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Genetic diversity of the Leptospiral immunoglobulin-like (Lig) genes in pathogenic *Leptospira* spp.

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ABSTRACT

Recent serologic, immunoprotection, and pathogenesis studies identified the Lig proteins as key virulence determinants in interactions of leptospiral pathogens with the mammalian host. We examined the sequence variation and recombination patterns of *ligA*, *ligB*, and *ligC* among 10 pathogenic strains from five *Leptospira* species. All strains were found to have intact *ligB* genes and genetic drift accounting for most of the *ligB* genetic diversity observed. The *ligA* gene was found exclusively in *L. interrogans* and *L. kirschneri* strains, and was created from *ligB* by a two-step partial gene duplication process. The aminoterminal domain of LigB and the LigA paralog were essentially identical (98.5 ± 0.8% mean identity) in strains with both genes. Like *ligB*, *ligC* gene variation also followed phylogenetic patterns, suggesting an early gene duplication event. However, *ligC* is a pseudogene in several strains, suggesting that LigC is not essential for virulence. Two *ligB* genes and one *ligC* gene had mosaic compositions and evidence for recombination events between related *Leptospira* species was also found for some *ligA* genes. In conclusion, the results presented here indicate that Lig diversity has important ramifications for the selection of Lig polypeptides for use in diagnosis and as vaccine candidates. This sequence information will aid the identification of highly conserved regions within the Lig proteins and improve upon the performance characteristics of the Lig proteins in diagnostic assays and in subunit vaccine formulations with the potential to confer heterologous protection.

1. Introduction

Pathogenic spirochaetes belonging to the genus *Leptospira* are the agents of leptospirosis, which is considered to be the most widespread zoonosis in the world (Faine et al., 1999; Levett, 2001; Bharti et al., 2003). Susceptible animals, including humans, are infected by direct contact with urine from a reservoir host, usually rats or other rodents, or indirectly through contaminated water. Transmission occurs via dermal abrasions or inoculation of the mucous or conjunctival membranes (Faine et al., 1999). In the majority of infected individuals, leptospirosis is a self-limited disease characterized by flu-like symptoms (Faine et al., 1999). However, hepatorenal manifestations, as observed in Weil's disease, are frequent complications and are associated with significant (10–15%) mortality (Bharti et al., 2003; McBride et al., 2005). In addition, leptospirosis causes severe pulmonary haemorrhage syndrome (SPHS), for which case fatality is >50% (Segura et al., 2005; Gouveia et al., 2008). Leptospirosis is considered to be an emerging infectious disease in endemic regions of Asia (Karande et al., 2003, 2005; LaRocque et al., 2005; Yanagihara et al., 2007; Peacock and Newton, 2008) and Latin America (Ko et al., 1999; Sarkar et al., 2002; Romero et al., 2003; Johnson et al., 2004) and is a major public health concern in

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poverty stricken regions of the world (McBride et al., 2005; Ganoza et al., 2006; Riley et al., 2007).

The *Leptospira* genus is sub-classified into 18 genomospecies that includes both saprophytic and pathogenic species (Levett, 2001; Levett et al., 2006; Matthias et al., 2008). Classification based on serologic methods has identified ~300 serovars, of which more than 200 are considered to be pathogenic (Faine et al., 1999; Levett, 2001; Bharti et al., 2003). The availability of genomic sequence data from five *Leptospira* strains, *L. interrogans* serovars Lai (Ren et al., 2003) and Copenhageni (Nascimento et al., 2004), *L. borgpetersenii* serovar Hardjo strains L550 and JB197 (Bulach et al., 2006), and the saprophyte *L. biflexa* serovar Patoc I (Picardeau et al., 2008), is driving the discovery of new diagnostic tools and vaccines for leptospirosis. Considerable effort has been expended towards identifying conserved surface-exposed antigenic determinants that could improve diagnosis and provide heterologous protection via subunit or DNA vaccines.

A number of leptospiral outer membrane proteins (OMPs) have been characterized (Cullen et al., 2005), including OmpL1 (Haake et al., 1993), LipL41 (Shang et al., 1996), LipL36 (Haake et al., 1998), the major outer membrane protein, LipL32 (Haake et al., 2000), LipL21 (Cullen et al., 2003), LipL46 (Matsunaga et al., 2006), LenA (Verma et al., 2006), and the OmpA-like proteins Loa22 (Koizumi and Watanabe, 2003) and Omp52 (Hsieh et al., 2005). However, their performance in diagnostic assays for acute leptospirosis or as vaccine candidates has been problematic (Haake et al., 1999; Branger et al., 2001; Flannery et al., 2001; Guerreiro et al., 2001). LigA and LigB, belonging to a family of leptospiral immunoglobulin-like (Lig) proteins, appear to be promising antigens (Palaniappan et al., 2002; Matsunaga et al., 2003). The gene encoding a third Lig protein, *ligC*, was identified as a pseudogene in *L. interrogans* serovar Copenhageni and *L. kirschneri* serovar Grippotyphosa (Matsunaga et al., 2003), but was found to be intact in *L. interrogans* serovar Lai (Ren et al., 2003). The Lig proteins contain a series of bacterial immunoglobulin-like (Big) repeat domains that were originally identified in virulence determinants from *Escherichia coli* and *Yersina pseudotuberculosis* (Hamburger et al., 1999; Luo et al., 2000).

