

New insights into the pathogenicity of leptospires: evasion of host defences

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SUMMARY

Major progress has been made in the basic research of leptospirosis a global zoonotic disease. Recent knowledge on the genome of *L. interrogans* and the emergence of new genetic tools for comparative genetic studies have further developed research into the genetic pathogenesis of this illness. Many of these studies have compared the putative pathogenicity factors found in *L. interrogans*, with representative strains of saprophytic leptospires. Leptospires display a rich repertoire of adhesins endowed with multifunctional biological activities such as adhesion to host tissue components, plasminogen activation, resistance to complement. These adhesins are proteins or lipoproteins located on the outer membrane. Some of them (LenA) escape innate defence such as complement killing and some escape phagocytosis. Much work has to be done to elucidate many other aspects of *Leptospira* pathogenic factors such as those switched on in chronic infection.

KEY WORDS: Leptospires, Pathogenicity, Lipoproteins, Adhesion

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INTRODUCTION

Leptospirosis is an important zoonotic disease caused by spirochetes of the genus *Leptospira*. This genus includes free-living non pathogenic species as well as pathogenic species which can infect humans and animals. Leptospirosis has emerged as the most widespread zoonotic disease worldwide (CDC). In humans the disease varies from an asymptomatic flu-like illness to an acute life-threatening infection (known as Weil's Disease) with pulmonary haemorrhage, myocarditis and kidney and liver failure, the infection being mainly recorded in the tropics. In the past few years natural calamities such as cyclones and floods have often been the cause of severe outbreaks in places such as Nicaragua, Brazil and India (McBride *et al.*, 2005). Therefore leptospirosis is currently considered an emerging

global public health problem because of its increasing incidence in both developing and developed countries.

The sources of Leptospires are essentially wild and domesticated mammals harbouring the spirochetes in the proximal convolute tubules of the kidneys and chronically excreting the leptospires with urine into the environment. Humans become infected directly through exposure to urine and/or indirectly through fresh water contaminated with urine. Occasional exposure due to occupational activities to contaminated tissues and body fluids is also possible (Faine *et al.*, 1999). Leptospires enter the body through mucous membranes of the eyes, nose or throat and via cuts or abrasions of the skin and invade the host tissues and fluids. In humans the severity of the disease varies with the *Leptospira* species, the health and immune status of the patient.

Only recently have the pathogenetic mechanisms underlying the symptoms of leptospirosis been investigated on the basis of the new knowledge on host/parasite interactions and on the basis of genetics, that is genetic maps of putative genes of virulence and comparative analysis of

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Leptospira proteomas among different serovars of pathogenic and non-pathogenic organisms (Cullen *et al.*, 2002; Nally *et al.*, 2005; Thongboonkerd *et al.*, 2009). Though the molecular mechanisms underlying the pathogenesis of leptospirosis remains poorly understood new knowledge has been discovered regarding the first steps of *Leptospira* infection, which are *Leptospira* adherence and invasion of the mammalian host.

THE GENUS *LEPTOSPIRA*: TAXONOMY

Leptospirae belong to a unique genus of the phylum Spirochaete. The genus is quite heterogeneous, divided into different branches on the basis of genetic classification. The subgroups of saprophytic species *Leptospira biflexa*, *Leptospira wolbachii*, *Leptospira kmetyi*, *Leptospira yanagawa*, *Leptospira terpstreae* and *Leptospira vantielii* include microorganisms living free in the surface water, not pathogenic for humans.

Another group includes the pathogenic species differentiated into 8 genospecies *Leptospira interrogans*, *Leptospira kirschneri*, *Leptospira*

noguchi, *Leptospira borgpeterseni*, *Leptospira santarosae*, *Leptospira weilii*, *Leptospira alexanderi*, *Leptospira alstoni*. Another subgroup comprises species of “intermediate behaviour” whose role in pathogenicity could not be demonstrated: *Leptospira inada*, *Leptospira broomi*, *Leptospira fainei*, *Leptospira wolfii* and *Leptospira licerasiae* (International Committee on Systematics of Prokaryotes, 2008). A phylogenetic tree derived from 16S rRNA sequence analysis of the 18 representative species is reported in Figure 1 (Slack *et al.*, 2009).

