Journal of Microbiology, Immunology and Infection (2020) **53**, 163–175

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jmii.com

Original Article

Identification of a novel protein in the genome sequences of *Leptospira interrogans* with the ability to interact with host's components

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Received 22 June 2018; accepted 24 December 2018
Available online 22 January 2019

KEYWORDS
Recombinant protein;
Leptospira;
Laminin;
Plasminogen;
Host-pathogen interaction

Abstract *Background:* Leptospirosis is an infectious disease that affects humans and animals worldwide. The etiological agents of this disease are the pathogenic species of the genus *Leptospira*. The mechanisms involved in the leptospiral pathogenesis are not full understood. The elucidation of novel mediators of host-pathogen interaction is important in the detection of virulence factors involved in the pathogenesis of leptospirosis.
Objective: This work focused on identification and characterization of a hypothetical protein of *Leptospira* encoded by the gene LIC10920.
Methods: The protein of unknown function was predicted to be surface exposed. Therefore, the LIC10920 gene was cloned and the protein expressed in *Escherichia coli* BL21 (DE3) Star pLysS strain. The recombinant protein was purified by metal affinity chromatography and evaluated with leptospirosis human serum samples. The interaction with host components was also performed.
Results: The recombinant protein was recognized by antibodies present in leptopsirosis human serum, suggesting its expression during infection. Immunofluorescence and intact bacteria assays indicated that the bacterial protein is surface-exposed. The recombinant protein interacted with

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https://doi.org/10.1016/j.jmii.2018.12.012

human laminin, in a dose-dependent and saturable manner and was named Lsa24.9, for Leptospiral surface adhesin, followed by its molecular mass. Lsa24.9 also binds plasminogen (PLG) in a dose-dependent and saturable fashion, fulfilling receptor ligand interaction. Moreover, Lsa24.9 has the ability to acquire PLG from normal human serum, exhibiting similar profile as observed with the human purified component. PLG bound Lsa24.9 was able of generating plasmin, which could increase the proteolytic power of the bacteria.

Conclusions: This novel leptospiral protein may function as an adhesin at the colonization steps and may help the invasion process by plasmin generation at the bacterial cell surface.

Introduction

Leptospirosis is an infectious disease caused by pathogenic species of the genus *Leptospira* that affects humans and animals.[1] Leptospires are highly motile spirochetes that penetrate abraded skin and mucous membranes, enter the bloodstream, and disseminate throughout the body, resulting in a systemic infection.[2] Leptospirosis clinical symptoms vary from mild flu-like to severe complications with multiple-organs failure, generating a high cost for the public health.[3,4]

The mechanisms involved in leptospiral pathogenesis are not well elucidated. Nevertheless, it is generally thought that to establish the initial step of infection pathogenic bacteria have to adhere to host tissues by interacting with surface molecules such as extracellular matrix components (ECM). Several of these leptospiral molecules, which are called adhesins have been reported to bind different ECM components *in vitro*[5,6]. Furthermore, some leptospiral adhesins exhibit multifunctional properties and show binding capacities with others host components, as PLG, fibrinogen, plasma fibronectin, C4BP, factor H and vitronectin.[7–11]

Proteolytic activity has been demonstrated to be important during the penetration of several pathogenic bacteria. After adherence, pathogenic bacteria have to overcome barriers imposed by epithelial tissues, and reach secondary sites of infection within the hosts [12–16]. The capacity of leptospires to interact with PLG on the surface and to be converted into plasmin by host activators has been previously demonstrated by our group.[17,18] Then, several PLG binding proteins were characterized and identified on the leptospires surface.[6,19–22] Moreover, plasmin generated in the presence of leptospiral proteins cleaves fibrinogen and human complement proteins, facilitating tissue penetration and immune evasion.[23–25]

Identification of new leptospiral proteins involved in bacteria virulence is important to understand the mechanisms of the disease and consequently, the identification of vaccine targets. In this work, we identified a probable lipoprotein encoded by the gene LIC10920 of *Leptospira interrogans* serovar Copenhageni. The recombinant protein was evaluated for their cellular location, reactivity with human leptospirosis serum samples and their ability to bind human host components. LIC10920 recombinant protein was capable of adhering to laminin and to interact with PLG generating plasmin. Our data suggest that this interaction could facilitate *Leptospira* in the host-penetration process.

Material and methods

ECM and biological components

Laminin, collagen, plasma and cellular fibronectin, elastin, vitronectin, fetuin and BSA were purchased (Sigma - 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; plasma fibronectin, vitronectin, and human complement serum were isolated from human plasma; elastin was derived from human aorta and collagen type I was isolated from rat tail. PLG was purified from plasma human and factor H were purchased from EMD Chemicals, Inc. (San Diego, CA, USA). C4BP isolated from normal human serum was purchased from Complement Technology, INC. (Tyler, TX, USA).

Leptospira strains

The pathogenic *Leptospira* strains used were *L. interrogans* serovar Copenhageni strain M20 (nonvirulent), *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 (virulent) and nonpathogenic *Leptospira biflexa* serovar Patoc strain Patoc 1. The strains were cultured at 28 °C under aerobic conditions in liquid EMJH medium (Difco, BD, Franklin Lakes, NJ) with 10% rabbit serum, enriched with ʟ-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).

In silico sequence analysis

Predicted coding sequence (CDS) LIC10920 was identified on *L. interrogans* serovar Copenhageni database http://bioinfo03.ibi.unicamp.br/leptospira/. CDS selection was based on predicted cellular localization by PSORT and CELLO web servers, http://psort.hgc.jp/form.html and http://cello.life.nctu.edu.tw/, respectively. The SMART, http://smart.embl-heidelbergde/, PFAM, http://www.sanger.ac.uk/Software/Pfam and LipoP, http://www.cbs.dtu.dk/services/LipoP/web servers were used to search for predicted functional and structural domains. Conservation analyses of the coding sequences were assessed using Clustal Omega multiple-sequence alignment, http://www.ebi.ac.uk/Tools/msa/clustalo/.

Cloning, expression and purification of LIC10920

The amplification of LIC10920 was performed by PCR with *L. interrogans* serovar Copenhageni strain FIOCRUZ L1-130 genomic DNA using specific primers. The gene sequence was amplified without the signal sequence. The PCR fragment of 756 bp was ligated into the *Escherichia coli* expression vector pAE. The DNA sequence was confirmed by sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems, Foster city, CA). Then, plasmid pAE-LIC10920 was used to transform *E. coli* BL21 (DE3) Star pLysS. Recombinant protein was expressed by addition of 1 mM IPTG for 3 h under constant agitation at 37 °C in the presence of 50 μg/mL ampicillin and 34 μg/mL chloramphenicol. The cells were harvested by centrifugation, and the resulting bacterial pellet was resuspended in lysis buffer (20 mM Tris/HCL-pH8.0, 200 mM NaCl, 200 mg/mL lysozyme, 2 mM PMSF and 1% Triton-X114). The bacteria cells were lysed on ice with the aid of a sonication apparatus (Ultrasonic processor; GE Healthcare Bio-Sciences). The insoluble fraction was recovered and resuspended in a buffer containing 20 mM Tris/HCL-pH8.0, 500 mM NaCl and 8 M urea. The protein was purified through Ni^{+2}-charged chelating chromatography in a Sepharose fast flow columns and dialyzed against buffer containing 500 mM NaCl and 20 mM Tris/HCL-pH 8.0. The efficiency of the purification and protein loss were evaluated after dialysis by 12% SDS-PAGE. Protein concentration was estimated by comparing with pre-determined concentrations of albumin (BSA-Bovine Serum Albumin).

