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Adhesin activity of *Leptospira interrogans* lipoprotein identified by *in vivo* and *in vitro* shotgun phage display

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ABSTRACT

Leptospira interrogans causes leptospirosis, one of the most common zoonotic diseases in the world. This pathogenic spirochete is able to bind to extracellular matrix, to express virulent factors and to cause host death. Until now, there is no effective human vaccine for the disease. Shotgun phage display genomic libraries of *L. interrogans* were constructed and used for *in vivo* biopanning in hamsters and screened for ligands able to bind to LLC-PK1 epithelial cells. In both panning procedures, clones coding for the putative lipoprotein LIC12976 were identified and, in order to confirm its adhesin activity, a recombinant protein was produced in *Escherichia coli* and showed to interact with A31 fibroblasts, LLC-PK1 and Vero epithelial cells *in vitro*. Moreover, rLIC12976 was shown to bind to laminin, indicating an adhesin function. This protein was also detected in extracts of *L. interrogans* from different serovars and it was found to be conserved among pathogenic leptospires. Further, the protein was tested as vaccine candidate and immunization of hamsters with LIC12976 did not confer protection against a lethal challenge with the homologous *L. interrogans* serovar Copenhageni. Nevertheless, LIC12976 seems to act as an adhesin, and may be important for the host–pathogen interaction, so that its study can contribute to the understanding of the virulence mechanisms in pathogenic leptospires.

1. Introduction

Leptospira is the etiologic agent of leptospirosis, a zoonosis recognized as an emerging infectious disease that is extremely spread throughout the world [1,2]. The disease transmission, directly or indirectly from animals to humans, is through cuts and abrasions in the skin or via the conjunctiva, and may also occur in intact skin after prolonged exposure to water contaminated with urine of infected animals [1].

The knowledge on the mechanism of pathogenicity of *Leptospira* is very limited [1,3]. Nowadays, the genomes of nine strains of the genus *Leptospira* were sequenced: *L. interrogans* serovar Lai [4], *L. interrogans* strain Fiocruz L1–130 serovar Copenhageni [5], JB197 and L550 strains from *Leptospira borgpetersenii* serovar Hardjo [6], *Leptospira santorosai* serovar Shermani [7], VAR010 and MMD0835 strains from *Leptospira licerasiae* serovar Varillal [8] and *Leptospira biflexa* serovar Patoc strains Paris and Ames [9]. Comparative genomic studies can provide knowledge about mechanisms of pathogenicity and thus help to establish strategies to control the disease and to identify potential candidates for drugs or vaccine development [2,5].

Bacteria interacting with eukaryotic hosts express adhesive molecules on their surfaces that mediate adhesion to a receptor on the target cell surface or with soluble macromolecules and what can head the pathogen to a specific location in the host [10]. Pathogenic bacteria utilize numerous mechanisms to cause disease in humans. The first step in host–pathogen interaction and tissue colonization is the adherence to the surface of the cell or to extracellular matrix proteins. Once attached, pathogens are able to initiate specific biochemical processes that include proliferation, secretion of toxins, immune evasion responses and cell invasion, culminating in the appearance of disease symptoms [11].

Jacobsson and Frykberg [12] were pioneers to identify adhesins using the shotgun phage display. In this technique, the phage library is constructed from random fragments from genomic DNA. Shotgun phage display has identified a number of genes coding for adhesins involved in host-pathogen interactions [13–15].

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In the present study, a new adhesin, LIC12976, was identified through *in vivo* and *in vitro* screening a shotgun phage display library constructed using genomic DNA from *L. interrogans* serovar Copenhageni. In agreement, we showed that the recombinant protein rLIC12976 is able to bind to mammalian cells, including kidney epithelial cells and laminin.

2. Materials and methods

2.1. Microorganisms and culture condition

L. interrogans serovar Copenhageni Fiocruz L1–130 was obtained from ATCC catalog BAA-1198. It was cultivated as described [16]. Virulence was maintained by iterative passages in Golden Syrian hamsters.

2.2. Library construction, biopanning and sequence analysis

The libraries construction and the *in vivo* biopanning were done as described [17–19]. Briefly, hamsters were anesthetized intraperitoneally with 2.5% Avertin [20] and the thoracic cavity was opened in order to expose the heart. 1×10^9 phages/mL (200 μL/animal) from the library were injected directly into the animal heart. After 5 min of phage circulation, the animal was perfused and the phages were recovered by removing the kidneys. The panning procedure was repeated for four rounds. This study has the approval of the Ethics Committee for Animal Use from Instituto Butantan, protocol number 564/08. The *in vitro* biopanning was performed according the BRASIL methodology [21] with modifications. Briefly, 1×10^{12} phages/mL (100 μL/tube) were incubated with 1×10^6 pig kidney epithelial LLC-PK1 cells. After centrifugation, the phages in the precipitate were eluted by low pH, neutralized with Tris–HCl pH 8.0, centrifuged after the addition of 1% BSA-DMEM to remove cellular debris and the supernatant was again incubated with LLC-PK1 cells. After panning procedures, clones of each round were analyzed as described [18].