Besides the genetic classification there is still in use the old phenotypic classification based on serology, which recognised more than 230 serovars among pathogenic leptospires (Faine *et al.*, 1999). This classification is maintained for epidemiological purposes.

PATHOGENESIS OF LEPTOSPIROSIS

Pathogenic leptospires can infect a wide range of animals, causing diseases in some of them- humans, dogs, cattle- and a chronic infection in others, mainly rodents who undergo a persistent renal carriage. In humans-who are incidental hosts- leptospires produce an acute disease, without chronic manifestations, which can be severe and fatal, characterized by jaundice, renal failure and/or pulmonary haemorrhage as shown in Figure 2 (Barthi *et al.*, 2003; Mc Bride *et al.*, 2005).

ENTRY AND INVASION OF THE HOST.

1. Adhesion

After exposure of mucous membranes or broken skin to water or soil contaminated with leptospires shed in animal urine, the microorganisms quickly establish a systemic infection by crossing tissue barriers and blood invasion (Faine *et al.*, 1999). Different strategies are carried on by the pathogenic leptospires for invasion. One is the capacity to adhere to host cell and cellular matrix (ECM) as many other pathogens do. Virulent pathogenic leptospires bind to endothelial, fibroblast, kidney epithelial and monocyte/macrophage cell lines cultured in vitro, but not culture-attenuated organisms (Ito *et al.*; Meriem *et al.*, 1997; Merien *et al.*, 1998, Thomas

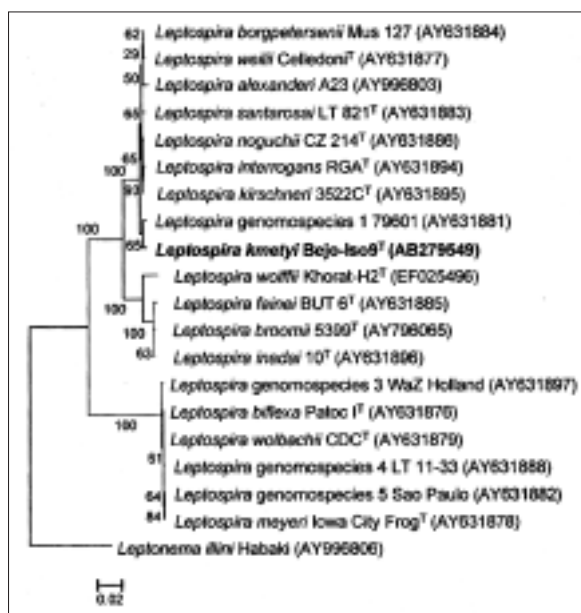


FIGURE 1 - 16S rRNA gene sequence-based phylogeny of 18 representative species of the genus *Leptospira*. *Leptonema illini* used as an outgroup. Bootstrap values are displayed as percentages. Bar, 0.02 inferred nucleotide substitution per 100 ml. (from Slack *et al.*, 2009).

