Jarisch-Herxheimer reaction, which is caused by the sudden release of these cytokines, is a complication of antimicrobial therapy for leptospirosis. Moreover, tumour necrosis factor may play a key part in disease progression, as levels of this cytokine are a predictor of poor clinical outcomes⁵⁷.

Leptospiral LPS has been shown to activate Toll-like receptor 2 (TLR2) in human cells rather than the TLR4 pathway⁵⁸, an unusual finding that may relate to a 1-methylphosphate moiety that is not found in the lipid A of other bacteria⁵⁹. In addition, leptospiral lipoproteins induce innate immune responses by activating the TLR2 pathway^{58,60}. However, leptospiral LPS activates both TLR2 and TLR4 pathways in mouse cells, indicating that there are species-specific differences with respect to TLR activation⁶¹. Leptospire stimulate the expansion of $\gamma\delta$ T cell populations in naive peripheral blood mononuclear cells, and patients with leptospirosis have increased numbers of these specific T cells⁵⁴; this suggests that acquired cell-mediated responses may promote inflammation, in addition to the stimulation of inflammation by the innate and acquired humoral responses.

Leptospire can induce a peculiar hypokalaemic, non-oliguric form of acute renal failure that is characterized by impaired tubular Na^+ reabsorption⁶². Although non-esterified unsaturated fatty acids derived from leptospire

Human leukocyte antigen
Also known as the major histocompatibility complex, this is a key part of the human immune system.

Jarisch-Herxheimer reaction
An inflammatory reaction (characterized by the sudden onset of fever, chills and, in some cases, hypotension) that is induced in certain cases by antimicrobial therapy and believed to be due to the rapid release of bacterial antigens.

Box 2 | Genomes of *Leptospira* spp.

A major advance in our understanding of *Leptospira* spp. and their pathogenesis has come from the recent sequencing of the genomes of two pathogenic species, *Leptospira interrogans* and *Leptospira borgpetersenii*, and the saprophytic species *Leptospira biflexa*^{13–16}. Overall, the genomes have a GC content of between 35% and 41% and possess two circular chromosomes of approximately 4 Mb and 300 kb. An additional 74 kb replicon has been identified in *L. biflexa*¹⁶, which can also possess a fourth circular replicon, the 74 kb leptospiral bacteriophage LE1 (REFS 74, 146). A comparative analysis of *Leptospira* spp. genomes provides clues about the genetic determinants that are responsible for the different lifestyles of the spirochaetes¹⁷. A comparison of the proteins encoded by these genomes has revealed a common backbone of 2,052 proteins for this genus¹⁶. The *L. interrogans* and *L. borgpetersenii* genomes contain approximately 3,400 and 2,800 predicted coding regions (excluding transposases and pseudogenes), respectively, of which 656 are pathogen specific and not found in the saprophyte *L. biflexa*. The functions of most (59%) of these genes are unknown, suggesting the existence of pathogenic mechanisms that are unique to *Leptospira* spp. The saprophyte *L. biflexa*, which survives exclusively in the external environment, has many more genes encoding environment-sensing and metabolic proteins than the pathogenic leptospires¹⁶. Although *L. interrogans* and *L. borgpetersenii* share 2,708 genes, there are 627 and 265 genes from *L. interrogans* and *L. borgpetersenii*, respectively, that are not shared with the other pathogenic species. *L. interrogans* has retained more genes from its free-living ancestor, most of which relate to survival in the external environment¹⁶. *L. borgpetersenii* has a smaller genome (3.9 Mb compared with 4.6 Mb) and a much larger proportion of transposase genes or pseudogenes (20% compared with 2%) than *L. interrogans*. Together, these findings indicate that *L. borgpetersenii* is undergoing a process of genome reduction and specialization¹⁵. Gene loss seems to have impaired the ability of *L. borgpetersenii* to survive in the external environment, and therefore it relies on direct contact between host animals (cows) rather than indirect environmental exposures as its principle mode of transmission.

have been found to inhibit the kidney Na⁺–K⁺ ATPase⁶³, it seems more plausible that the renal manifestations of infection are the direct result of a focal tubulo-interstitial nephritis. Leptospiral outer membrane proteins, such as *LipL32*, activate TLR-dependent pathways, which lead to the activation of nuclear factor- κ B, mitogen-activated kinases and cytokines and, subsequently, to tubular damage⁶⁰. Furthermore, activation of these pathways may provide an explanation for the dysregulation of Na⁺ transporters in infected kidneys, a finding that has been shown to be associated with impaired Na⁺ reabsorption^{64,65}.

Leptospires can induce apoptosis in macrophages and hepatocytes^{22,66,67}, but the overall contribution of apoptosis to disease pathogenesis has not been clarified. Leptospirosis elicits the production of autoantibodies, such as cardiolipin-specific antibodies⁶⁸, and several reports suggest that autoimmune mechanisms may play a part in the development of uveitis³⁷ and LPHS (BOX 3)⁶⁹ during infection.

Genetic tools for *Leptospira* spp.

The virulence mechanisms and, more generally, the biology of the causative agents of leptospirosis remain largely unknown. Before 2000, the lack of genetic tools available for use in pathogenic or saprophytic leptospires precluded the full characterization of genes of interest. In the first genetic studies, carried out in the 1990s, several *Leptospira* spp. genes were isolated by the functional complementation of *Escherichia coli* mutants. This method led to the identification of the *L. biflexa* recombinase A (*recA*) gene⁷⁰, the *L. interrogans* *rfb* genes⁷¹

and a number of amino acid biosynthesis genes, such as aspartate semi-aldehyde dehydrogenase (*asd*) and the tryptophan biosynthesis gene *trpE*^{72,73}.

The origin of replication from the LE1 temperate leptophage⁷⁴, a 74 kb extrachromosomal element of *L. biflexa*¹⁶ and a genomic island that can excise from the *L. interrogans* chromosome⁷⁵ were used to generate a plasmid vector that can replicate autonomously in both *L. biflexa* and *E. coli*⁷⁶. However, although DNA can be introduced into leptospires by electroporation^{76,77} and conjugation⁷⁸, there is currently no replicative plasmid vector available for pathogenic leptospires.

Deletion of chromosomal genes (including *trpE*, *recA*, the haeme synthetase gene *hemH*, the flagellar filament core protein gene *flaB* and the methionine biosynthesis genes *metY*, *metX* and *metW*) was achieved by targeted mutagenesis in the saprophyte *L. biflexa* using a suicide plasmid⁷⁹. Recently the first gene to be successfully targeted, *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 for various *Leptospira* spp. strains^{77,81,82}. In *L. biflexa* an extensive library of mutants can be generated and screened for phenotypes that affect 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* (REF. 77). Transformation experiments with *L. interrogans* that were performed in two different laboratories resulted in approximately 1,000 random mutations, of which 721 affected the protein-coding regions of 551 different genes⁸¹ (TABLE 1). Further improvements to the methods and the identification of more readily transformable *L. interrogans* strains may allow the generation of a mutant library for high-throughput screening for specific processes that are known to be involved in pathogenesis.

Animal models of virulence

Guinea pigs and hamsters are the standard experimental models for acute leptospirosis¹. Infection with low inocula (<100 leptospires) produces similar disease kinetics (FIG. 3b) and severity to those observed in humans⁴⁸. 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 immunity⁸⁶ (TABLE 2). However, mice are relatively resistant to infection and high inocula (up to 10⁸ organisms) are required to produce disease, a situation that may not parallel natural exposure. Furthermore, when mice do develop disease it is more fulminant and they tend to die within markedly shorter intervals (5 days) than hamsters infected with low-inoculum lethal challenges (FIG. 3b). This finding raises concerns that this experimental animal model may not reproduce the disease dynamics and pathogenic processes that are observed in natural infections. Rats have been used as a model system to study persistent colonization but also require high inocula^{28,39}. Similar to the situation in mice, it is not understood why this common reservoir in nature is relatively difficult to infect experimentally. Natural

Figure 2 | The cycle of leptospiral infection. Mammalian species excrete leptospiral pathogens in their urine and serve as reservoirs for their transmission. The pathogens are maintained in sylvatic and domestic environments by transmission among rodent species. In these reservoirs, infection produces chronic, asymptomatic carriage. Leptospire can then infect livestock and domestic and wild animals and cause a range of disease manifestations and carrier states. Maintenance of leptospirosis in these populations is due to their continued exposure to rodent reservoirs or to transmission within animal herds. Leptospirosis is transmitted to humans by direct contact with reservoir animals or by exposure to environmental surface water or soil that is contaminated with their urine. Leptospire penetrate abraded skin or mucous membranes, enter the bloodstream and disseminate throughout the body tissue. Infection causes an acute febrile illness during the early 'leptospiraemic' phase and progresses during the late 'immune' phase to cause severe multisystem manifestations such as hepatic dysfunction and jaundice, acute renal failure, pulmonary haemorrhage syndrome, myocarditis and meningoencephalitis. Although the immune response eventually eliminates the pathogens, leptospire may persist for prolonged periods in immunoprivileged sites, such as the renal tubules and the anterior chamber and vitreous humor of the eye, where they can produce, respectively, urinary shedding weeks after resolution of the illness and uveitis months after exposure. Humans are an accidental host and do not shed sufficient numbers of leptospire to serve as reservoirs for transmission.

infection with leptospirosis occurs in non-human primates, which have been used as models to study the disease⁸⁷ and, more recently, the development of pulmonary haemorrhage syndrome⁸⁸.

