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Multifunctional and Redundant Roles of *Leptospira interrogans* Proteins in Bacterial-Adhesion and fibrin clotting inhibition

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A R T I C L E I N F O

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A B S T R A C T

Pathogenic *Leptopira* is the etiological agent of leptospirosis, the most widespread zoonotic infection in the world. The disease represents a major public health problem, especially in tropical countries. The present work focused on two hypothetical proteins of unknown function, encoded by the genes LIC13059 and LIC10879, and predicted to be surface-exposed proteins. The genes were cloned and the proteins expressed using *E. coli* as a host system. We report that the recombinant proteins interacted with extracellular matrix (ECM) laminin, in a dose-dependent fashion and are novel potential adhesins. The recombinant proteins were called Lsa25.6 (rLIC13059) and Lsa16 (rLIC10879), for Leptospiral surface adhesins, followed by the respective molecular masses. The proteins attached to plasminogen (PLG), generating plasmin, in the presence of PLG-activator uPA. Both proteins bind to fibrinogen (Fg), but only Lsa25.6 inhibited fibrin clotting by thrombin-catalyzed reaction. Moreover, Lsa16 interacts with the mammalian cell receptor E-cadherin, and could contribute to bacterial attachment to epithelial cells. The proteins were recognized by confirmed leptospirosis serum samples, suggesting that they are expressed during infection. The corresponding leptospiral proteins are surface exposed based on proteinase K accessibility assay, being LIC10879 most probably exposed in its dimer form. The data of this study extend the spectrum of surface-exposed proteins of *L. interrogans* and indicate a possible role of the originally annotated hypothetical proteins in infection processes.

1. Introduction

Leptospirosis is an emerging globally spread zoonosis, resulting in a systemic infection with high incidence and mortality rates in tropical and developing countries. The etiological agent of leptospirosis is pathogenic spirochete of the genus *Leptospira*. There are more than 200 serovars of *Leptospira* distributed among both pathogenic and non-pathogenic species. Transmission of pathogenic leptospires among mammalian hosts typically involves dissemination via soil or water contaminated by the urine of carrier animals. Leptospires penetrate through mucous membranes and cuts or abrasions on the skin (Bharti et al., 2003; Faine et al., 1999).

Although many studies have been performed focusing on leptospiral pathogenesis, the mechanisms involved are poorly elucidated. It is known that the adhesion of pathogenic *Leptospira* to host tissue components is an essential step for infection. Our group has demonstrated the attachment of virulent *Leptospira* to extracellular matrix (ECM) components and several leptospiral surface proteins described as possible mediators for these interactions (Barbosa et al., 2006; Fernandes et al., 2016, 2014; Teixeira et al., 2015; Vieira et al., 2014). Furthermore, it has been shown that pathogenic *Leptospira* have the ability to bind *in vitro* to a variety of cell lines (Ballard et al., 1986; Cinco et al.,

Abbreviations: ECM, extracellular matrix; PLG, plasminogen; Fg, fibrinogen; Lsa, leptospiral surface adhesins; ELISA, enzyme-linked immunosorbent assay; EMJH, culture medium Ellinghausen-McCullough/Johnson-Haris; uPA, plasminogen activator of the urokinase type; CDS, coding sequence; OD, optical density; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; OPD, o-phenylenediamine; PK, proteinase K; IPTG, isopropyl-β-D thiogalactopyranoside; LB, Luria-Bertani; PMSF, phenylmethylsulfonyl fluoride; CD, circular dichroism; PBS-T, phosphate-buffered saline Tween 20; MAT, microscopic agglutination test; ACA, aminocaproic acid; BSA, bovine serum albumin; CAM, cell adhesion molecule; LIC, *Leptospira interrogans* serovar Copenhageni genome nomenclature

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Table 1
Features of the coding sequences LIC13059 and LIC10879 employed in these studies, primer sequences, restriction cloning sites and molecular mass.

Gene Locus	Accession Number	Recombinant protein (given name)	Sequence of primers for PCR amplification	Recombinant protein molecular mass (kDa)
LIC13059* LIC_RS15745	**WP_000672495.1**	Lsa25.6	F:5′CTCGAGTGCAAAGATGATAGA *XhoI* ATTAAAATAA 3′ R: 5′AAGCTTTTAATTTACTGGAA *HindIII* AAAGCATTTGA 3′	25.6
LIC10879* LIC_RS04545	**WP_000827840**	Lsa16	F:5′CTCGAGCATAAAAATTGTGAT *XhoI* CCTTCCTTA 3′ R:5′AAGCTTTTACTTTTTTAACTCA *HindIII* GGCGCAAG 3′	16

* LIC: *Leptospira interrogans* serovar Copenhageni genome nomenclature.

2006; Evangelista et al., 2014a, 2014b; Martinez-Lopez et al., 2010; Merien et al., 1998, 1997; Robbins et al., 2015; Zhang et al., 2012).

After adhesion, pathogenic leptospires are expected to have the competence to penetrate, disseminate and reach mammalian host tissues of target organs. In this sense, we have reported the ability of *Leptospira* to recruit PLG from human plasma, generating plasmin, capable to degrading laminin, fibronecting, IgG and C3b, contributing to host tissue penetration and immune evasion (Vieira et al., 2013, 2011, 2009; Vieira and Nascimento, 2016). Moreover, binding of leptospires to human Fg resulted in inhibition of fibrin clotting by thrombin-catalyzed reaction, a mechanism that could facilitate bleeding, helping bacteria dissemination (Choy et al., 2011; Oliveira et al., 2013). To handle all these interactions, several recombinant proteins of *Leptospira* have been isolated and their ability to act as Fg- and PLG- binding proteins demonstrated (Oliveira et al., 2013; Silva et al., 2016; Siqueira et al., 2016; Vieira et al., 2010).