Circular dicrhoism (CD) spectroscopy

Purified recombinant protein was dialyzed against sodium phosphate buffer pH 7.4 and CD spectroscopy measurements were performed at 20 °C using a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Tokyo) equipped with a Peltier unit for temperature control. Far-UV CD spectra were measured using a 1 mm-path-length cell at 0.5 nm intervals. The spectrum is presented as an average of five scans recorded from 180 to 260 nm. The residual molar ellipticity was expressed in degree cm2 dmol-1. Spectrum data were evaluated with CAPITO software (http://capito.nmr.fli-leibniz.de/) that calculates the secondary structure content from the ellipticity experimental data.

Antiserum production against rLIC10920

BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg of the recombinant protein mixed with 10% (v/v) Alhydrogel (2% Al(OH)3, BrenntagBiosector) as an adjuvant. Negative control mice were injected with PBS mixed with adjuvant. Two weeks after each immunization, the mice were bled from the retro-orbital plexus, and the resulting pooled sera analyzed by ELISA for the determination of antibody titre.

Immunoblotting assay

The purified recombinant protein was loaded into 12% SDS-PAGE and transferred to nitrocellulose membrane (Hybond ECL; GE Healthcare) in a semidry equipment. Membrane was blocked with 10% non-fat dried milk in PBS containing 0.05% Tween 20 (PBS-T) and then incubated with anti-Lsa24.9 (1:800) polyclonal serum for 2 h at room temperature. Lsa46 protein serum was used as control at 1:800 dilution.[20] The membrane was incubated with HRP-conjugated anti-mouse IgG (1:3000, Sigma) for 1 h. Monoclonal HRP-conjugated His tag antibodies (1:10000, Sigma) were also used. The protein reactivity was revealed by ECL reagent kit (GE Healthcare).

Evaluation of cellular location of the CDS LIC10920

(i) Immunofluorescence assay (IFA) The localization of the native protein by IFA was performed with suspensions of *L. interrogans* (M20) containing approximately 10^9 cells/mL of live leptospires harvested at 3800 g for 15 min and washed twice with PBS (with 50 mM NaCl). Leptospires were resuspended in 200 μl of PBS with 2% paraformaldehyde for 40 min at 30 °C under gentle shaking. After incubation, the leptospires were washed gently with PBS and incubated for 1 h at 30 °C with polyclonal mouse anti-serum against LipL46, rLIC10920 and PBS at a 1:50 dilution. The leptospires were washed with PBS containing 1% BSA and incubated with anti-mouse IgG antibodies conjugated to fluorescein isothiocyante (FITC, Sigma) at a 1:50 dilution for 50 min. Leptospires were then washed and resupended in 50 μl of PBS containing 0.03 μg propidium iodide (Sigma–Aldrich) and 50 μl anti-fading solution (ProLong Gold, Molecular Probes). The immunofluorescence-labeled leptospires were examined using a confocal LSM 510 META immunofluorescence microscope (Zeiss, Germany); **(ii) Intact Bacterial Cell Test.** *L. interrogans* serovar Copenhageni, culture-attenuated strain (M20) and *L. biflexa* serovar Patoc 1, non-pathogenic strain were centrifuged at 3800 g for 15 min and the bacterial pellet washed with low salt PBS (containing 50 mM NaCl) and 1% BSA. Leptospires were incubated with antibody against DnaK (cytoplasmic protein), PBS (no immune serum) at 1: 1000 dilution or recombinant protein antiserum at 1: 200 dilution for 2 hs at room temperature. The samples were washed with 1% PBS and incubated for 1 h and 30 min with anti-mouse IgG secondary antibody conjugated to fluorescein isothiocyanate (FITC, Sigma–Aldrich), diluted 1: 100 in a solution of PBS *low salt* with 1% BSA. After this time, the solution was centrifuged and the bacterial pellet was washed twice with 1% PBS. Bacteria were resuspended in 400 μl of PBS *1ow salt* and distributed on a black plate (Corning), specific for the reading equipment. Fluorescence intensity was read in Fluoroskan Ascent FL (Thermo Scientific) equipment, λ excitation: 485 nm, λ emission: 538 nm.

Microscopic agglutination test (MAT)

The microscopic agglutination test was performed according to Faine et al.[3] In brief, an array of serovars of

Leptospira spp. as antigens were employed, as previously described.[9] A laboratory-confirmed case of leptospirosis was defined by demonstration of a four-fold microagglutination titer rise between paired serum samples. The serovar was considered to be the one with the highest dilution that could cause 50% of agglutination. MAT was considered negative when the titer was below 100.

Binding of recombinant protein to ECM and serum components

Protein attachment to individual macromolecules of ECM and serum components was performed as described in Siqueira et al. [10]

Dose—response curves and K_D values

ELISA plates were coated overnight with 1 μg laminin or PLG. Plates were then blocked and increasing concentrations of purified recombinant protein was added, ranging from 0 to 1500 nM, followed by incubation for 2 h at 37 °C. The assessment of bound protein was performed with polyclonal antiserum raised in mice against the protein followed by HRP- conjugated anti-mouse IgG. When the reaction reached on a saturation point, equilibrium dissociation constant (K_D) was calculated, according to a method described elsewhere.[26] The equation K_D = (Amax [protein])/A)-[protein], where A is the absorbance at a given protein concentration, Amax is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and K_D is the equilibrium dissociation constant for a given protein concentration (ELISA data point).

Evaluation of recombinant protein denaturation on binding with the components

In order to verify the effect of recombinant protein denaturation in the interaction with PLG and laminin, the protein (10 μg/ml) was submitted to 96 °C for 10 min. Next, recombinant protein was incubated in a microdilution plate, where 1 μg per well of components were pre-immobilized. BSA and fetuin were used as negative control. Detection of the protein bound to the components was performed with a pool of polyclonal serum, used at 1: 5000 dilution. The plates were incubated for 2 hs at 37 °C. Anti-mouse IgG secondary antibody at 1:5000 dilution was added for 1 h at 37 °C. The results were expressed as the mean absorbance and values were compared to those obtained by the non-denatured protein binding.