Virulence factors

The virulence factors that have been identified 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 a dedicated protein secretion pathway similar to the type III and type IV secretion machinery that is used by Gram-negative bacteria to inject proteins into host cells.

Virulent leptospire, but not culture-attenuated or saprophytic organisms, adhere to and enter mammalian host cells^{22,24,25,89} (FIG. 1c). Proteins that are present on the surface of leptospire, including several that have been shown to bind *in vitro* to various components of the extracellular matrix^{36,90–94}, are thought to mediate the

host cell–leptospire interaction (FIG. 4). Virulent leptospire have different protein and LPS profiles from those of culture-attenuated strains and have fewer protein particles on the outer membrane surface, as determined by freeze-fracture electron microscopy⁹⁵.

Like those of other spirochaetes, the genomes of *Leptospira* spp. encode more lipoproteins than non-spirochaete genomes. The *L. interrogans* genome contains approximately 145 genes that encode putative lipoproteins⁹⁶ in addition to putative extracellular and outer membrane proteins^{97,98}.

Consistent with the predicted ability of *Leptospira* spp. to migrate through host tissues, the genomes of these organisms encode a wide range of putative haemolysins and proteases that may facilitate this process. An analysis of the *L. interrogans* genome identified nine genes that encode putative haemolysins, including a pore-forming protein⁹⁹ and a sphingomyelinase¹⁶ that are not found in the saprophyte *L. biflexa*. The *L. interrogans* genome also contains a microbial collagenase that is proposed to be involved in the destruction of host tissues.

Koch's molecular postulates

A series of conditions that must be met to establish that a gene is a virulence factor. For example, the gene must be present in strains that are associated with the disease but not in those which are not, the mutation of the gene must reduce or abolish bacterial virulence and complementation of the mutant must restore it.

Figure 3 | Disease kinetics of leptospirosis. a | The kinetics of leptospiral infection and disease. Infection produces leptospiroemia in the first few days after exposure, which is followed by migration of leptospires to the tissues of multiple organs by the third day of infection. In humans, a fever develops, with the appearance of agglutinating antibodies 5–14 days after exposure. Leptospires are cleared from the bloodstream and organs as the titres of serum agglutinating antibodies increase. Although early-phase illness is mild and resolves in most infected individuals, a subset of patients develop severe late-phase manifestations 4–6 days after the onset of illness, during the period of immune-mediated destruction and clearance of leptospires. **b** | Survival curves for hamsters during experimental leptospirosis. Inoculation with increasing amounts of *Leptospira interrogans* strain Fiocruz L1-130 is associated with a shortening of the incubation period and decreased survival among Golden Syrian hamsters.

Few proteins have been shown experimentally to be present on the leptospiral surface¹⁰⁰. Approximately 12 outer membrane proteins, including *OmpL1* (REF. 101), *LipL32* (REF. 102), immunoglobulin-like repeat containing protein B (*LigB*)¹⁰³, *LenA*³⁶, *LenD*³⁶ and *Loa22* (REF. 104) have been identified. Our knowledge of the outer surface of leptospires therefore remains limited, and further development of tools for the accurate localization of surface-associated determinants is required.

***Loa22*.** The only gene found to date that fulfils Koch's molecular postulates for a virulence factor gene is *loa22*. In *L. interrogans*, disruption of *loa22* by *Himar1* insertion led to a complete loss of virulence in the guinea pig disease model¹⁰⁴ (TABLE 1). *Loa22* is exposed on

the bacterial surface¹⁰⁴ and recognized by sera from human patients with leptospirosis¹⁰⁵, and its expression is upregulated in an acute model of infection¹⁰⁶. The *in vitro* binding between *Loa22* and components of the extracellular matrix is weak¹⁰⁷. The carboxyl terminus 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* (REF. 16), differential expression of this gene in pathogenic and non-pathogenic leptospires or pathogen-specific sialylation of *Loa22*, which is dependent on pathogen-specific sialic acid modification pathways (J. Ricaldi and J. Vinetz, personal communication), may explain why this protein is a crucial determinant of *L. interrogans* virulence.

Box 3 | Leptospirosis and pulmonary haemorrhage syndrome

Leptospirosis-associated pulmonary haemorrhage syndrome (LPHS), first described in Korea and China¹⁴⁷, was brought to the attention of the world by a large outbreak in Nicaragua in 1995 (REF. 148). Subsequently, LPHS has emerged as a major cause of haemorrhagic fever in developing countries^{30,149–151}. LPHS is striking for its fulminant presentation of massive pulmonary bleeding and acute lung injury and is associated with poor clinical outcomes⁶, indicating that the pathogenesis of LPHS may be different from that of Weil's disease. Patients with LPHS have high amounts of leptospiral DNA (indicative of $\geq 10^6$ organisms per g) in lung tissues³⁰. However, few intact leptospires are found in the lung⁴⁹. The main lesion associated with LPHS is damage to 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 spaces of affected lung tissues^{69,152,153}, suggesting the involvement of an underlying autoimmune process. The sudden appearance of LPHS in certain settings¹⁵¹ suggests that the introduction of clones with enhanced virulence may be a contributing factor to the recent emergence of this syndrome.

***LipL32*.** *LipL32* (REF. 102) (also known as haemolysis-associated protein 1 (*Hap1*)¹⁰⁸) is surface exposed¹⁰² and accounts for 75% of the outer membrane proteome¹⁰⁹. This lipoprotein is highly conserved among pathogenic *Leptospira* spp.¹¹⁰, whereas there are no orthologues in the saprophytic *L. biflexa*¹⁶. *LipL32* was long thought to be a putative virulence factor. Higher levels of *LipL32* are expressed in leptospires during acute lethal infections than during *in vitro* culture¹⁰⁶. In addition, the C terminus of *LipL32* binds *in vitro* to laminin, collagen I, collagen IV, collagen V and plasma fibronectin^{92,93}. Furthermore, the crystal structure of *LipL32* was recently elucidated and shown to have structural homologies with proteins such as collagenase that bind to components of the extracellular matrix¹¹¹. However, a *LipL32*-mutant strain, obtained by *Himar1* insertion mutagenesis, was found to be as efficient as the wild-type

Table 1 | **Selected mutations obtained in pathogenic leptospires**

Inactivated gene	Strain	Method	Phenotype	Ref.
<i>loa22</i>	Lai 56601	<i>Himar1</i>	Attenuation of virulence*	106
<i>hemO</i>	L495	<i>Himar1</i>	Hemin growth deficiency and attenuation of virulence	124
<i>ligB</i>	Fiocruz L1-130	Allelic exchange	No attenuation of virulence	80
<i>lipL32</i>	L495	<i>Himar1</i>	No attenuation of virulence	114
<i>lenB</i>	L495	<i>Himar1</i>	No attenuation of virulence	81
<i>uvrB</i>	Lai 56601	<i>Himar1</i>	UV sensitivity	81
<i>ligC</i>	L495	<i>Himar1</i>	No attenuation of virulence	81
LA1641 [†]	L495	<i>Himar1</i>	Attenuation of virulence	81
LA0615 [†]	L495	<i>Himar1</i>	Attenuation of virulence	81

*Complementation of the mutant *loa22* results in restoration of virulence in animal models¹⁰⁶. [†]Transposon insertions were mapped onto the genome of *Leptospira interrogans* serovar Lai strain 56601. Fiocruz L1-130, *L. interrogans* serovar Copenhageni strain Fiocruz L1-130; Lai 56601, *L. interrogans* serovar Lai strain 56601; *lenB*, leptospiral endostatin-like protein B gene; *lipL32*, lipoprotein L32 gene; L495, *L. interrogans* serovar Manilae strain L495; UV, ultraviolet light.

strain in causing acute disease and chronic colonization in experimental animals¹¹² (TABLE 1). The role of this major outer membrane protein in pathogenesis remains unclear.