The *lig* genes are of great interest because emerging serologic, vaccine, and pathogenesis studies indicate that Lig proteins are key virulence determinants involved in host–pathogen interactions. Lig proteins mediate interaction with multiple host extracellular matrix proteins, including fibronectin, fibrinogen, collagen, and laminin (Choy et al., 2007). Several studies have provided evidence that the Lig proteins are protective immunogens in animal models of leptospirosis (Koizumi and Watanabe, 2004; Palaniappan et al., 2006; Silva et al., 2007). In addition, we recently demonstrated that a recombinant polypeptide containing Big domains 2–6 from LigB was able to protect hamsters against homologous challenge by *L. interrogans* serovar Copenhageni (unpublished data). Virulent forms of *L. interrogans* and *L. kirschneri* strains express higher levels of Lig proteins than culture-attenuated forms (Matsunaga et al., 2003). Lig expression is strongly induced by shifting the osmolarity from low levels used in EMJH culture medium to osmolarity levels found in host tissues (Matsunaga et al., 2005). Up-regulation during early host infection is consistent with the strong serologic response to Lig proteins observed during acute leptospirosis (Croda et al., 2007).

Considering the large number of pathogenic *Leptospira* serovars and the broad distribution of leptospiral host reservoirs, the potential effect of selective pressure on the genetic diversity of the Lig proteins was unclear. Given the potential of the Lig proteins as diagnostic antigens and vaccine candidates, we examined their sequence diversity in the serovars most often associated with leptospirosis.

2. Materials and methods

2.1. Leptospira strains and culture conditions

Virulent leptospiral strains (Table 1) were obtained from culture collections maintained by the authors. The isolation conditions of a number of the strains used in this study were previously described (Ko et al., 1999; Haake et al., 2002; Silva et al., 2008). The identity of each of the strains used in this study was confirmed by 16S rRNA gene sequencing (Hookey et al., 1993) and serogrouping based on the microscopic agglutination test (MAT) (Cole et al., 1973). Strains were cultured in liquid Ellinghausen–McCullough–Johnson–Harris modified tween 80-bovine albumin medium (Ellinghausen and McCullough, 1965; Johnson and Harris,

Table 1
Leptospira strains, the status of their *lig* genes and level of identity compared to that of serovar Copenhageni.

Leptospira species	Serogroup	Serovar	Strain	*lig* gene status (PCR/SB/Seq)			% *lig* DNA sequence identity vs. Copenhageni		
				ligA	*ligB*	*ligC*	*ligA*	*ligB*	*ligC*
L. borgpetersenii		Hardjo	L550[a]	ND/ND/−	+/ND/+	ND/ND/−	NA	68.7	NA
		Hardjo	JB197[a]	ND/ND/−	+/ND/+	ND/ND/−	NA	67.9	NA
L. interrogans	Icterohaemorrhagiae	Copenhageni	Fiocruz L1-130[b]	+/+/+	+/+/+	+/+/+	100	100	100
	Icterohaemorrhagiae	Lai	56601[c]	−/ND/−	+/+/+	+/+/+	NA	97.1	99.5
	Canicola	ND	Kito[d]	+/+/+	+/+/+	+/ND/+	90.3	96.7	98.5
	Pomona	Pomona	Kennewicki Cornell[e]	+/ND/+	+/+/+	+/ND/+	90.1	97.1	99.5
	Pomona	Pomona	Kennewicki PO-06-047[f]	+/+/+	+/+/+	+/ND/+	90.3	96.7	98.8
L. kirschneri	Grippotyphosa	Grippotyphosa	RM52[g]	+/+/+	+/+/+	+/+/+	91.4	93.2	90.5
L. noguchii	Bataviae	ND	Cascata[h]	−/−/−	+/+/+	−/ND/−	NA	74.3	NA
L. weilii	Hebdomadis	ND	Eco-Challenge[i]	−/−/−	+/+/+	+/ND/+	NA	69.6	78

PCR: PCR screening assay; SB: Southern blot; Seq: gene sequenced; ND: not determined; NA: not applicable.
[a] Isolated from a chronically infected bovine, Australia, genome sequenced (Bulach et al., 2006).
[b] Isolated from a patient during a leptospirosis epidemic, Brazil (Ko et al., 1999), genome sequenced (Nascimento et al., 2004).
[c] Causative agent of rural leptospirosis in China, genome sequenced (Ren et al., 2003).
[d] Isolated from an infected canine, Brazil (Silva et al., 2008).
[e] Isolated from an aborted equine foetus, USA (Palaniappan et al., 2002).
[f] Isolated from an aborted swine foetus, USA.
[g] Isolated from an infected swine, USA (Shang et al., 1996).
[h] Isolated from a patient, Brazil (Silva et al., 2008).
[i] Isolated from a triathlete diagnosed with leptospirosis, Malaysia (Haake et al., 2002).