Binding characterization of recombinant protein to PLG and PLA generation assay

To determine the role of lysine residues in PLG-recombinant protein interaction, lysine analogue, 6-aminocaproic acid (ACA) (Sigma), and PLA generation, we followed the protocols described in Vieira et al., 2009.

Antibody inhibition assay

The effect of anti-rLIC10920 sera on the binding of the corresponding recombinant protein to laminin and PLG was evaluated in a dose-dependent manner. Plates were coated with 1 μg of each component and blocked with 10% non-fat dried milk in PBS-T. At the same time, 1 μg of recombinant protein was incubated with different concentrations of the antiserum (from 1:50 to 1:400 dilutions) or anti-PBS/adjuvant (used as control) for 2 h at 37 °C. Blocked or unblocked recombinant protein was then allowed to interact with the coated component for more 2 h at 37 °C. After washing with PBS-T, 1:5000 dilution of HRP-conjugated His tag tag mAbs was added. The detection of bound protein was performed as described previously. The results were expressed as the percentage of binding and the mean absorbance values were compared to those obtained in the absence of antibodies.

Detection of IgG antibodies in human serum samples

Reactivity of rLIC10920 with leptospirosis serum samples was evaluated by ELISA. The recombinant protein was immobilized on microdilution plate (250 ng/well) and incubated for 16 h at 4 °C. Next, plates was washed 3 times with PBS-T (PBS + 0.05% Tween-20), blocked with 200 μl per well of PBS-T + 10% milk and incubated for 2 h at 37 °C. The serum reactivity with the recombinant protein was evaluated by incubation for 1 h at room temperature. Anti-human IgG secondary antibody (Sigma—Aldrich) at the 1: 5000 dilution was added. Cut-off value is based on the optical density of the commercial human serum pool (Sigma—Aldrich) of healthy subjects and was used to determine the frequency of antibody positive patients. Cutoff values were set at three standard deviations above the mean OD_{492} of control sera.

Acquisition of PLG from normal human serum

Microtitre plates wells were coated overnight with LIC10920 or gelatin at 4 °C. The plates were washed three times with PBS-T and blocked for 2 h at 37 °C with PBS plus BSA. Next, normal human serum was diluted in 100 μl PBS (0—30%) for 90 min at 25 °C. After washing, binding between rLIC10920 and PLG was detected by adding mouse anti-PLG diluted 1:5000 followed peroxidase conjugated anti-mouse (1:5000). The detection of bound protein was performed as described previously.

Ethics statements

All animal studies were approved by the Ethics Committee of the Instituto Butantan, Sao Paulo, SP, Brazil, under protocol number 3905120116. The Committee in Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation. Confirmed-leptospirosis human serum samples were from Instituto Adolfo Lutz collection, Sao Paulo, Brazil, and were donated for research purposes. The Ethics Committee for Research with Human Beings of ICB/University of Sao Paulo has deliberated that this project is exempt of ethics approval because it does not involve human manipulation.

Statistical analysis

All results are expressed as the ±SD. Student's paired *t*-test was used to determine the significant difference among means and $p < 0.05$ was considered statistically significant. Three or two independent experiments were performed, each one in triplicate.

Results

Bioinformatics analysis of LIC10920 CDS

The selected coding sequence LIC10920 is genome annotated as a probable lipoprotein, having signal that is recognized by the enzyme signal peptidase II. According to LipoP server, the cleavage site for signal peptidase II is between amino acids 20–21.[27] No putative conserved domains within LIC10920 sequence was detected by SMART and PFAM web servers [28–30]. CELLO Server predicts LIC10920 to be extracellular.[31,32] Multiple sequence alignment was performed with Clustal Omega, comparing LIC10920 with the leptospiral sequence strains available in GenBank.[33] The depicted phylogram in Fig. 1 shows a high level of sequence conservation among pathogenic leptospiral strains, in black (percentage of identity are indicated), while sequences present in saprophytic strains shows lower levels of similarity, in grey.

Cloning of LIC10920 gene and characterization of recombinant protein

LIC10920 was amplified without the signal peptide and cloned into pAE vector. The recombinant protein was expressed as full-length with His-tag sequence at its N-terminal. Induction of expression was performed in the host strain *E. coli* BL21DE3 Star pLysS after addition of 1 mM of IPTG and the protein was expressed in its insoluble form. Recombinant protein was recovered from inclusion bodies after 8 M urea solubilization and purified by metal chelating chromatography under denaturation condition, and refolded by gradually removal of urea. The given name for purified recombinant protein is Lsa24.9 and an aliquot of purified Lsa24.9 was analyzed by SDS-PAGE (Fig. 2A, lane 1). Lsa46 was employed as a recombinant protein control (Fig. 2A, lane 2). Both recombinant proteins were recognized by western blotting probed with His tag mAbs (Fig. 2B, lanes 1 and 2), due to the presence of 6XHis tag at N-terminus, while only Lsa24.9 was recognized by its homolog antiserum (Fig. 2C, lane 1). Likewise, only Lsa46 was recognized by its homolog antiserum (Fig. 2D, lane 2), showing the specificity of the antiserum raised against each protein. The structural integrity of Lsa24.9 was evaluated by CD spectroscopy in order to evaluate the secondary structure content after the refolding. Analysis of the spectra data (Fig. 2E) was performed by CAPITO software.[34] According to data, Lsa24.9 showed 39% of beta-strands, 19% of alpha helix and 42% of random secondary structures.

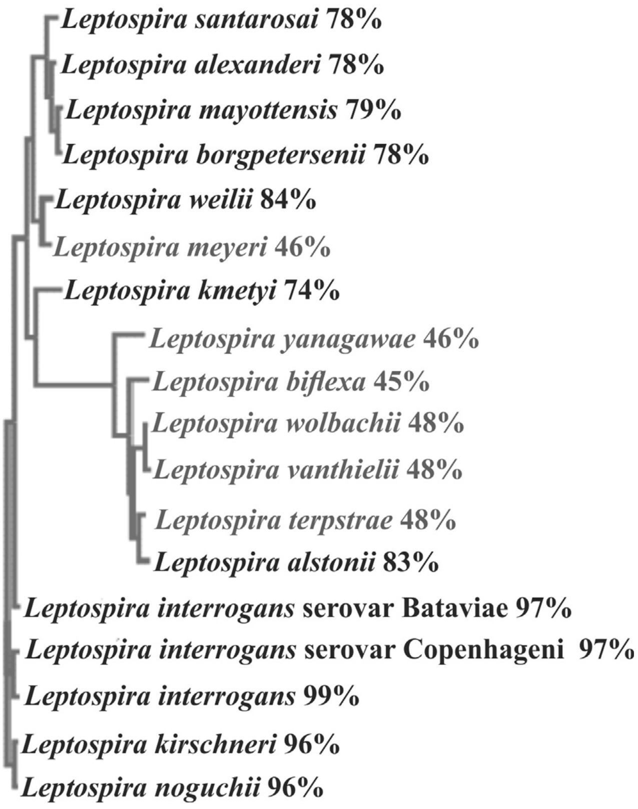

Fig. 1. Sequence conservation among species and serovars of leptospires by Clustal Omega. Blast analysis was performed to evaluate similarity among the LIC10920 CDS with leptospiral sequences available in GenBank database. These sequences were submitted to Clustal Omega multiple sequence alignments. The phylogram show higher degree of sequence conservation of LIC10920 among pathogenic strains (in black, 74–99%) when compared to saprophyte strains (in grey, 45–48%).