Leptospiral immunoglobulin-like proteins. Three high-molecular-mass *Leptospira* spp. proteins — *LigA*, *LigB* and *LigC* — are recently characterized members of the bacterial immunoglobulin-like protein superfamily^{86,103,113}. Lig proteins are anchored to the outer membrane and have 12 to 13 tandem bacterial immunoglobulin-like repeat domains. Like *lipL32*, lig genes are exclusively present in pathogenic *Leptospira* spp. Recombinant Lig proteins bind *in vitro* to host extracellular matrix proteins, including fibronectin, fibrinogen, collagen and laminin^{94,114}. Furthermore, the repeat domain portion of the *LigB* molecule binds Ca²⁺, which seems to enhance its ability to adhere to fibronectin¹¹⁵. The lig genes are upregulated under physiological osmolarity¹¹⁶ and encode surface-exposed proteins that are strongly recognized by sera from patients with leptospirosis^{103,117,118}. Lig proteins are considered to be putative virulence factors¹⁰³, as members of the bacterial immunoglobulin-like protein 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* gene⁸⁰, does not affect the ability of the bacterium to cause acute leptospirosis in hamsters or persistent renal colonization in rats. 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 bacteria may be of relevance to their 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 than strains passaged *in vitro*¹¹⁹. Their corkscrew motility allows these organisms to swim through gel-like media

such as connective tissues¹². However, it has not been determined whether loss of motility results in attenuation of virulence for pathogenic leptospires. *L. biflexa flaB* mutants cannot form functional endoflagella, but their cell bodies remain intact and helical¹²⁰. The endoflagella are therefore not responsible for dictating the helical shape of the cell body in *Leptospira* spp., as they are in *Borrelia burgdorferi*¹²¹. Proteins that are involved in the morphogenetic system of rod-shaped bacteria, such as MreB, MreC, MreD and penicillin-binding proteins, are encoded in the leptospiral genome. Leptospiral cell morphology may therefore be determined by the cytoskeleton and maintained by the rigid murein layer.

Multiple methyl-accepting chemotaxis proteins have been identified in *Leptospira* spp., suggesting that chemotactic responses to various chemoattractants and repellents occurs. Unlike saprophytic strains, *L. interrogans* displays positive chemotaxis towards haemoglobin¹²².

Iron acquisition is important for virulence in many bacterial pathogens, and *Leptospira* spp. contain several iron-uptake systems, including TonB-dependent outer membrane receptors⁸³. *Leptospira* spp. possess a haem oxygenase, encoded by *hemO*, that 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 leptospirosis¹²³ (TABLE 1), suggesting that *Leptospira* spp. use haem as their principal source of iron during infection.

Two attenuated *L. interrogans* mutants have disruptions in genes that encode hypothetical proteins that may be virulence factors⁸¹, but these findings need to be confirmed with complementation studies.

Previous microarray studies have shown that exposing *L. interrogans* to the osmolarity conditions found in host tissues induces a profound shift in its global transcription profile. Therefore, osmolarity and temperature^{116,124} are important factors for regulating the expression of proteins that mediate the infection of mammalian hosts. Nineteen of the twenty-five most strongly salt-induced *L. interrogans* genes encode hypothetical proteins¹¹⁶. These proteins

Table 2 | **Subunit vaccine candidates for leptospirosis***

Antigen	Adjuvant	Animal model	Inocula	Serovar	Vaccine efficacy (%) [†]	Ref.
LipL41 and OmpL1	<i>Escherichia coli</i> OMVs	Hamsters	10 ²	Grippotyphosa	40–100 [§]	135
LipL32	Adenovirus	Gerbils	10 ⁴	Canicola	73–75	138
LigA and LigB	Freunds	Mice	10 ⁶	Manilae	90–100	86
LipL32	DNA	Gerbils	10 ⁷	Canicola	39	136
LigA	Alum	Hamsters	10 ⁸	Pomona	100	139
Carboxyl terminus of LigA	Freunds	Hamsters	250	Copenhageni	67–100	140
LipL32	BCG	Hamsters	10 ²	Copenhageni	50 [§]	137
LigA	DNA	Hamsters	10 ⁸	Pomona	100	156
LigB	Alum	Hamsters	10 ⁵	Pomona	67–86	141
C terminus of LigA	Liposomes	Hamsters	10 ⁵	Pomona	88 [#]	157

*These studies evaluated immunization with subunit vaccine candidates for protection against host mortality or for host survival.

[†]Vaccine efficacy against overall mortality is shown for studies that demonstrated significant protection in multiple experiments, except when otherwise noted. [§]Significant protection ($p < 0.05$) against mortality in one of three experiments. ^{||}Immunization did not confer significant protection against overall mortality but was associated with a significant increase in survival rates.

^{||}Immunization did not confer significant protection against overall mortality but was associated with significant increase in survival rates when results were combined for three experiments. [#]Significant protection against mortality in one experiment. BCG, *Mycobacterium bovis* bacille Calmette–Guérin; LipL32, lipoprotein L32; LipL41, lipoprotein L41; OmpL1, outer membrane protein L1; OMV, outer membrane vesicle.

may be response regulators and environment-sensing proteins that are involved in survival or persistence in the environment or in the infected host. In addition, sphingomyelinase C is upregulated by increases in osmolarity to the levels that are found in mammalian host tissues¹¹⁶.

Immunity

The humoral response is thought to be the primary mechanism of immunity to leptospirosis¹²⁵. LPS seems to be the main target for the protective antibody response: passive transfer of immunity correlates with levels of agglutinating LPS-specific antibodies in transferred sera¹²⁶, and LPS-specific monoclonal antibodies passively protect naive animals from leptospirosis¹²⁷. However, it is not known whether antibody responses against leptospiral antigens other than LPS also confer protection.

Recent work has confirmed that immunity to leptospirosis is not limited to the humoral response. Mice require intact TLR2 (REF. 128) and TLR4 (REF. 85) activation pathways to control a lethal infection. In contrast to immunity in hosts that are susceptible to acute leptospirosis, protective immunity against *L. borgpetersenii* serovar Hardjo in bovine reservoirs is cell mediated. Immunization trials in cattle found that protection against this serovar, conferred by whole-leptospire-based vaccines, correlated with T helper 1 cell responses and not with agglutinating antibody titres^{129–131}.

Vaccines

In 1916, Ido *et al.* provided the first demonstration that immunization with killed leptospires protects against experimental infection¹³². Since then, whole-leptospire-based vaccines have been routinely administered to livestock and domestic animals

and used for immunization of human populations⁶. However, there are serious concerns about their use¹³³. Whole-leptospire-based vaccines are associated with high rates of adverse reactions and confer only short-term, serovar-specific immunity¹. Polyvalent vaccines are used to provide coverage for circulating serovar agents and must be reformulated at substantial cost when new serovars emerge¹³⁴. Furthermore, whole-leptospire-based vaccines are not universally effective in preventing carriage, which limits their use as a transmission-blocking intervention.

Owing to these limitations, efforts have focused on developing subunit vaccine candidates (TABLE 2) — more specifically, on identifying surface-associated proteins that are conserved among serovars, and targets for bactericidal immune responses. The first evidence for the feasibility of this approach was the demonstration that immunization with *E. coli* outer membrane vesicles containing recombinant LipL41 and OmpL1 partially protected against an otherwise lethal challenge of leptospires in hamsters¹³⁵. Subsequently, LipL32 has been shown to elicit immunoprotection when administered in naked DNA¹³⁶, *Mycobacterium bovis* bacille Calmette–Guérin (BCG)¹³⁷ and adenoviral¹³⁸ delivery systems. However, the overall efficacy of these candidate vaccines is low (40–75%) in experimental animals. The most promising subunit vaccine candidates are the Lig proteins, which have been shown to confer high-level protection (TABLE 2) approaching 100% in mice⁸⁶ and hamsters^{139–141}. The ability of Lig proteins to elicit cross-protective immunity to a range of serovar agents must be determined, as amino acid sequence identities for these proteins are 70–100% among *Leptospira* spp.¹⁴².