In the present work, we have focused on two hypothetical proteins of unknown function, encoded by the genes LIC13059 and LIC10879, and predicted to be surface-exposed proteins. The genes were cloned and the proteins expressed using *E. coli* as a host system. We report that the recombinant proteins are novel potential surface exposed adhesins, called Lsa25.6 (rLIC13059) and Lsa16 (rLIC10879), for Leptospiral surface adhesins, followed by the respective molecular masses. The proteins attach to PLG and Fg, but only Lsa25.6 inhibited fibrin clotting by thrombin-catalyzed reaction. Moreover, Lsa16 interacted with the mammalian cell receptor E-cadherin, and could contribute to bacterial attachment to epithelial cells. This study also describes the binding characterization of the putative adhesins and provides evidence of their expression during leptospirosis.

2. Material and methods

2.1. Biological components

Collagen, laminin, plasma and cellular fibronectin, elastin, e-cadherin (recombinant), fibrinogen and the control protein BSA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Laminin-1 and collagen type IV were derived from the basement membrane of Engelbreth-Holm-Swarm mouse sarcoma; cellular fibronectin was derived from human foreskin fibroblasts; elastin was derived from human aorta, and collagen type I was isolated from rat-tail. Native Plasminogen, purified from human plasma, and factor H were purchased from EMD chemicals (San Diego, CA, USA). C4BP, isolated from normal human serum, was purchased from Complement Technology (Tyler, TX, USA).

2.2. Bacterial strains and serum samples

The non-pathogenic *L. biflexa* (serovar Patoc strain Patoc 1), the pathogenic attenuated *L. interrogans* (serovars Canicola strain Hond Utrecht IV, Copenhageni strain M20, Hardjo strain Hardjoprajitno, Icterohaemorrhagiae strain RGA), *L. kirschneri* (serovars Cynopteri strain 3522C, Grippotyphosa strain Moskva V), *L. borgpetersenii* (serovar Whitticombi strain Whitticombi), *L. noguchii* (serovar Panama strain CZ214), *L. santarosai* (serovar Shermani strain 1342 K) were cultured 28 °C under aerobic conditions in liquid EMJH medium containing asparagine (0.015% w/v), sodium pyruvate (0.001% w/v) calcium chloride (0.001% w/v), magnesium chloride (0.001% w/v), peptone (0.03% w/v) and meat extract (0.02% w/v) (Meri et al., 2005). Leptospira cultures are maintained in the Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brazil. Human serum samples from patients with confirmed leptospirosis were from the Serum Collection of the Instituto Adolfo Lutz, São Paulo, Brazil, and were donated for research purposes only. *Escherichia coli* DH5a and *E. coli* BL21(DE3) Star pLysS (Mendes et al., 2011) were used as cloning and recombinant protein expression hosts, respectively.

2.3. In silico analysis of the coding sequence LIC13059 and LIC10879

Predicted coding sequence (CDS) LIC13059 (LIC_RS15745) and LIC10879 (LIC_RS04545) were selected from *L. interrogans* serovar Copenhageni genome sequences (Costa et al., 2015; Kanagavel et al., 2014) based on its cellular localization prediction by PSORT, http://psort.hgc.jp/form.html (Miller et al., 2011) and CELLO, http://cello.life.nctu.edu.tw/ (Croda et al., 2008). The signal peptide sequence was assessed by SignalP, http://cbs.dtu.dk/services/SignalP-3.0/ (Minke et al., 2009). The Smart, http://smart.embl-heidelberg.de/ (Hauk et al., 2008; Miyaki et al., 2010), PFAM, http://pfam.xfam.org/ (Hauk et al., 2011), and LipoP, http://www.cbs.dtu.dk/services/LipoP/ (Croda et al., 2007), web servers were used to search for predicted functional and structural domains within the amino acid sequence.

2.4. Cloning and expression of recombinant proteins in E. coli

The genes LIC13059 and LIC10879 without the signal peptide were amplified from the genomic DNA of *L. interrogans* serovar Copenhageni by PCR with specific primers (Table 1). The PCR amplified products were cloned into pGEM-T easy vector (Promega) and positive clones were selected by DNA restriction analysis, PCR of colonies and further confirmed by nucleotide sequencing analysis. After confirmation of the sequences, the DNA inserts were removed by digestion with the specific restriction enzyme and ligated into the protein expression pAE vector (Cullen et al., 2003), previously digested with the same enzymes. This plasmid includes a 6XHis sequence tag at the N-terminus of recombinant proteins. The plasmids pAE-LIC13059 and pAE-LIC10879, confirmed by sequencing, were employed to transform BL21 (DE3) Star pLysS expression host cells. *E. coli* BL21 (DE3) Star pLysS cells containing the construction were grown at 37 °C in Luria-Bertani (LB) broth with 100 µg/ml ampicillin and 34 µg/ml chloramphenicol. The bacterial growth was achieved by continuous shaking until an optical

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