Recognition of native protein on the leptospiral surface by anti-Lsa24.9 serum

Assessment of the cellular localization of the selected CDS on the pathogenic *Leptospira* was performed by immunofluorescence confocal microscopy and intact bacteria assays. Leptospires were visualized by propidium iodide staining (Fig. 3A-PI), followed by protein detection with anti-Lsa24.9 polyclonal antibodies raised in mice against recombinant protein and presence of anti-mouse IgG antibodies conjugated to FITC (Fig. 3A-FITC). As positive and negative controls, we used LipL46 outer membrane protein[35] and non-immune serum, respectively. The localization of the protein on the leptospires was achieved by merging both fields (Fig. 3A-merged). We also evaluated the presence of native protein on the bacterial surface after intact leptospires treatment with anti-Lsa24.9 polyclonal antibodies, followed of IgG antibodies conjugated to FITC and fluorescence intensity detected by Fluoroskan. We have included *L. biflexa*, a saprophyte strain that exhibit CDS of 45% identity with the sequence present in pathogenic strains. Fluorescence intensity was observed

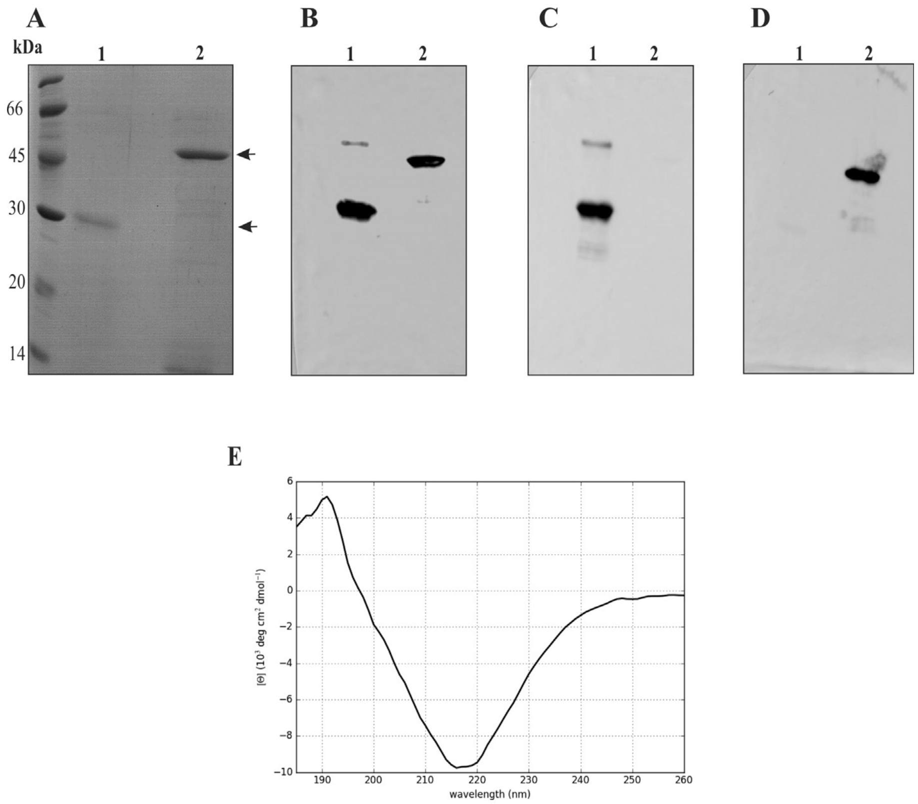

Fig. 2. Analysis of recombinant protein Lsa24.9 purification, western blotting recognition by antibodies and CD analysis. (A) Purification analysis of recombinant protein was performed by SDS-PAGE after Comassie blue stained, Lsa24.9 (lane 1) and Lsa46 (lane 2), employed as a control. M: molecular mass protein marker. In (B) western blotting of Lsa24.9 and Lsa46 probed with His tag mAbs (lanes 1 and 2, respectively); in (C) proteins were probed with the polyclonal antiserum raised in mice against Lsa24.9 (lane 1) and, in (D) proteins were probed with polyclonal antiserum against Lsa46. (E) CD spectrum of Lsa24.9 showing a mixed secondary structure. The far-UV CD spectrum is presented as the mean of five scans.

for the CDS LIC10920 in pathogenic strain after anti-Lsa24.9 treatment, but not for DnaK, a cytoplasmic protein used as a negative control.[36] The data suggest the presence of this protein on the pathogenic leptospiral surface (Fig. 3B).

Detection of human antibodies in leptospirosis serum samples against Lsa24.9

To examine whether the native protein is expressed during infection, we assessed the presence of total IgG antibodies against Lsa24.9 in a set of confirmed leptospirosis human serum samples by ELISA. We used a total of 40-paired samples, half for each at the onset (MAT-) and convalescent (MAT+) phases of the disease. Normal human serum samples were employed to determine the cut-off. The results show the presence of IgG for Lsa24.9 in both phases of the disease. The reactivity of the protein at the convalescent phase was 60% (Fig. 4B), while at the early phase of leptospirosis, when MAT is still negative, 50% of evaluated samples were reactive (Fig. 4A). The results suggest that Lsa24.9 has the potential to be used as an early marker of leptospirosis. Moreover, these results indicate that Lsa24.9 is capable of inducing an immune response in infected host and hence expressed during infection.

Identification of extracellular matrix ligands for Lsa24.9 protein

To evaluate whether the Lsa24.9 protein could mediate host colonization by adhering to extracellular matrix proteins, we immobilized on a microplate laminin, cellular fibronectin, collagen I and IV, elastin, E-cadherin, and the control proteins BSA and fetuin. Binding to Lsa24.9 recombinant protein was evaluated by ELISA, using polyclonal antibodies raised in mice against the recombinant protein. The results obtained show that Lsa24.9 protein exhibited adhesiveness to laminin (Fig. 5A). No statistical significance was observed when others components were assessed. The interaction of Lsa24.9 to laminin was confirmed with His tag mAbs (Fig. 5B). To assess the interaction of Lsa24.9 protein to laminin on a quantitative basis, a dose—response curve was evaluated by ELISA, in which increasing protein concentration was allowed to interact to fixed laminin concentration. Dose-dependent and saturable curve was observed. Binding saturation level was achieved at protein concentration around 1 μM, as shown (Fig. 5C). The calculated dissociation equilibrium constant (K_D) for the Lsa24.9 protein with laminin is 0.1 ± 0.06 μM. The involvement of structural epitopes was also evaluated when the Lsa24.9 protein was submitted heat-denaturing condition prior to the binding reaction