The availability of multiple genome sequences provides an opportunity to use high-throughput strategies to identify new vaccine candidates¹⁰⁵. The ultimate

T helper 1 cell

A type of activated T helper cell that promotes responses associated with the production of a particular set of cytokines, including tumour necrosis factor and interferon- γ , the main function of which is to stimulate phagocytosis-mediated defences against pathogens.

Figure 4 | The cell wall of leptospires. Leptospires have an inner membrane (IM) and an outer membrane (OM). The peptidoglycan cell wall is associated with the IM¹⁰⁰ and together these contain the FeoA–FeoB-type iron transporter (FeoAB)⁸³, penicillin-binding proteins (PBPs) and the lipoprotein LipL31. The leptospiral OM contains lipopolysaccharide (LPS), the transmembrane porin outer membrane protein L1 (OmpL1) and the lipoproteins LipL32, LipL36 (on the inner surface of the OM), LipL41 and LigB. Several TonB-dependent receptors (TBDRs) were identified by genome analysis, of which three are involved in the transport of iron citrate, the siderophore desferrioxamine and hemin^{83,154}. Transport requires energy transduction from the TonB–ExbB–ExbD complex in the inner membrane (for simplicity, only one TonB–ExbB–ExbD–TBDR system is shown). *Leptospira* spp. possess orthologues of the *Escherichia coli* export systems that transport OMPs and lipoproteins¹⁵⁵, including the IM lipoprotein signal peptidase I (SPase I) and SPase II. Lipoproteins are first transported through the Sec system and then bind to the ABC transporter formed by LoIC, LoID and LoIE. OMPs are transported through the Sec translocon, bound by the periplasmic chaperone Skp and then bound by Omp85 before being integrated into the lipid bilayer. An incomplete set of type II secretion-like genes is also present in the *Leptospira* spp. genomes. Several cytoplasmic membrane ABC transporters are found in leptospires, including a metallic cation uptake family ABC transporter⁸³. As in other spirochaetes, the endoflagella are located in the periplasm. The surface-exposed Loa22, leptospiral endostatin-like protein A (LenA), LenD, LigA and LigC proteins are not shown but are also known to be present at the surface of leptospires.

goal for vaccine development is to identify a candidate that protects against multiple *Leptospira* species. The *L. interrogans* and *L. borgpetersenii* genomes share 2,708 open reading frames, of which 656 are not present in the *L. biflexa* genome^{15,16} (BOX 2). Strategies to refine the number of target candidates include the sequencing of a wider representation of pathogenic *Leptospira* spp. genomes and the bioinformatic analysis and selection of open reading frames that are highly conserved among these genomes and that encode outer membrane proteins⁹⁸. The main barrier to 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 determine whether infection with leptospirosis protects against subsequent reinfection in high-risk populations and to identify the mechanisms of immunity that are involved. Until epidemiologically validated immune correlates are identified, the discovery of vaccine candidates will probably 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 are required to explain why it is so difficult to introduce DNA into pathogenic leptospires by methods that are commonly used for other bacteria. More efficient methods are needed to test the roles of putative virulence factors. The presence of prophage-like loci in the genomes of pathogenic *Leptospira* spp.^{75,143} suggests that transduction may occur and that 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 2,000 genes¹⁶; *L. biflexa* could therefore be used as a model bacterium to identify the functions of these common genes and to gain an insight into the general biology of *Leptospira* spp.

The development of genetic tools to transform leptospires has circumvented a substantial barrier to the elucidation of pathogen-related determinants of virulence and has led to the identification of Loa22 as the first virulence factor in *Leptospira* spp.¹⁰⁴. LipL32 and Lig proteins were long thought to be virulence factors, but mutagenesis of the corresponding genes did not result in attenuation of virulence. This suggests 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, proteomic 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 to learn more about leptospiral gene regulation and the interactions among leptospiral proteins. Microarrays are a valuable tool to identify regulatory networks or analyse the pleiotropic effects of a mutation. The use of genetically distinct (or engineered) laboratory rodents together with microarrays or proteomic studies should permit researchers to better delineate the mechanisms leading to chronic renal shedding. Ecological and metagenomic 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 will allow us to determine whether severe disease manifestations, such as LPHS, are due to strain-specific factors that enhance the pathogen's virulence or to innate or acquired host immune responses and susceptibility factors. Elucidation of the molecular mechanisms of pathogenesis will contribute to the development of novel strategies for the treatment and prevention of leptospirosis. Such advances are urgently needed to address the large disease burden that is attributable to this emerging infectious disease in impoverished populations.