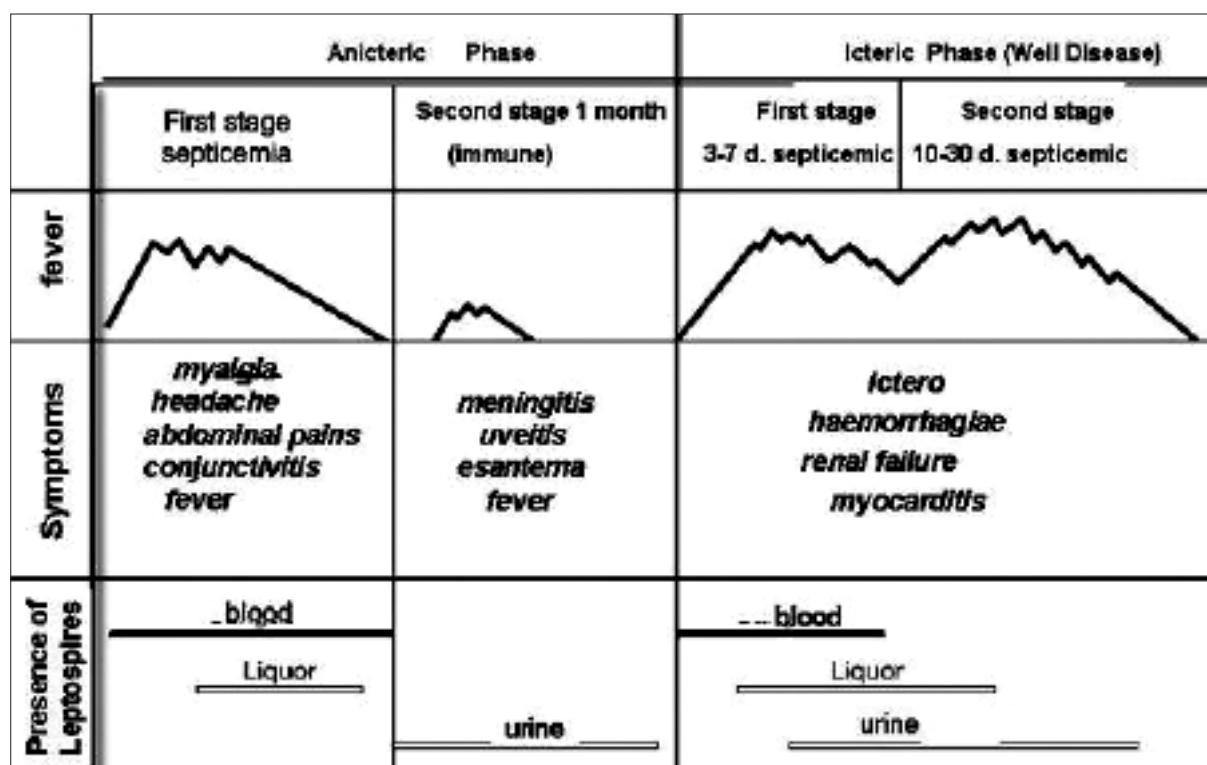


FIGURE 2 - Sequelae of anicteric and icteric leptospirosis: relationships between clinical symptoms and the presence of leptospires in organic fluids.

et al., 1990). Through they are not intracellular parasites, they efficiently enter host cells *in vitro* (Merien *et al.*, 1997; Merien *et al.*, 2007) and rapidly translocate across polarized cell monolayers without altering the trans-epithelial electrical resistance (Thomas *et al.*, 1990; Barocchi *et al.*, 2002). They reside only transiently within these cells. In normal non-phagocytic cells internalized microorganisms have been observed in cytoplasmic and phagosomal compartments (Thomas *et al.*, 1990; Barocchi *et al.*, 2002; Liu *et al.*, 2007), therefore leptospires appear to use a novel cell entry mechanism which permits a rapid translocation to spread to target organs, evading immune killing. Most studies on *Leptospira* adherence, however, have focused on the attachment of bacteria to ECM components as suggested by observations during acute leptospirosis in animal models, in which leptospires have been demonstrated within the interstitium between hepatocytes and tubular epithelial cells (Merien *et al.*, 1988). Infectious strains of *Leptospira* adhere to ECM components such as collagen type I, Type

IV, laminin and also fibronectin (Barbosa *et al.*, 2006; Atzinger *et al.*, 2008). In some cases one or more adhesins binding these substrates have been identified, namely the 36 kDa outer surface isolated protein which binds to fibronectin (Merien *et al.*, 2000). With the aid of functional genomic studies based on the sequence of genome of *L. interrogans* serovar Copenhageni, (Nascimento *et al.*, 2004), more than 200 outer membrane proteins have been predicted which may be implicated in pathogenesis. On this basis a 24 kDa laminin binding protein named Lsa24, an Lsa21 protein binding collagen IV laminin and fibronectin have been described along with the LigA and LigB proteins binding mainly fibronectin (Barbosa *et al.*, 2006; Choy *et al.*, 2007; Atzinger *et al.*, 2008). More recently, the endostatin-like protein A (Len A) was shown to bind human plasminogen (Verma *et al.*, 2010), and the Len-A bound plasminogen could be converted to plasmin, which in turn degraded fibrinogen, suggesting that acquisition of host-derived plasmin by LenA may aid *Leptospira* dissemination

through host tissues. It is worth mentioning that LenA appears to be a multifunctional surface protein since it also binds the complement cascade regulator factor H and laminin.