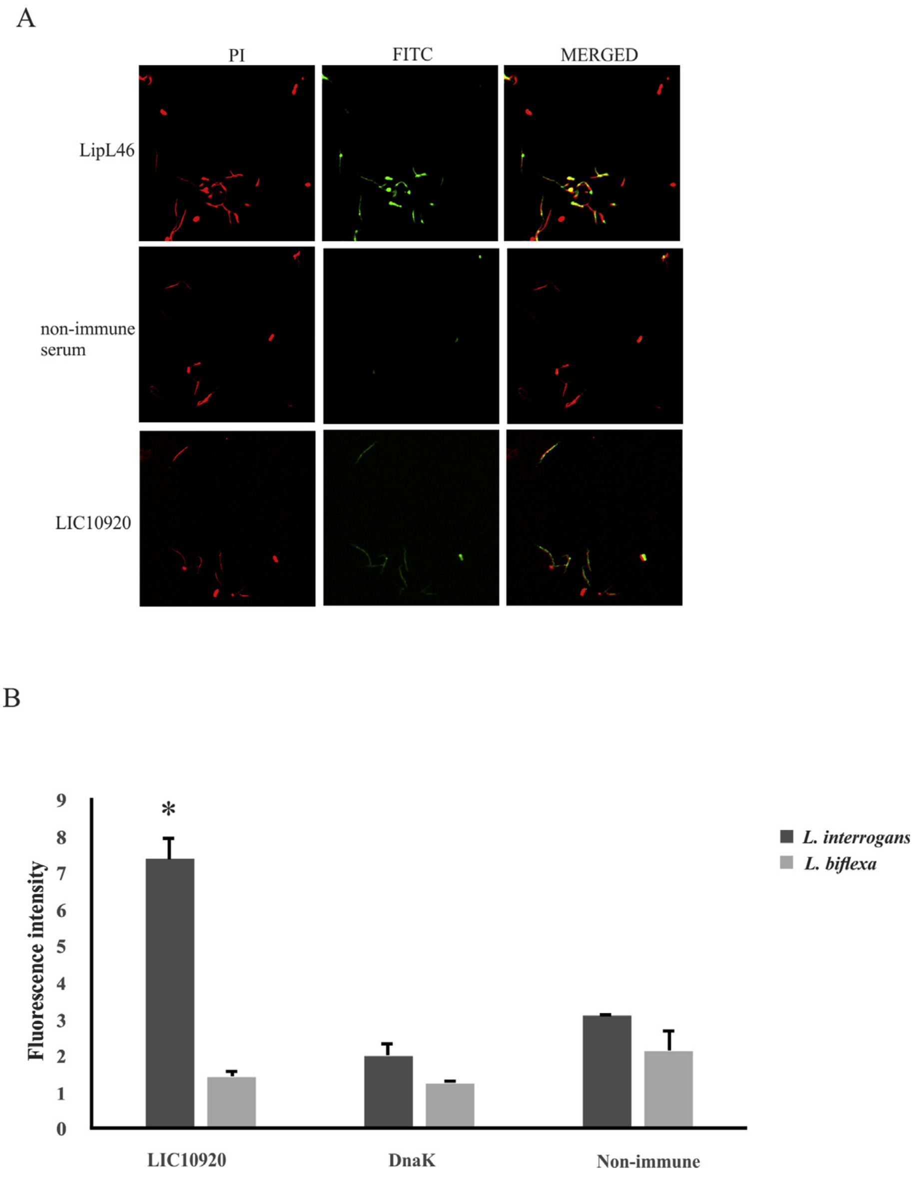

Fig. 3. **Cellular localization of native protein in *Leptospira* by immunofluorescence and intact leptospires assays.** (A) Attenuated strain of *Leptospira interrogans* serovar Copenhageni were fixed with paraformaldehyde and polyclonal anti-Lsa24.9 was used to identify surface-exposed protein. Leptopsires probed with non-immune serum or serum anti-LipL46 was used as negative control and positive, respectively. Leptospires were probed by propidium iodide staining (PI). FITC-conjugated secondary antibodies were used to reveal the surface-bound antibodies (FITC). The merge of images is showed in the last column (merged). (B) Intact attenuated pathogenic and saprophytic leptospires were treated with antiserum against Lsa24.9, followed by the FITC-conjugated secondary antibodies. Non-immune serum was used as a negative control and serum against DnaK was used as a marker for cytoplasmic protein. Treated leptospires were placed on microdilution plates and readings were performed in a Fluoroskan at λ 485 nm excitation and λ 538 nm emission. For statistical analyses, the fluorescence signal was compared to the negative controls by two-tailed t-test (*$p < 0.05$).

(Fig. 5D). As depicted, protein denaturation promoted a reduction of attachment, suggesting the importance of protein structure in this interaction. To assess the participation of immunogenic epitopes of Lsa24.9 on laminin binding, recombinant protein was incubated with their respective antiserum prior to the binding reaction. A reduction dependent on serum dilution was observed on the attachment of Lsa24.9 to laminin, contrasting with the results obtained after the treatment of Lsa24.9 with non-immune serum, used as control. The data suggest the involvement of immunogenic epitopes on the binding reactions (Fig. 5E).

Identification of human plasma proteins that bind to Lsa24.9 recombinant protein

Several leptospires proteins exhibit multifunctional properties and could act as human plasma components

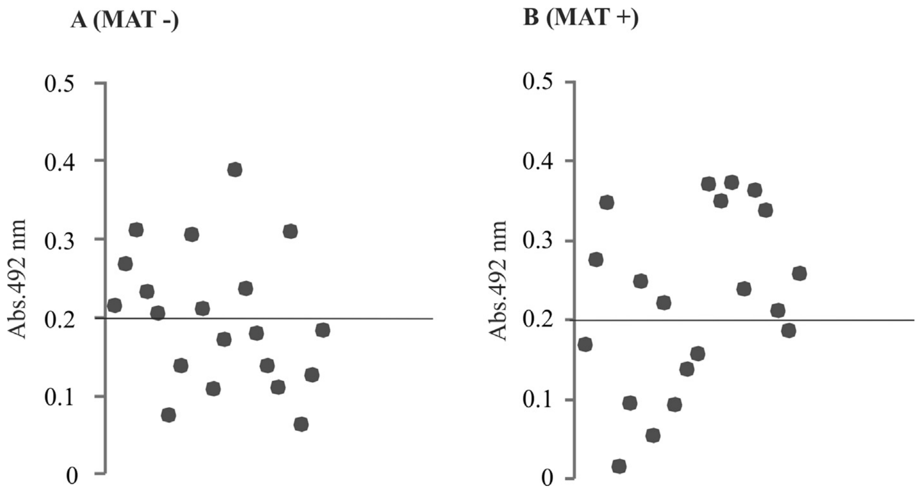

Fig. 4. Detection of IgG antibodies in leptospirosis human serum samples. Reactivity of Lsa24.9 was evaluated in confirmed leptospirosis serum samples at the onset (MAT-) and convalescent (MAT+) phases of the disease. The reactivity of Lsa24.9 was 50% at the initial (MAT-) and 60% at the convalescent (MAT+) phases. The cutoff values were defined as the mean plus 3 standard deviations obtained with normal human serum samples.