1. Faine, S., Adler, B., Bolin, C. & Perolat, P. *Leptospira and Leptospirosis* (MedScience, Melbourne, 1999).
2. Weil, A. Ueber eine eigenthümliche, mit Milztumor, Icterus und Nephritis einhergehende acute Infektionskrankheit. *Dtsch. Arch. Klin. Med.* **39**, 209–232 (1886) (in German).
3. Inada, R., Ido, Y., Hoki, R., Kakeno, R. & Ito, H. The etiology, mode of infection and specific therapy of Weil's disease (*Spirochaetosis icterohaemorrhagica*). *J. Exp. Med.* **23**, 377–403 (1916).
A landmark paper that describes the discovery of *Leptospira* spp. as the aetiological agents of leptospirosis.
4. Levett, P. N. Leptospirosis. *Clin. Microbiol. Rev.* **14**, 296–326 (2001).
This comprehensive review provides wide coverage of our knowledge of leptospirosis.
5. Bharti, A. R. *et al.* Leptospirosis: a zoonotic disease of global importance. *Lancet Infect. Dis.* **3**, 757–771 (2003).
This review provides important complementary information to reference 4 and discusses the main issues facing the field of leptospirosis.
6. McBride, A. J., Athanazio, D. A., Reis, M. G. & Ko, A. I. Leptospirosis. *Curr. Opin. Infect. Dis.* **18**, 376–386 (2005).
7. Ko, A. I., *et al.* Urban epidemic of severe leptospirosis in Brazil. *Lancet* **354**, 820–825 (1999).
8. Riley, L. W., Ko, A. I., Unger, A. & Reis, M. G. Slum health: diseases of neglected populations. *BMC Int. Health Hum. Rights* **7**, 2 (2007).
9. Reis, R. B. *et al.* Impact of environment and social gradient on *Leptospira* infection in urban slums. *PLoS Negl. Trop. Dis.* **2**, e228 (2008).
10. WHO. Leptospirosis worldwide, 1999. *Wkly Epidemiol. Rec.* **74**, 237–242 (1999).
11. Paster, B. J. *et al.* Phylogenetic analysis of the spirochetes. *J. Bacteriol.* **173**, 6101–6109 (1991).
12. Li, C., Motaleb, M. A., Sal, M., Goldstein, S. F. & Charon, N. Gyration, rotation, periplasmic flagella: the biology of spirochete motility. *J. Mol. Microbiol. Biotechnol.* **2**, 345–354 (2000).
13. Ren, S. *et al.* Unique and physiological pathogenic features of *Leptospira interrogans* revealed by whole genome sequencing. *Nature* **422**, 888–893 (2003).
This article describes the first complete genome sequence of a leptospiral species.
14. Nascimento, A. L. *et al.* Comparative genomics of two *Leptospira interrogans* serovars reveals novel insights into physiology and pathogenesis. *J. Bacteriol.* **186**, 2164–2172 (2004).
15. Bulach, D. M. *et al.* Genome reduction in *Leptospira borgpetersenii* reflects limited transmission potential. *Proc. Natl Acad. Sci. USA* **103**, 14560–14565 (2006).
This work showed that the *L. borgpetersenii* genome is undergoing a process of reduction that seems to have impaired its ability to survive in the external environment and has caused it to rely on direct contact as its mode of transmission.
16. Picardeau, M. *et al.* Genome sequence of the saprophyte *Leptospira biflexa* provides insights into the evolution of *Leptospira* and the pathogenesis of leptospirosis. *PLoS ONE* **3**, e1607 (2008).
This paper describes the genome of the model bacterium *L. biflexa* and its comparison to the pathogenic *L. interrogans* and *L. borgpetersenii*.
17. Xue, F., Yan, J. & Picardeau, M. Evolution and pathogenesis of *Leptospira* spp.: lessons learned from the genomes. *Microbes Infect.* **11**, 328–333 (2008).
18. Thiermann, A. B. The Norway rat as a selective chronic carrier of *Leptospira icterohaemorrhagiae*. *J. Wildl. Dis.* **17**, 39–43 (1981).
19. Trueba, G., Zapata, S., Madrid, K., Cullen, P. & Haake, D. Cell aggregation: a mechanism of pathogenic *Leptospira* to survive in fresh water. *Int. Microbiol.* **7**, 35–40 (2004).
20. Ristow, P. *et al.* Biofilm formation by saprophytic and pathogenic leptospires. *Microbiology* **154**, 1309–1317 (2008).
References 19 and 20 describe key findings about the survival mechanisms of pathogenic *Leptospira* spp. outside the host.
21. Haake, D. A. & Lovett, M. A. Interjunctional invasion of endothelial cell monolayers. *Methods Enzymol.* **236**, 447–463 (1994).
22. Merien, F., Baranton, G. & Perolat, P. Invasion of Vero cells and induction of apoptosis in macrophages by pathogenic *Leptospira interrogans* are correlated with virulence. *Infect. Immun.* **65**, 729–738 (1997).
23. Liu, Y., Zheng, W., Li, L., Mao, Y. & Yan, J. Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with *in vitro* cultured mammalian cells. *Med. Microbiol. Immunol.* **196**, 233–239 (2007).
24. Thomas, D. D. & Higbie, L. M. *In vitro* association of leptospires with host cells. *Infect. Immun.* **58**, 581–585 (1990).
25. Barocchi, M. A., Ko, A. I., Reis, M. G., McDonald, K. L. & Riley, L. W. Rapid translocation of polarized MDCK cell monolayers by *Leptospira interrogans*, an invasive but nonintracellular pathogen. *Infect. Immun.* **70**, 6926–6932 (2002).
This study found that leptospires rapidly translocate across polarized cell monolayers by invading host cells and transiently residing in the cytoplasmic compartment.
26. Faine, S. Virulence in *Leptospira*. I. Reactions of guinea-pigs to experimental infections with *Leptospira icterohaemorrhagiae*. *Br. J. Exp. Pathol.* **38**, 1–7 (1957).
27. Faine, S. Virulence in *Leptospira*. II. The growth *in vivo* of virulent *Leptospira icterohaemorrhagiae*. *Br. J. Exp. Pathol.* **38**, 8–14 (1957).
28. Athanazio, D. A. *et al.* *Rattus norvegicus* as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. *Acta Trop.* **105**, 176–180 (2008).
29. Lourdault, K., Aviat, F. & Picardeau, M. The use of quantitative real-time PCR to study the dissemination of *Leptospira interrogans* in the guinea pig infection model of leptospirosis. *J. Med. Microbiol.* **58**, 648–655 (2009).
30. Segura, E. R. *et al.* Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. *Clin. Infect. Dis.* **40**, 343–351 (2005).
31. Truccolo, J., Serais, O., Merien, F. & Perolat, P. Following the course of human leptospirosis: evidence of a critical threshold for the vital prognosis using a quantitative PCR assay. *FEMS Microbiol. Lett.* **204**, 17–321 (2001).
32. Cinco, M. & Banfi, E. Activation of complement by leptospires and its bactericidal activity. *Zentralbl. Bakteriologie. Mikrobiol. Hyg. A* **254**, 261–265 (1983).
33. Johnson, R. C. & Muschel, L. H. Antileptospiral activity of serum. I. Normal and immune serum. *J. Bacteriol.* **91**, 1403–1409 (1966).
34. Meri, T., Murgia, R., Stefanel, P., Meri, S. & Cinco, M. Regulation of complement activation at the C3-level by serum resistant leptospires. *Microb. Pathog.* **39**, 139–147 (2005).
35. Verma, A. *et al.* LfhA, a novel factor H-binding protein of *Leptospira interrogans*. *Infect. Immun.* **74**, 2659–2666 (2006).
This study is the first report of a leptospiral complement factor H-binding protein that potentially contributes to both *Leptospira* spp. resistance to complement-mediated killing and the evasion of the immune response as the pathogen disseminates in the bloodstream.
36. Stevenson, B. *et al.* *Leptospira interrogans* endostatin-like outer membrane proteins bind host fibronectin, laminin and regulators of complement. *PLoS ONE* **2**, e1188 (2007).
37. Verma, A. *et al.* LruA and LruB antibodies in sera of humans with leptospirosis uveitis. *Clin. Vaccine Immunol.* **15**, 1019–1023 (2008).
38. Barbosa, A. S. *et al.* Immune evasion of *Leptospira* species by acquisition of human complement regulator C4BP. *Infect. Immun.* **77**, 1137–1143 (2009).
39. Nally, J. E., Chow, E., Fishbein, M. C., Blanco, D. R. & Lovett, M. A. Changes in lipopolysaccharide O antigen distinguish acute versus chronic *Leptospira interrogans* infections. *Infect. Immun.* **73**, 3251–3260 (2005).
This interesting study suggests that differential expression of LPS O antigen influences the ability of the pathogen to cause an acute lethal infection versus chronic renal carriage.
40. Sterling, C. R. & Thiermann, A. B. Urban rats as chronic carriers of leptospirosis: an ultrastructural investigation. *Vet. Pathol.* **18**, 628–637 (1981).
41. Monahan, A. M., Callanan, J. J. & Nally, J. E. Proteomic analysis of *Leptospira interrogans* shed in urine of chronically infected hosts. *Infect. Immun.* **76**, 4952–4958 (2008).
42. Ganoza, C. A. *et al.* Determining risk for severe leptospirosis by molecular analysis of environmental surface waters for pathogenic *Leptospira*. *PLoS Med.* **3**, e308 (2006).
This investigation found that an outbreak of severe leptospirosis was associated with exposure to high concentrations of pathogenic leptospires in environmental surface waters. The work was pioneering in that it developed new molecular tools to quantify leptospires in the environment.
43. Matthias, M. A. *et al.* Human leptospirosis caused by a new, antigenically unique *Leptospira* associated with a *Rattus* species reservoir in the peruvian Amazon. *PLoS Negl. Trop. Dis.* **2**, e213 (2008).
44. Thaipadungpanit, J. *et al.* A dominant clone of *Leptospira interrogans* associated with an outbreak of human leptospirosis in Thailand. *PLoS Negl. Trop. Dis.* **1**, e56 (2007).
45. Slack, A., Symonds, M., Dohnt, M. & Smythe, L. An improved multiple-locus variable number of tandem repeats analysis for *Leptospira interrogans* serovar Australis: a comparison with fluorescent amplified fragment length polymorphism analysis and its use to redefine the molecular epidemiology of this serovar in Queensland, Australia. *J. Med. Microbiol.* **55**, 1549–1557 (2006).