All these adhesins are expressed in virulent leptospires and some of them only under physiologically relevant conditions of temperature, pH or osmolarity. Recently binding activity to human cell surface receptors such as proteoglycans has been reported (Breiner *et al.*, 2009). The majority of these surface proteins involved in the interactions with host components appear to be induced by environmental conditions such as temperature and the presence of serum (Patarakul *et al.*, 2010). Since kidney proximal-tubule epithelial cells produce proteoglycan GAGs motifs, this can facilitate the colonization of kidneys, especially in animal hosts. Evidence of kidney colonization by leptospires has been documented by Ristow, as biofilm formation in the proximal renal tubule lumen of *Rabbit norvegicus* (Ristow *et al.*, 2008).

2. Evading natural defences: phagocytosis

Leptospires can be isolated from the bloodstream within minutes after inoculation (Faine *et al.*, 1999) and detected in multiple organs by the third day after infection. They may reach 10^6 – 10^7 organisms per ml or g in the blood and tissue of patients and infected animals (Truccolo *et al.*, 2001). Therefore leptospires evade the host innate immune response during the initial stages of infection mainly through clearance by phagocytosis and killing by complement.

In non immune host pathogenic leptospires are slowly internalized by both neutrophils and macrophages derived from different animal species: the organisms appear to survive unless specific antibodies are present (Cinco *et al.*, 1981; Banfi *et al.*, 1982; Cinco *et al.*, 1983). One of the receptors involved in the adhesion to neutrophils, in non-opsonic condition, is the CR3 integrin (the complement receptor), via its fibronectin binding domain (Cinco *et al.*, 2002). This would confirm the capacity of leptospires to absorb fibronectin, as already documented (Merien *et al.*, 2000). Once phagocytosed by neutrophils, which play a key role in the clearance of bacteria, the intracellular killing proceeds scantily through the oxygen-dependent machinery, including mainly the effect of H_2O_2 (Murgia *et al.*, 2002). Instead, leptospires seem more susceptible to the bacte-

ricidal activity of cationic peptides of neutrophils which play an important role in oxygen-independent killing (Scocchi *et al.*, 1993; Sambri *et al.*, 2002). More in detail, cathelicin-derived peptides shared antileptospiral activity varying among the different *Leptospira* strains. Overall these studies indicate that leptospires are scarcely phagocytosed and killing occurs only in the presence of specific antibodies.

Since leptospires show a predilection for central nervous system (CNS) like other pathogenic Spirochetes, up to 25% of the patients experience mild neurological symptoms. Some studies have focused on the interactions of leptospires with microglial cells, the resident phagocytes of the CNS, known to locally mediate surveillance and defence against noxae; BV2 murine microglial cells were able to internalize but not kill pathogenic leptospires - in non-opsonic conditions. These monocytes, however, are stimulated by the leptospires - and their lipoprotein extracts - to respond with molecular signals such as p38 phosphorylation and NF- κ B activation, as well as release of cytokines and nitric oxide (Cinco *et al.*, 2006; Blasi *et al.*, 2007). As with microglial cells, interaction with monocytes, especially in immune hosts, triggers the activation of cells and the release of pro-inflammatory cytokines (Klimpel *et al.*, 2003; Vernel-Pauillac *et al.*, 2006). Tumor necrosis factor (TNF) is one of these mediators, as level of this cytokine is a predictor of poor clinical outcomes (Tajiki *et al.*, 2006): *Leptospira* components such as Peptidoglycan and LPS are able to induce TNF release (Cinco *et al.*, 1996). It is noteworthy that LPS of leptospires is unique in its ability to activate Toll-like receptor 2 (TLR2) in human cells rather than the TLR4, as the other lipopolisaccharides do (Werts *et al.*, 2001). This unusual finding has been correlated to the 1-methyl phosphate moiety that is not found in the Lipid A of other bacteria (Que, *et al.*, 2004).