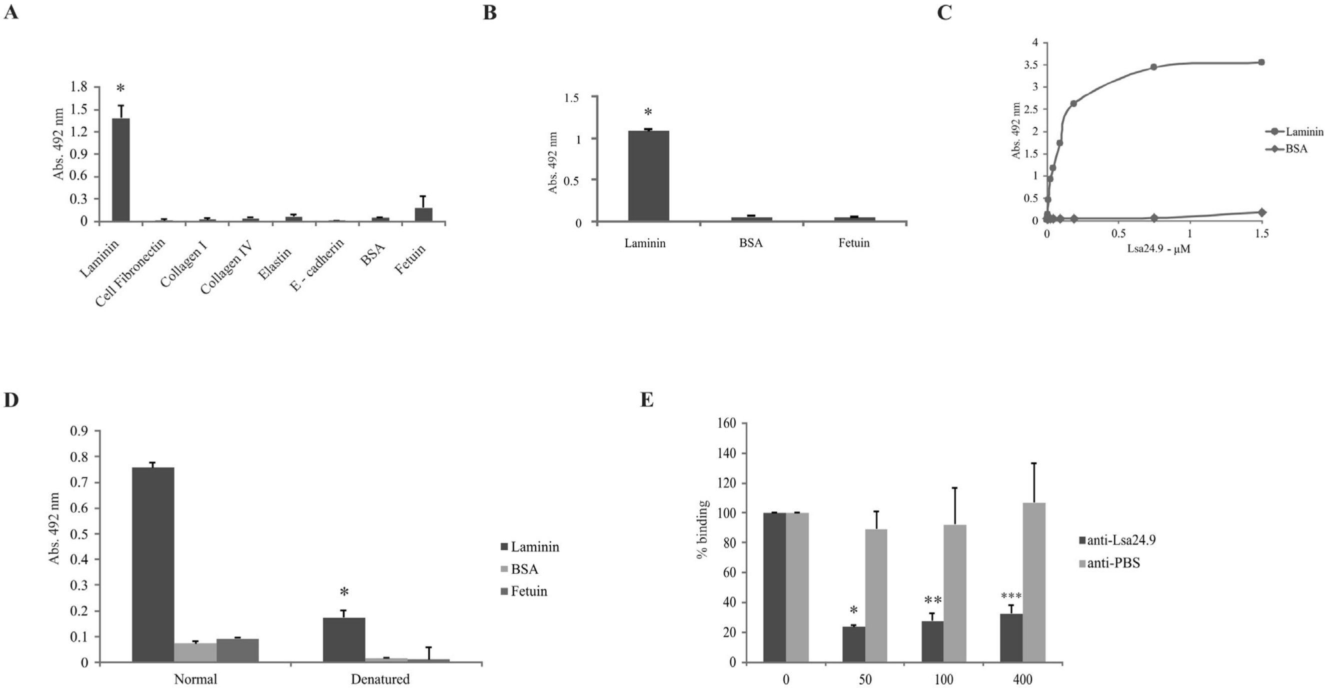

Fig. 5. Interaction and characterization of Lsa24.9 with extracellular components by ELISA. (A) Wells were coated with 1 μg of laminin, cellular fibronectin, collagen I and IV, elastin, E-cadherin and the controls proteins BSA and fetuin. Lsa24.9 was added and the binding was evaluated probing the reactions with polyclonal serum anti-Lsa24.9. (B) The interaction of Lsa24.9 to laminin was confirmed when the reaction was probed with His tag mAbs. (C) Effect of increasing concentration of Lsa24.9 on the binding reaction to a constant laminin concentration. BSA was used as a negative control. The saturation level of the binding was reached around 1.0 μM of Lsa24.9 and the calculated $K_D = 0.1 \pm 0.06$ μM. (D) Effect of heat-inactivated Lsa24.9 upon binding to laminin. For statistical analyses, the binding of Lsa24.9 to laminin was compared with the binding to BSA by the two-tailed t-test ($^*p < 0.05$). (E) Effect of non-immune and polyclonal anti-Lsa24.9 serum upon the binding of Lsa24.9 with laminin in comparison with the binding in the absence of antibodies by the two-tailed t-test ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Results were expressed as percentage of the binding.

receptors.[6] To evaluate the ability of Lsa24.9 protein to interact with the plasma proteins, we immobilized onto ELISA plates PLG, plasma fibronectin, vitronectin, fibrinogen, C4BP, FH and the control proteins BSA and fetuin, followed by incubation with the Lsa24.9 protein. Our data showed that protein binds to PLG and plasma fibronectin, after using polyclonal antibodies raised in mice against recombinant protein (Fig. 6A). This binding was confirmed by anti his mAbs only in the case of PLG (Fig. 6B). Similarly, dose-dependent binding and saturation was only achieved for PLG, saturation reached at protein concentration of 0.75 μM, with a K_D of 0.032 ± 0.01 μM (Fig. 6C). To assess whether the Lsa24.9 recombinant protein could recruit PLG from normal human serum, one microgram of Lsa24.9 was immobilized onto ELISA microplates and incubated with different percentage of human serum. The acquirement of PLG from serum by Lsa24.9 was indirectly evaluated by ELISA and the reactions were probed with anti-PLG antibodies. The data showed that Lsa24.9 can acquire PLG from serum when compared to gelatin, used as negative control for non-specific binding. The interaction of Lsa24.9 to PLG occurred in a dose-dependent fashion and saturation was reached around 20% of serum dilution (Fig. 6D). This result suggests that the CDS LIC10920 at the leptospiral surface has the potential to recruit PLG in a more physiological condition.