46. Zuermer, R. L. & Alt, D. P. Variable nucleotide tandem repeat analysis reveals a unique group of *Leptospira interrogans* serovar Pomona isolates associated with California sea lions. *J. Clin. Microbiol.* **47**, 1202–1205 (2009).
47. Lingappa, J. *et al.* HLA-DQ6 and ingestion of contaminated water: possible gene-environment interaction in an outbreak of Leptospirosis. *Genes Immun.* **5**, 311–314 (2004).
This is the first report of a host genetic susceptibility factor for leptospirosis and also of a gene–environment interaction for an infectious disease.
48. Silva, E. F. *et al.* Characterization of virulence of *Leptospira* isolates in a hamster model. *Vaccine* **26**, 3892–3896 (2008).
49. Nicodemo, A. C. *et al.* Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. *Am. J. Trop. Med. Hyg.* **56**, 181–187 (1997).
50. De Brito, T., Böhm, G. M. & Yasuda, P. H. Vascular damage in acute experimental leptospirosis of the guinea-pig. *J. Pathol.* **128**, 177–182 (1979).
51. Chierakul, W. *et al.* Activation of the coagulation cascade in patients with leptospirosis. *Clin. Infect. Dis.* **46**, 254–260 (2008).
This study found that activation of the coagulation cascade, including disseminated intravascular coagulation, is a prominent feature in leptospirosis.
52. Wagenaar, J. F. *et al.* What role do coagulation disorders play in the pathogenesis of leptospirosis? *Trop. Med. Int. Health* **12**, 111–122 (2007).
53. Diamant, D., Brunialti, M. K. C., Romero, E. C., Kallas, E. G. & Salomao, R. Peripheral blood mononuclear cell activation induced by *Leptospira interrogans* glycolipoprotein. *Infect. Immun.* **70**, 1677–1683 (2002).
54. Klimpel, G. R., Matthias, M. A. & Vinetz, J. M. *Leptospira interrogans* activation of human peripheral blood mononuclear cells: preferential expansion of TCR $\gamma\delta^+$ T cells vs TCR $\alpha\beta^+$ T cells. *J. Immunol.* **171**, 1447–1455 (2003).
55. Vernel-Pauillac, F. & Merien, F. Proinflammatory and immunomodulatory cytokine mRNA time course profiles in hamsters infected with a virulent variant of *Leptospira interrogans*. *Infect. Immun.* **74**, 4172–4179 (2006).
56. de Fost, M., Hartskeerl, R. A., Groenendijk, M. R. & van der Poll, T. Interleukin 12 in part regulates gamma interferon release in human whole blood stimulated with *Leptospira interrogans*. *Clin. Diagn. Lab. Immunol.* **10**, 332–335 (2003).
57. Tajiki, M. H. & Salomao, R. Association of plasma levels of tumor necrosis factor alpha with severity of disease and mortality among patients with leptospirosis. *Clin. Infect. Dis.* **23**, 1177–1178 (1996).
58. Werts, C. *et al.* Leptospiral lipopolysaccharide activates cells through a TLR2-dependent mechanism. *Nature Immunol.* **2**, 346–352 (2001).
59. Que-Gewirth, N. L. *et al.* A methylated phosphate group and four amide-linked acyl chains in *Leptospira interrogans* lipid A. The membrane anchor of an unusual lipopolysaccharide that activates TLR. *J. Biol. Chem.* **279**, 25420–25429 (2004).
References 58 and 59 show that *Leptospira* spp. possess an unusual LPS that uses TLR2 rather than TLR4 for signalling in human cells.
60. Yang, C. W. *et al.* Toll-like receptor 2 mediates early inflammation by leptospiral outer membrane proteins in proximal tubule cells. *Kidney Int.* **69**, 815–822 (2006).
61. Nahori, M. A. *et al.* Differential TLR recognition of leptospiral lipid A and lipopolysaccharide in murine and human cells. *J. Immunol.* **175**, 6022–6031 (2005).
62. Seguro, A. C., Lomar, A. V. & Rocha, A. S. Acute renal failure of leptospirosis: nonoliguric and hypokalemic forms. *Nephron* **55**, 146–151 (1990).
63. Burth, P., Younes-Ibrahim, M., Santos, M. C., Castro-Faria Neto, H. C. & de Castro Faria, M. V. Role of nonesterified unsaturated fatty acids in the pathophysiological processes of leptospiral infection. *J. Infect. Dis.* **191**, 51–57 (2005).
64. Wu, M. S., Yang, C. W., Pan, M. J., Chang, C. T. & Chen, Y. C. Reduced renal Na⁺-K⁺-Cl⁻ co-transporter activity and inhibited NKCC2 mRNA expression by *Leptospira shermani*: from bed-side to bench. *Nephrol. Dial. Transplant.* **19**, 2472–2479 (2004).
65. Andrade, L., Rodrigues, A. C. Jr, Sanches, T. R., Souza, R. B. & Seguro, A. C. Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung. *Am. J. Physiol. Renal Physiol.* **292**, F586–F592 (2007).
66. Isogai, E. *et al.* Apoptosis of lymphocytes in mice administered lipopolysaccharide from *Leptospira interrogans*. *Zentralbl. Veterinarmed. B* **45**, 529–537 (1998).
67. Merien, F., Truccolo, J., Rougier, Y., Baranton, G. & Perolat, P. *In vivo* apoptosis of hepatocytes in guinea pigs infected with *Leptospira interrogans* serovar Icterohaemorrhagiae. *FEMS Microbiol. Lett.* **169**, 95–102 (1998).
68. Rugman, F. P., Pinn, G., Palmer, M. F., Waite, M. & Hay, C. R. Anticardiolipin antibodies in leptospirosis. *J. Clin. Pathol.* **44**, 517–519 (1991).
69. Nally, J. E. *et al.* Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. *Am. J. Pathol.* **164**, 1115–1127 (2004).
This study raises the potential for the involvement of an autoimmune process in the development of this severe disease form.
70. Stamm, L. V., Parrish, E. A. & Gherardini, F. C. Cloning of the *recA* gene from a free-living Leptospira and distribution of RecA-like protein among spirochetes. *Appl. Environ. Microbiol.* **57**, 183–189 (1991).
71. Mitchison, M. *et al.* Identification and characterization of the dTDP-rhamnose biosynthesis and transfer genes of the lipopolysaccharide-related *rfb* locus in *Leptospira interrogans* serovar Copenhageni. *J. Bacteriol.* **179**, 1262–1267 (1997).
72. Yelton, D. B. & Cohen, R. A. Analysis of cloned DNA from *Leptospira biflexa* serovar patoc which complements a deletion of the *Escherichia coli* *trpE* gene. *J. Bacteriol.* **165**, 41–46 (1986).
73. Baril, C., Richaud, C., Fournie, E., Baranton, G. & Saint Girons, I. Cloning of *dapD*, *aroD* and *asd* of *Leptospira interrogans* serovar Icterohaemorrhagiae, and nucleotide sequence of the *asd* gene. *J. Gen. Microbiol.* **138**, 47–53 (1992).
74. Saint Girons, I., Margarita, D., Amouriaux, P. & Baranton, G. First isolation of bacteriophages for a spirochaete: potential genetic tools for *Leptospira*. *Res. Microbiol.* **141**, 1131–1138 (1990).
75. Bourhy, P. *et al.* A genomic island of the pathogen *Leptospira interrogans* serovar Lai can excise from its chromosome. *Infect. Immun.* **75**, 677–683 (2007).