3. Resistance to complement

More than resistance to phagocytosis the invasiveness of leptospires seems to be related to another innate host defence that is the complement. Complement is a major component of the innate immune system, and is involved in protection against invading microorganisms due to its opsonic, inflammatory and lytic activities (Bloom, 2009). Once activated complement can destroy

microorganisms in a few minutes, unless they inhibit the C activity by acquiring C control proteins from the host or express their own C inhibitory molecules (Rautemaa, *et al.*, 1999). Moreover, some microorganisms can prevent the access of C components or the cytolytic membrane attack complex (MAC) into the outer membrane by producing capsules or outer membrane constituents that form a protective barrier against C attack. Pioneering reports by Johnson (Johnson *et al.*, 1966; Johnson *et al.*, 1967) and our own studies on C resistance within the genus *Leptospira* (Cinco *et al.*, 1983) have demonstrated that several strains of pathogenic leptospires activated complement through the alternative pathway, but remained viable after exposure to human C, whereas the non-pathogenic leptospires were killed to various degrees. Further studies to elucidate the mechanism of C resistance pointed out that there are different levels of human serum sensitivities among leptospires: the fully and intermediate resistant strains belonging to the pathogenic ones and a fully sensitive leptospire corresponding to the non-pathogenic Patoc1 strain. Complement resistance was found to correlate to the capacity to bind the factor H and factor H related protein 1 (FHR-1), which is the main alternative complement pathway regulator by preventing binding of factor B to C3b, accelerating decay of the C3-convertase C3bBb and acting as a cofactor for the cleavage of C3b by factor I (Meri *et al.*, 2005).

Surface proteins participating in the binding of factor H and factor H-like (FHL-1) have been identified by different groups of authors they are: outer membrane protein LenA (Leptospiral endostatin-like proteinA) formerly called LfhA, LS24 (Leptospiral surface adhesin) and LenB (Verm *et al.*, 2006; Barbosa *et al.*, 2006). Interestingly the Len A protein was also shown to bind the host component laminin (Stevenson *et al.*, 2007) and to belong to a family of proteins named Len B, C, D, E and F all exhibiting affinities for fibronectin. Therefore these surface components all facilitate host invasion and colonisation. Recent findings indicate that both serum resistant and serum intermediate pathogenic leptospires are able to bind C4BP, whereas the serum sensitive strain Patoc1 is not (Barbosa *et al.*, 2009). C4BP is a Complement cascade regulator, which plays a key role in the classical pathway, by interfering with

the assembly of C3 convertase and acting as a cofactor for Factor I in the proteolytic inactivation of C4Bp (Gigli *et al.*, 1979). Surface bound C4Bp promotes factor I mediated cleavage of C4b: this interaction contributes further to complement resistance by Leptospires, acting through the classical pathway. It is worth mentioning here that C resistance emerged from these studies does not depend on either the number of passages *in vitro* nor on the virulence of the pathogenic strain; C resistance appears as an intrinsic property of leptospires been preserved after cultivation *in vitro*. Resistant strains maintain the expression of Factor H and C4BP ligands. To date only a few pathogens have been reported to recruit both factor H and C4BP, as *Neisseria gonorrhoeae*, *Streptococcus pyogenes*, *Candida albicans*, and the relapsing fever spirochetes *Borrelia recurrentis* and *Borrelia duttonii*.

LEPTOSPIRAL LIPOPROTEINS: THE KEY OF VIRULENCE

As reported before, LPS of leptospires is not endowed with major endotoxicity. By contrast, leptospiral outer membrane lipoproteins act as the main virulence factors towards host tissues. The genomes of *Leptospira interrogans* encode more lipoproteins than non-spirochetes genomes: approximately 145 genes have been detected which encode putative lipoproteins in addition to putative extracellular and outer membrane proteins (Setubal, *et al.*, 2006). Proteomics has become a feasible strategy for identifying surface-exposed proteins now that the genome sequences of some *Leptospira* species are available. One of the basis of genome information (Nascimento, *et al.*, 2004) over 260 membrane-associated proteins are predicted and subsequent studies have been developed to identify such proteins in relation to virulence, putative candidates for developing subunit vaccine and single antigen to use in serology.