Binding characterization of Lsa24.9 to PLG

It has been previously shown the involvement of PLG kringle domains in the interaction with lysine residues present in the binding protein.[17] To evaluate the participation of lysine residues in the binding of Lsa24.9 to PLG, an analogue of lysine residue, ACA, was added to the reaction. The results confirmed that interaction occurs via lysine residues, because 2 mM of ACA concentration was able to inhibit this interaction (Fig. 7A). We have demonstrated that bound PLG to the surface of leptospires could be converted to enzymatically active plasmin when its activator is present.[17] To assess whether PLG bound to Lsa24.9 protein generates proteolytic activity, a microplate was coated with Lsa24.9, incubated with PLG, uPA type PLG activator and a plasmin specific chromogenic substrate. The cleavage of the specific plasmin substrate was measured at 405 nm and indirectly demonstrated plasmin activity, when Lsa24.9 is bound to PLG, in the presence of PLG activator. No cleavage of the chromogenic substrate was observed in the reaction mixtures lacking at least one of the components (Fig. 7B). Aiming to evaluate the participation of structural epitopes on the binding of Lsa24.9 to PLG, the protein was heat-denatured prior to the binding reaction. As shown in Fig. 7C, a statistically significant reduction was observed in this interaction, suggesting the involvement of structured regions on the binding. Likewise, the participation of immunogenic epitopes seems to be also important for the attachment of Lsa24.9 protein to PLG, since after incubation with its homolog antiserum, a significant reduction was observed. The reduction was dependent on serum dilution (Fig. 7D) but, non-immune serum also had an inhibition effect on the binding. Possibly this effect observed is due to the plasminogen presence in the human serum. The results suggest the participation of immunogenic epitopes on the binding (Fig. 7D).

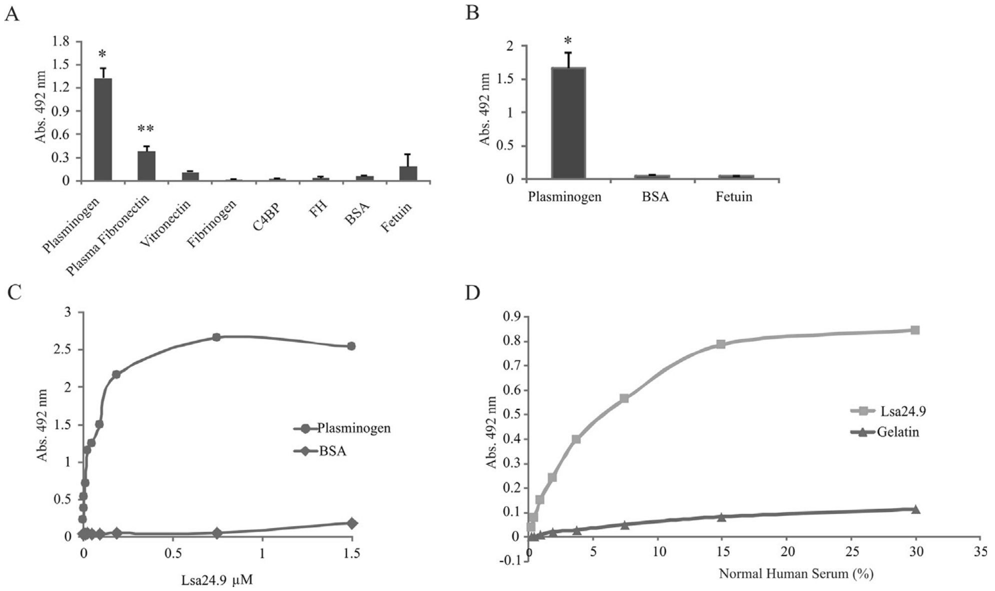

Fig. 6. Interaction of Lsa24.9 with plasma components by ELISA assay. (A) Wells were coated with 1 μg of PLG, plasma fibronectin, vitronectin, fibrinogen, C4BP, factor H and the control proteins BSA and fetuin. Lsa24.9 was added and binding was evaluated by probing the reaction with anti-Lsa24.9 serum. (B) The interaction of Lsa24.9 to PLG when the reaction was probed with His tag mAbs. (C) Effect of increasing concentration of Lsa24.9 on the binding to a constant PLG concentration. BSA was used as a negative control. The saturation level of the binding was reached at 0.75 μM of Lsa24.9 and the calculated $K_D = 0.032 \pm 0.01$ μM. For statistical analyses, the binding of Lsa24.9 to PLG was compared with the binding to BSA by the two-tailed t-test (*$p < 0.05$, **$p < 0.01$). (D) Lsa24.9 was immobilized and incubated with different amounts of normal human serum (0—30%). Binding was detected with anti-PLG. Gelatin was employed as a negative control. Each point represents the data determined in triplicate and results were expressed as mean±standard deviations of absorbance at 492 nm for each point.

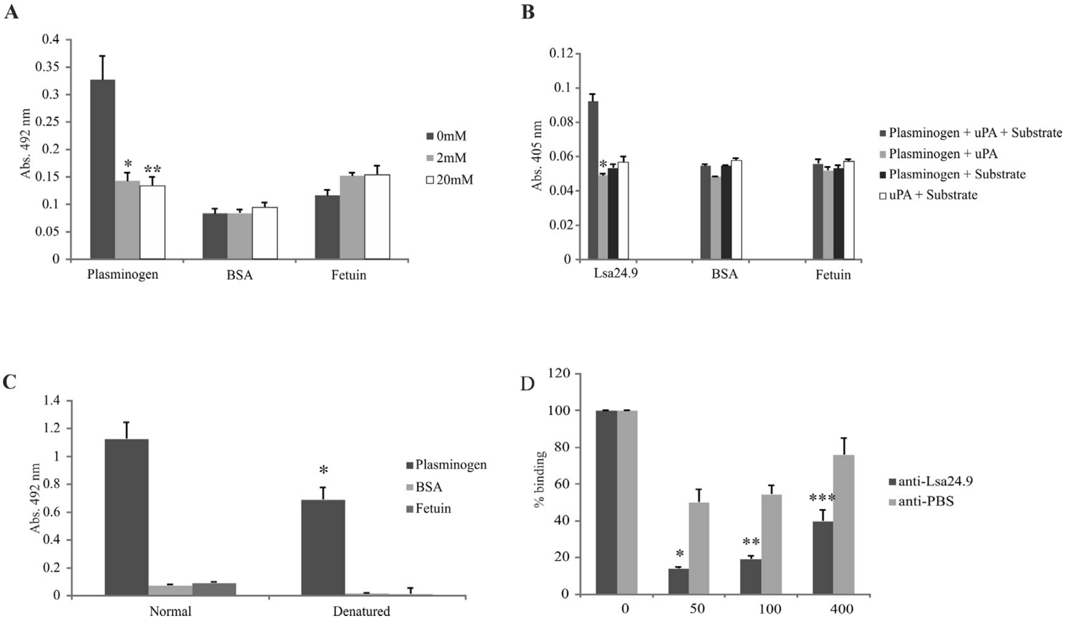

Fig. 7. **Characterization of binding of Lsa24.9 to PLG and plasmin generation.** (A) Binding of Lsa24.9 to PLG in the presence of the lysine analogue ACA was evaluated by ELISA assay. The binding of recombinant protein was detected by anti-Lsa24.9 serum. For statistical analyses, the binding in the presence of the lysine analogue was compared to the binding to PLG without analogue by the two-tailed t-test (*$p < 0.1$, **$p < 0.01$). (B) Plasmin generation by PLG bound to Lsa24.7 was measured indirectly by the cleavage of plasmin-specific substrate using a modified ELISA assay. Lsa24.9 was immobilized followed by adding PLG, urokinase-type PLG activator (uPA) and the plasmin substrate. Controls lacking at least one component and BSA protein were used. Bars represent mean absorbance at 405 nm (OD_{405nm}) as a measure of relative substrate cleavage ± the standard deviation of three replicates. For statistical analyses, significant differences were observed relative to control BSA Lsa24.9 and when was treated with PLG, uPA and substrate (*$p < 0.05$). (C) The effect of heat-inactivated Lsa24.9 on the binding to PLG. For statistical analyses, the binding of Lsa24.9 to PLG was compared with the binding to BSA by the two-tailed t-test (*$p < 0.05$). (D) The effect of non-immune and anti-Lsa24.9 serum on the binding of Lsa24.9 to PLG was compared to the binding in the absence of antibodies by the two-tailed t-test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Results were expressed in percentage of the binding.