76. Saint Girons, I. *et al.* The LE1 bacteriophage replicates as a plasmid within *Leptospira biflexa*: construction of an *L. biflexa*-*Escherichia coli* shuttle vector. *J. Bacteriol.* **182**, 5700–5705 (2000).
The first study to show the successful introduction of DNA into *Leptospira* spp.
77. Bourhy, P., Louvel, H., Saint Girons, I. & Picardeau, M. Random insertional mutagenesis of *Leptospira interrogans*, the agent of leptospirosis, using a *mariner* transposon. *J. Bacteriol.* **187**, 3255–3258 (2005).
This paper describes the first evidence of gene transfer in pathogenic *Leptospira* spp. and the generation of the first mutants in these pathogens.
78. Picardeau, M. Conjugative transfer between *Escherichia coli* and *Leptospira* spp. as a new genetic tool. *Appl. Environ. Microbiol.* **74**, 319–322 (2008).
79. Louvel, H. & Picardeau, M. Genetic manipulation of *Leptospira biflexa*. *Curr. Protoc. Microbiol.* **12**, 12E.4 (2007).
80. Croda, J. *et al.* Targeted mutagenesis in pathogenic *Leptospira*: disruption of the *ligB* gene does not affect virulence in animal models of leptospirosis. *Infect. Immun.* **76**, 5826–5833 (2008).
The first and so far only description of targeted inactivation in pathogenic *Leptospira* spp.
81. Murray, G. L. *et al.* Genome-wide transposon mutagenesis in pathogenic *Leptospira* spp. *Infect. Immun.* **77**, 810–816 (2009).
82. Louvel, H., Saint Girons, I. & Picardeau, M. Isolation and characterization of FecA- and FecB- mediated iron acquisition systems of the spirochete *Leptospira biflexa* by random insertional mutagenesis. *J. Bacteriol.* **187**, 3249–3254 (2005).
83. Louvel, H. *et al.* Comparative and functional genomic analyses of iron transport and regulation in *Leptospira* spp. *J. Bacteriol.* **188**, 7893–7904 (2006).
84. Pereira, M. M., Andrade, J., Marchevsky, R. S. & Ribeiro dos Santos, R. Morphological characterization of lung and kidney lesions in C3H/HeJ mice infected with *Leptospira interrogans* serovar Icterohaemorrhagiae: defect of CD4⁺ and CD8⁺ T-cells are prognosticators of the disease progression. *Exp. Toxicol. Pathol.* **50**, 191–198 (1998).
85. Viriyakosol, S., Matthias, M. A., Swancutt, M. A., Kirkland, T. N. & Vinetz, J. M. Toll-like receptor 4 protects against lethal *Leptospira interrogans* serovar Icterohaemorrhagiae infection and contributes to
- in vivo* control of leptospiral burden. *Infect. Immun.* **74**, 887–895 (2006).
- This article highlights the importance of TLR pathway activation in innate immunity against leptospirosis.**
86. Koizumi, N. & Watanabe, H. Leptospiral immunoglobulin-like proteins elicit protective immunity. *Vaccine* **22**, 1545–1552 (2004).
87. Minette, H. P. & Shaffer, M. F. Experimental leptospirosis in monkeys. *Am. J. Trop. Med. Hyg.* **17**, 202–212 (1968).
88. Pereira, M. M. *et al.* Experimental leptospirosis in marmoset monkeys (*Callithrix jacchus*): a new model for studies of severe pulmonary leptospirosis. *Am. J. Trop. Med. Hyg.* **72**, 13–20 (2005).
89. Ito, T. & Yanagawa, R. Leptospiral attachment to extracellular matrix of mouse fibroblast (L929) cells. *Ver. Microbiol.* **15**, 89–96 (1987).
90. Merien, F., Truccolo, J., Baranton, G. & Perolat, P. Identification of a 36-kDa fibronectin-binding protein expressed by a virulent variant of *Leptospira interrogans* serovar Icterohaemorrhagiae. *FEMS Microbiol. Lett.* **185**, 17–22 (2000).
91. Atzingen, M. V. *et al.* A novel leptospiral protein binding adhesive matrix molecules and present during human infection. *BMC Microbiol.* **8**, 70 (2008).
92. Hauk, P. *et al.* In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. *Infect. Immun.* **76**, 2642–2650 (2008).
93. Hoke, D. E., Egan, S., Cullen, P. A. & Adler, B. LipL32 is an extracellular matrix-interacting protein of *Leptospira* spp. and *Pseudomonas aeruginosa*. *Infect. Immun.* **76**, 2063–2069 (2008).
94. Choy, H. A. *et al.* Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infect. Immun.* **75**, 2441–2450 (2007).
The first study to determine that Lig proteins bind to extracellular matrix moieties.
95. Haake, D. A. *et al.* Changes in the surface of *Leptospira interrogans* serovar grippityphosa during *in vitro* cultivation. *Infect. Immun.* **59**, 1131–1140 (1991).
96. Setubal, J. C., Reis, M. G., Matsunaga, J. & Haake, D. A. Lipoprotein computational prediction in spirochaetal genomes. *Microbiology* **152**, 113–121 (2006).
97. Viratyosin, W., Ingsriswang, S., Pacharawongsakda, E. & Palittapongarnpim, P. Genome-wide subcellular localization of putative outer membrane and extracellular proteins in *Leptospira interrogans* serovar Lai genome using bioinformatics approaches. *BMC Genomics* **9**, 181 (2008).
98. Yang, H. L. *et al.* *In silico* and microarray-based genomic approaches to identifying potential vaccine candidates against *Leptospira interrogans*. *BMC Genomics* **7**, 293 (2006).
99. Lee, S. H., Kim, S., Park, S. C. & Kim, M. J. Cytotoxic activities of *Leptospira interrogans* hemolysin SpH as a pore-forming protein on mammalian cells. *Infect. Immun.* **70**, 315–322 (2002).
100. Cullen, P. A., Haake, D. A. & Adler, B. Outer membrane proteins of pathogenic spirochetes. *FEMS Microbiol. Rev.* **28**, 291–318 (2004).
101. Haake, D. A. *et al.* Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic *Leptospira* spp. *J. Bacteriol.* **175**, 4225–4234 (1993).
102. Haake, D. A. *et al.* The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. *Infect. Immun.* **68**, 2276–2285 (2000).
103. Matsunaga, J. *et al.* Pathogenic *Leptospira* species express surface-exposed proteins belonging to the bacterial immunoglobulin superfamily. *Mol. Microbiol.* **49**, 929–945 (2003).
104. Ristow, P. *et al.* The OmpA-like protein Loa22 is essential for leptospiral virulence. *PLoS Pathog.* **3**, e97 (2007).
The first identification of a virulence determinant according to Koch's molecular postulates.
105. Gamberini, M. *et al.* Whole-genome analysis of *Leptospira interrogans* to identify potential vaccine candidates against leptospirosis. *FEMS Microbiol. Lett.* **244**, 305–313 (2005).
106. Nally, J. E., Whitelegge, J. P., Bassilian, S., Blanco, D. R. & Lovett, M. A. Characterization of the outer membrane proteome of *Leptospira interrogans* expressed during acute lethal infection. *Infect. Immun.* **75**, 766–773 (2007).