2.3. Cloning, expression, purification of rLIC12976 and antisera production

Leptospira's genomic DNAs were extracted using DNAzol Reagent (Invitrogen). The gene *lic12976* was amplified by PCR using specific primers forward 5′ TAGG**CTCGAG**GAACGTCATTCTCTTC 3′ and reverse 5′ ACC**AAGCTT**CTAAAAACGTTCCGAATT 3′ containing *Xho* I and *Hin*d III restriction sites (bold). The amplified product from *L. interrogans* serovar Copenhageni genomic DNA was cloned into the pGEM-T easy vector (Promega) and subcloned into the pAE vector [22], as described [18]. *E. coli* BL21 Star (DE3) pLysS was used for protein expression overnight at 20 °C and the purification of the recombinant protein was performed as described [23]. The presence of the recombinant protein was evaluated by 12% SDS–PAGE, the fractions of interest were dialysed against PBS and the protein was quantified by Bradford (Bio-Rad). The antisera against rLIC12976 and immunoblot assay were performed as described [23,24], using BSA 2% in PBST during incubation with antiserum.

2.4. Bioinformatic analysis

The PSORT program (http://psort.nibb.ac.jp/) was used to predict the sub cellular localization of the protein. Public and custom sequence-specific search algorithms were used for identification of sequence motifs including signal peptides, lipoprotein cleavages sites and transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM) and (http://www.cbs.dtu.dk/services/SignalP), as described [25].

2.5. Binding of rLIC12976 to mammalian cells and extracellular matrix components

A31 (mouse BALB/c3T3 clone A31 fibroblast), Vero (african green monkey kidney epithelial cell – *Cercopithecus aethiops*) and LLC-PK1 (pig kidney epithelial cell – *Sus scrofa*) cells were cultivated and fixed as described [18]. The binding procedure was performed according [16] with modifications. Briefly, Nunc MaxiSorp microtiter plates were coated with 1.0 μg of plasma fibronectin, fibrinogen, BSA, laminin, cellular fibronectin and monosialoganglioside GM1 (all purchased from Sigma–Aldrich) and were incubated at 4 °C overnight and washed three times with PBST (0.1% Tween). The cells and the ECM proteins were blocked with 200 μL of 1% BSA in PBST and 10% BSA in PBST, respectively. The incubation were proceed at 37 °C for 1 h and then at 4 °C for 16 h. After three washes with PBST, 1.0 μg of rLIC12976 protein was diluted in PBS, incubated for 1 h and 30 min at 37 °C and washed six times with PBST. The detection was performed by incubation with polyclonal antisera anti-LIC12976 1:5000 dilution for 1 h at 37 °C followed by three washes with PBST and incubation with anti-mouse IgG peroxidase conjugate 1:10,000 dilution. After three washes with PBST, the plate was developed as described [23]. For competition experiments, rLIC12976 was incubated with laminin (0, 0.5, 1.0 and 5.0 μg) for 1 h and 30 min before the incubation with the cells.

2.6. Immunization of hamsters and leptospiral challenge

All procedures were approved by the Committee for the Use of Experimental Animals of the Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil. Immunization and challenge were done as described [26].

3. Results

3.1. LIC12976 was selected by in vivo and in vitro biopanning and it is conserved in pathogenic leptospiral genomes

After four rounds of *in vivo* biopanning through hamster perfusion combined with the recovering of the phages from kidneys in parallel with *in vitro* biopanning against kidney epithelial LLC-PK1 cells, two and one, respectively, phage clones in frame coding for a partial sequence of LIC12976 were selected. LIC12976 encodes a putative lipoprotein with 47.7 kDa of molecular mass corresponding to 418 amino acids residues and pI 8.12. This protein possesses a signal peptide, but functionally motifs besides the lipobox, were not identified (Supplementary Fig. 1). A PCR using primers designed on *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 DNA sequence was used to investigate the presence of this gene in other *Leptospira* serovars. Indeed, this gene was amplified in all pathogenic serovars assayed of the species *L. interrogans*, comprising a fragment of approximately 1.2 kb, but not in the saprophytic *L. biflexa* serovar Patoc (Fig. 1), confirming that *lic12976* is conserved in pathogenic leptospires species. The recombinant LIC12976 (rLIC12976) with 45.8 kDa corresponding to amino acid 29–418 was expressed and purified.

3.2. rLIC12976 is expressed in pathogenic Leptospira serovars

The polyclonal antiserum against rLIC12976 was able to specifically recognize the recombinant protein (Fig. 2A) but not other *L. interrogans* recombinant proteins. Immunoblot analysis were also performed on a panel of *Leptospira* serovar extracts and the antiserum against rLIC12976 reacted with a band corresponding to a molecular mass of LIC12976 in all pathogenic serovar samples