Discussion

PLG is a 90 kDa inactive zymogen that is abundantly present in plasma with concentrations ranging from 180 to 200 μg/mL. Its active form, plasmin, plays an important physiological role cleaving insoluble fibrin polymers into soluble fragments. Moreover, it degrades extracellular matrix proteins and act in other proteins involved in immunity and tissue repair.[37,38] Several bacterial pathogens have developed strategies to interact with PLG and use the host proteolytic system to their own advantage. It has been demonstrated that *Haemophilus influenza*,[39,40] *Neisseria meningitides*, *Neisseria gonorrhoeae*,[41] *Yersinia pestis*,[42] *Borrelia burgdorferi*[12] and *L. interrogans*[17] can acquire PLG on their surfaces and promote its conversion to plasmin. The ability of these pathogens to interact with the host PLG is due to presence of PLG-binding protein and to the stimulation of host or bacterial activators. In *Leptospira* several PLG-binding proteins have been identified, contributing to clarify the pathogenesis mechanism of these bacteria.[6,20–22,43–48] However, there are still many proteins with unknown function at the bacterial cell surface that are possibly involved in pathogenesis. The characterization of these proteins is critical to understanding leptospirosis disease.

In this work, we identified and characterized a new protein of unknown function, encoded by the gene LIC10920 and genome annotated as a lipoprotein. The CDS LIC10920 is well conserved among pathogenic species of *Leptospira* and show lower identity in saprophyte strains. The conservation of this sequence among pathogenic species may indicate a functional importance. Thus, we have cloned the gene LIC10920 and expressed in *E. coli*, as a 24.9 kDa recombinant protein. We show that this recombinant protein is recognized by confirmed leptospirosis serum samples, suggesting its expression during infection. Moreover, antibodies obtained against recombinant protein were capable to recognize native protein on leptospiral surface, indicating that this protein may interact with host components. Several leptospiral proteins that were characterized as surface exposed showed extracellular matrix binding properties.[5,8,10,20,43,48–50] Likewise, the recombinant protein rLIC10920 could bind to laminin in a dose-dependent manner, and thus, was called Lsa24.9. Many proteins that mediates attachment to laminin have been characterized: Lmb of *Streptococcus agalactiae*,[51] enolase de *Staphylococcus aureus*,[52] Rck and PagC of *Salmonella*,[53] BmpA and ErpX of *B. burgdorferi*[54,55] are all laminin-binding proteins that may play a role in mammalian infection. In general, extracellular matrix components are attractive targets for adherence and invasion by pathogens, due to the capacity of the microorganisms to bind and degrade matrix proteins using extracellular matrix degrading proteases, surface-bound PLG or matrix metalloproteinases recruited from the host.[56]

Previous works of our group has reported that the binding of leptopsires to PLG and that plasmin generation promoted laminin and fibronectin degradation, favoring the dissemination of the bacteria through the host tissues. Moreover, it has been shown that plasmin generation on leptospires surface interfere with complement C3b and IgG depositions on the bacterial surface, decreasing opsonization process, representing a potential immune escape strategy for pathogenic leptopsires.[19,23] Interesting, leptospires are capable of stimulating the expression of PLG activators by endothelial cells and promoted a high migration activity through these cells in the presence of PLG/plasmin. Moreover, it has been observed a transcriptional upregulation of matrix metalloprotease 9, suggesting an increase in proteolytic ability of the leptospires to penetrate the hosts.[18] To date, several leptospiral proteins have been described as PLG binding and many these ligands interact with multiple host molecules.[57] In this work, we show that Lsa24.9 protein also have capacity bind to PLG via lysine residues, suggesting that the kringle domains of PLG are involved in this interaction. The involvement of lysine residues in PLG binding had already been showed for leptospires[43] as well as for the others PLG binding proteins.[9,20–22,44,47,50]

PLG bound to Lsa24.9 protein could be converted to plasmin, in the presence of PLG activator, although we did not detected fibrinogen and laminin degradation products when plasmin was generated in the presence of Lsa24.9. The interaction of Lsa24.9 with PLG was dose-dependent and saturable, suggesting high affinity binding among receptor-ligand. Moreover, Lsa24.9 can acquire PLG from normal human serum, in a dose-dependent manner, suggesting that this binding can occur in a more physiological condition. It has been described that Mce protein can participate in several *Leptospira* infection processes, due their capacity of interact with laminin, plasma and cellular fibronectin, collagen IV, integrins and recruit PLG directly from normal human serum.[58] Lsa23, another multifunctional surface protein of *Leptospira* has also demonstrated the ability of recruit PLG, FH and C4BP from normal human serum. Interesting that, both regulators FH and C4BP and plasmin generation through the binding to Lsa23, can promote C3b and C4b degradation, contributing to leptospiral immune evasion.[24]

In conclusion, we show indirectly that Lsa24.9 recombinant protein binds to host molecules. We have characterized this novel leptospiral protein having adhesiveness properties to laminin and the ability to interact with PLG, generating plasmin. The production of plasmin on leptospiral surface could contribute to invasion, dissemination and immune evasion processes within the hosts. Although these results are based on binding to purified molecules, Lsa24.9 protein has shown ability to acquiring PLG directly from normal human serum, suggesting the biological relevance of this interaction. Furthermore, this protein is recognized by human antibodies present in confirmed leptospirosis samples, suggesting its expression during the infection process. It is anticipated that the construction of mutants of LIC10920/Lsa24.9 in *L. biflexa*, undergoing in our laboratory, would enlighten the information on its biological role. Lastly, this protein originally annotated of unknown function may have a role at different level of leptospiral infection.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of date and material

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

The following Brazilian agencies: FAPESP (grant 14/50981-0), CNPq (grants 302758/2013-5, and 441449/2014-0) and Fundação Butantan, financially supported this work; ADR and AFT have fellowships from CAPES and FAPESP (2016/11541 0). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

All authors participated in the literature revision, discussion and preparation of manuscript, including figures.

Authors' information

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Acknowledgments

We are grateful to all the members for support on this project.

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