Leptospiral outer membrane proteins (OMPs) are generally well conserved and would have the potential advantage of inducing comprehensive immunity and play a role in virulence. Only few transmembrane OMPs have been described: the first is the Omp1 L protein (Haake *et al.*, 2000) potentially acting as a porin; OmpL36, OmpL37, OmpL47 and OmpL54 have been recently de-

scribed as novel membrane spanning proteins, whose role has yet to be investigated (Pinne *et al.*, 2009).

Most of the OMP proteins have been characterized using Triton X-114 extracts of OMPs, biotinylated surface proteins (Cullen *et al.*, 2002; Cullen *et al.*, 2005), outer membrane vesicles (Nally *et al.*, 2005) and proteins from the outer membrane proteome of *L. interrogans*. Of the majority of the OMPs identified the function is unknown. Here we report the main lipoproteins expressed only by pathogenic leptospire and of which a role in pathogenicity was demonstrated (Figure 3).

Lip 32 represents the major component of the outer membrane proteome. *Lip 32* is highly conserved among pathogenic leptospire during acute lethal infection (Nally *et al.*, 2007) and its C terminus was found to bind laminin, collagen II, IV V, and plasma fibronectin (Hoke *et al.*, 2008). *Lip 32* is associated with Hap1 haemolysin (Lee *et al.*, 2000). However this protein is not a prerequisite for virulence since mutant *Lip 32* deficient strain still retains its virulence in experimental animals (Murray *et al.*, 2009).

Loa22. To date, the only OmpA-like protein which behaves like a true virulence factor is *Loa22*. This surface protein elicits an immune response in human patients (Nally *et al.*, 2007), is upregulated during acute infection and slightly binds to ECM. When the *loa22* gene is disrupted in *L. interrogans* by Himar 1 insertion, there is a complete loss of virulence in the guinea pig disease model (Ristow *et al.*, 2007). Therefore this protein completely fulfils Koch's postulates for virulence.

LenA, *LenB*, *Len C*, *Len D*, *Len E* and *Len F* are proteins evolved in invasion and colonization; *lenA* was firstly described as *LfhA* and *Lsa24* by two groups (Werma *et al.*, 2006 Barbosa *et al.*, 2006), and was found to bind human factor H, FHR-1 and laminin. Recent studies have revealed that this protein binds to human plasminogen, which may aid bacterial dissemination through host tissue (Verma *et al.*, 2010). *Len B*, *C*, *D*, *E*, *F*, are the products of 5 additional genes homologous to *LfhA/Lsa24*. They were found to bind Factor H (*LenB*), laminin, and fibronectin. All six genes encode domains predicted to bear structural and functional similarities with mammalian endostatin.