107. Barbosa, A. S. *et al.* A newly identified leptospiral adhesin mediates attachment to laminin. *Infect. Immun.* **74**, 6356–6364 (2006).
108. Lee, S. H. *et al.* Identification and partial characterization of a novel hemolysin from *Leptospira interrogans* serovar lai. *Gene* **254**, 19–28 (2000). **Together with reference 102, the first description of LipL32, the major surface-exposed protein of pathogens.**
109. Cullen, P. A., Cordwell, S. J., Bulach, D. M., Haake, D. A. & Adler, B. Global analysis of outer membrane proteins from *Leptospira interrogans* serovar Lai. *Infect. Immun.* **70**, 2311–2318 (2002).
110. Haake, D. A. *et al.* Molecular evolution and mosaicism of leptospiral outer membrane proteins involves horizontal DNA transfer. *J. Bacteriol.* **186**, 2818–2828 (2004).
111. Vivian, J. P. *et al.* Crystal structure of LipL32, the most abundant surface protein of pathogenic *Leptospira* spp. *J. Mol. Biol.* **387**, 1229–1238 (2009).
112. Murray, G. L. *et al.* The major surface protein LipL32 is not required for either acute or chronic infection with *Leptospira interrogans*. *Infect. Immun.* **77**, 952–958 (2009).
113. Palaniappan, R. U. *et al.* Cloning and molecular characterization of an immunogenic LigA protein of *Leptospira interrogans*. *Infect. Immun.* **70**, 5924–5930 (2002). **This study and that in reference 103 identified LigA, LigB and LigC as members of the bacterial immunoglobulin-like superfamily of proteins.**
114. Lin, Y. P. & Chang, Y. F. A domain of the *Leptospira* LigB contributes to high affinity binding of fibronectin. *Biochem. Biophys. Res. Commun.* **362**, 443–448 (2007).
115. Lin, Y. P., Raman, R., Sharma, Y. & Chang, Y. F. Calcium binds to leptospiral immunoglobulin-like protein, LigB, and modulates fibronectin binding. *J. Biol. Chem.* **283**, 25140–25149 (2008).
116. Matsunaga, J. *et al.* Response of *Leptospira interrogans* to physiologic osmolality: relevance in signaling the environment-to-host transition. *Infect. Immun.* **75**, 2864–2874 (2007). **References 94 and 116 both found that physiological osmolality is a key environmental signal for the differential regulation of leptospiral genes.**
117. Srimanote, P. *et al.* Recombinant ligA for leptospirosis diagnosis and ligA among the *Leptospira* spp. clinical isolates. *J. Microbiol. Methods* **72**, 73–81 (2008).
118. Croda, J. *et al.* *Leptospira* immunoglobulin-like proteins as a serodiagnostic marker for acute leptospirosis. *J. Clin. Microbiol.* **45**, 1528–1534 (2007).
119. Ellis, W. A., Hovind-Hougen, K., Möller, S. & Birch-Andersen, A. Morphological changes upon subculturing of freshly isolated strains of *Leptospira interrogans* serovar hardjo. *Zentralbl. Bakteriol. Mikrobiol. Hyg. A* **255**, 323–335 (1983).
120. Picardeau, M., Brenot, A. & Saint Girons, I. First evidence for gene replacement in *Leptospira* spp. Inactivation of *L. biflexa* flaB results in non-motile mutants deficient in endoflagella. *Mol. Microbiol.* **40**, 189–199 (2001). **The first report of targeted gene inactivation in Leptospira spp.; unlike that of the spirochaete B. burgdorferi, the cell body of the leptospiral flaB mutant remains helical.**
121. Motaleb, M. A. *et al.* *Borrelia burgdorferi* periplasmic flagella have both skeletal and motility functions. *Proc. Natl Acad. Sci. USA* **97**, 10899–10904 (2000).
122. Yuri, K. *et al.* Chemotaxis of leptospires to hemoglobin in relation to virulence. *Infect. Immun.* **61**, 2270–2272 (1993).
123. Murray, G. L. *et al.* *Leptospira interrogans* requires heme oxygenase for disease pathogenesis. *Microbes Infect.* **11**, 311–314 (2009).
124. Lo, M. *et al.* Effects of temperature on gene expression patterns in *Leptospira interrogans* serovar Lai as assessed by whole-genome microarrays. *Infect. Immun.* **74**, 848–859 (2006).
125. Adler, B. & Faine, S. Host immunological mechanisms in the resistance of mice to leptospiral infections. *Infect. Immun.* **17**, 67–72 (1977).
126. Adler, B. & Faine, S. The antibodies involved in the human immune response to leptospiral infection. *J. Med. Microbiol.* **11**, 387–400 (1978).
127. Jost, B. H., Adler, B., Vinh, T. & Faine, S. A monoclonal antibody reacting with a determinant on leptospiral lipopolysaccharide protects guinea pigs against leptospirosis. *J. Med. Microbiol.* **22**, 269–275 (1986).
128. Chassin, C. *et al.* TLR4- and TLR2-mediated B cell responses control the clearance of the bacterial pathogen, *Leptospira interrogans*. *J. Immunol.* **183**, 2669–2677 (2009).
129. Naiman, B. M. *et al.* Evaluation of type 1 immune response in naive and vaccinated animals following challenge with *Leptospira borgpetersenii* serovar Hardjo: involvement of WC1 + $\gamma\delta$ and CD4 T cells. *Infect. Immun.* **70**, 6147–6157 (2002). **This study raises the possibility that cell-mediated immune responses confer protection against leptospirosis in certain hosts, such as cows.**
130. Brown, R. A. *et al.* Comparison of three different leptospiral vaccines for induction of a type 1 immune response to *Leptospira borgpetersenii* serovar Hardjo. *Vaccine* **21**, 4448–4458 (2003).
131. Blumberman, S. L., Herzig, C. T. & Baldwin, C. L. WC1 + $\gamma\delta$ T cell memory population is induced by killed bacterial vaccine. *Eur. J. Immunol.* **37**, 1204–1216 (2007).
132. Ido, Y., Hoki, R., Ito, H. & Wani, H. The prophylaxis of Weil's disease (*Spirochaetosis Icterohaemorrhagica*). *J. Exp. Med.* **24**, 471–483 (1916).
133. Koizumi, N. & Watanabe, H. Leptospirosis vaccines: past, present, and future. *J. Postgrad. Med.* **51**, 210–214 (2005).
134. Gonzalez, A. *et al.* Immunogenicity and protective capacity of leptospiral whole-cell monovalent serogroup Ballum vaccines in hamsters. *Rev. Argent. Microbiol.* **37**, 169–175 (2005).
135. Haake, D. A. *et al.* Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. *Infect. Immun.* **67**, 6572–6582 (1999). **The first evidence that immunization with a recombinant protein confers protection against experimental leptospirosis.**
136. Branger, C. *et al.* Protection against *Leptospira interrogans* sensu lato challenge by DNA immunization with the gene encoding hemolysin-associated protein 1. *Infect. Immun.* **73**, 4062–4069 (2005).
137. Seixas, F. K. *et al.* Recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen of *Leptospira interrogans* protects hamsters from challenge. *Vaccine* **26**, 88–95 (2007).
138. Branger, C. *et al.* Identification of the hemolysin-associated protein 1 as a cross-protective immunogen of *Leptospira interrogans* by adenovirus-mediated vaccination. *Infect. Immun.* **69**, 6831–6838 (2001).
139. Palaniappan, R. U. *et al.* Immunoprotection of recombinant leptospiral immunoglobulin-like protein A against *Leptospira interrogans* serovar Pomona infection. *Infect. Immun.* **74**, 1745–1750 (2006). **References 86 and 139 were the first to report that Lig proteins induce high-level immunoprotection against a lethal L. interrogans infection in experimental animals.**
140. Silva, E. F. *et al.* The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine* **25**, 6277–6286 (2007).
141. Yan, W. *et al.* Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (LigB) in a hamster challenge model. *Microbes Infect.* **11**, 230–237 (2009).
142. McBride, A. J. *et al.* Genetic diversity of the Leptospiral immunoglobulin-like (Lig) genes in pathogenic *Leptospira* spp. *Infect. Genet. Evol.* **9**, 196–205 (2009).
143. Qin, J. H. *et al.* Identification of a novel prophage-like gene cluster actively expressed in both virulent and avirulent strains of *Leptospira interrogans* serovar Lai. *Infect. Immun.* **76**, 2411–2419 (2008).
144. Salaün, L., Mérien, F., Gurianova, S., Baranton, G. & Picardeau, M. Application of multilocus variable-number tandem-repeat analysis for molecular typing of the agent of leptospirosis. *J. Clin. Microbiol.* **44**, 3954–3962 (2006).
145. Ahmed, N. *et al.* Multilocus sequence typing method for identification and genotypic classification of pathogenic *Leptospira* species. *Ann. Clin. Microbiol. Antimicrob.* **5**, 28 (2006).
146. Bourhy, P. *et al.* Complete nucleotide sequence of the LE1 prophage from the spirochaete *Leptospira biflexa* and characterization of its replication and partition functions. *J. Bacteriol.* **187**, 3931–3940 (2005).
147. Park, S. K. *et al.* Leptospirosis in Chonbuk Province of Korea in 1987: a study of 93 patients. *Am. J. Trop. Med. Hyg.* **41**, 345–351 (1989).
148. Trevejo, R. T. *et al.* Epidemic leptospirosis associated with pulmonary hemorrhage — Nicaragua, 1995. *J. Infect. Dis.* **178**, 1457–1463 (1998).
149. Panaphut, T., Domrongkitthaiporn, S. & Thinkamrop, B. Prognostic factors of death in leptospirosis: a prospective cohort study in Khon Kaen, Thailand. *Int. J. Infect. Dis.* **6**, 52–59 (2002).
150. Marotto, P. C. F. *et al.* Acute lung injury in leptospirosis: clinical and laboratory features, outcome, and factors associated with mortality. *Clin. Infect. Dis.* **29**, 1561–1563 (1999).
151. Gouveia, E. L. *et al.* Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg. Infect. Dis.* **14**, 505–508 (2008).
152. Yang, G. G. & Hsu, Y. H. Nitric oxide production and immunoglobulin deposition in leptospiral hemorrhagic respiratory failure. *J. Formos. Med. Assoc.* **104**, 759–763 (2005).
153. Croda, J. *et al.* Leptospirosis pulmonary hemorrhage syndrome is associated with linear deposition of immunoglobulin and complement on the alveolar surface. *Clin. Microbiol. Infect.* (in press).
154. Asuthkar, S., Velineni, S., Stadlmann, J., Altmann, F. & Sriharan, M. Expression and characterization of an iron-regulated hemin-binding protein, HbpA, from *Leptospira interrogans* serovar Lai. *Infect. Immun.* **75**, 4582–4591 (2007).
155. Bos, M. P., Robert, V. & Tommassen, J. Biogenesis of the Gram-negative bacterial outer membrane. *Annu. Rev. Microbiol.* **61**, 191–214 (2007).
156. Faisal, S. M. *et al.* Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine* **26**, 277–287 (2008).
157. Faisal, S. M., Yan, W., McDonough, S. P. & Chang, Y. F. *Leptospira* immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. *Vaccine* **27**, 378–387 (2009).

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DATABASES

CDC Diseases and Conditions: <http://www.cdc.gov/DiseasesConditions/Leptospirosis>
Entrez Gene: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&asq=flaB|hemH|ligB|metW|metX|metY|recA|trpE>
Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/sites/entrez?db=genomeprj&term=Borrelia%20burgdorferi%20Escherichia%20coli%20Leptospira%20biflexa%20Leptospira%20borgpetersenii%20Leptospira%20interrogans%20Mus%20musculus%20Rattus%20norvegicus>
UniProtKB: <http://www.uniprot.org/C4BPA|complement%20factor%20H|LenA|LenD|LigA|LigC|LipL32|LipL41|OmpL1|TLR2|TLR4>

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Mathieu Picardeau's homepage: <http://www.pasteur.fr/research/spiro>

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