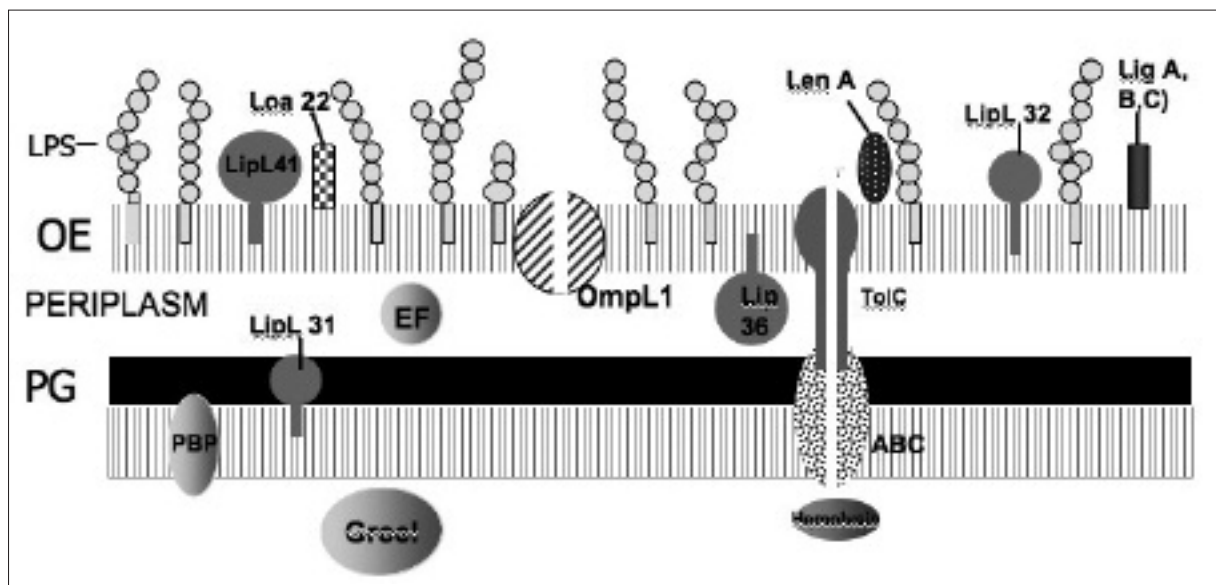


FIGURE 3 - Proteins and Lipoproteins in leptospiral membranes. IM = inner membrane; PG = peptidoglycan closely associated with IM; OE = outer membrane; LPS = lipopolysaccharide. Subsurface proteins include GroEl, the periplasmic flagellum EF, lipoprotein LpL31, penicillin binding proteins PBP. The OE contains the transmembrane proteins including porin OmpL1. Type 1 efflux system is represented by TolC transmembrane protein, forming a complex with the ATP binding cassette transporter ABC to export cytoplasmic component such as hemolysin (from Nascimento *et al.*, 2004; Ko *et al.* 2009). LipL41, LipL36, LipL32, Len A, Lig A,B,C, are the surface exposed proteins mentioned in the text.

LigA,B,C, (Leptospiral Immunoglobulin-Like Proteins), members of the bacterial immunoglobulin-like protein super family, mediate interactions with host cell such as invasion and cell attachment in other bacteria. They were found exclusively in pathogenic leptospires (Choy, *et al.*, 2007).

Lig proteins are anchored to the outer membrane and have 12 to 13 tandem bacterial immunoglobulin-like repeated domains. They strongly adhere to ECM, including fibronectin, fibrinogen, collagen and laminin (Lin *et al.*, 2007). The *lig* genes are upregulated under physiological osmolarity and encode surface proteins which are recognized by sera of patients with leptospirosis (Matsunaga *et al.*, 2007; Croda *et al.*, 2007). They confer a high level of cross-protection approaching 100% in mice (Kaizumi *et al.*, 2004). Though appearing putative virulence factors, mutants at LigB and LigA genes do not affect the capacity of the organisms to cause acute infection in experimental animals. Therefore other important adhesins participate in *Leptospira* adhesion in cooperation with Lig-proteins.

CONCLUSIONS

During the past 10 years a number of studies have aimed to clarify the virulence factors of leptospires on the basis of known genomic sequences of some serovars of *L. interrogans* and one serovar of *L. biflexa*. Most of these studies also compared the proteome similarities between pathogenic and saprophytic leptospires, detecting a number of proteins present only in the pathogenic, virulent serovars.

To date, only one lipoprotein appears to be a true virulence factor among the other candidates. Difficulties arise from the need to ascertain not only the presence of putative virulence proteins, but also their expression during infection. Some authors carried out studies mimicking in vivo conditions (iron limitations and serum presence), detecting the up regulation of 5 novel proteins, in additions to the well known Loa22, which are new putative virulence factors to study (Eshghi *et al.*, 2009). Therefore recent approaches aim to identify which proteins - among those considered putative virulence factors - present only on pathogenic leptospires are expressed during acute in-

fection or in conditions which mimic the natural infection: that will be a hard task.

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