Characterization of two new putative adhesins of *Leptospira interrogans*

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**Abstract**
We here report the characterization of two novel proteins encoded by the genes LIC11122 and LIC12287, identified in the genome sequences of *Leptospira interrogans*, annotated, respectively, as a putative sigma factor and a hypothetical protein. The CDSs LIC11122 and LIC12287 have signal peptide SPII and SPI and are predicted to be located mainly at the cytoplasmic membrane of the bacteria. The genes were cloned and the proteins expressed using *Escherichia coli*. Proteinase K digestion showed that both proteins are surface exposed. Evaluation of interaction of recombinant proteins with extracellular matrix components revealed that they are laminin binding and they were called Lsa19 (LIC11122) and Lsa14 (LIC12287), for *Leptospiral-surface adhesin* of 19 and 14 kDa, respectively. The bindings were dose-dependent on protein concentration, reaching saturation, fulfilling the ligand-binding criteria. Reactivity of the recombinant proteins with leptospirosis human sera has shown that Lsa19 and, to a lesser extent, Lsa14, are recognized by antibodies, suggesting that, most probably, Lsa19 is expressed during infection. The proteins interact with plasminogen and generate plasmin in the presence of urokinase-type plasminogen activator. Plasmin generation in *Leptospira* has been associated with tissue penetration and immune evasion strategies. The presence of a sigma factor on the cell surface playing a secondary role, probably mediating host –pathogen interaction, suggests that LIC11122 is a moonlighting protein candidate. Although the biological significance of these putative adhesins will require the generation of mutants, our data suggest that Lsa19 is a potential candidate for future evaluation of its role in adhesion/colonization activities during *L. interrogans* infection.

**INTRODUCTION**
Leptospirosis is a worldwide zoonosis that affects several species of mammals, including humans, caused by pathogenic species of bacteria of the genus *Leptospira*. The estimated number of cases worldwide exceeds 1 million each year, and over 70% of them are reported from tropical regions [1].

In urban settings, rodents are the main reservoirs of the disease since they are asymptomatic carriers that keep shedding live bacteria in urine. Human contamination by leptospires occurs directly through contact with the urine of infected animals or indirectly by exposure to contaminated environments [2, 3]. Leptospires penetrate the individual’s mucosa through small wounds or sodden skin [4].

Due to the wide-ranging symptoms, the number of leptospirosis cases is normally underestimated. Fever, headache, muscle aches, nausea and vomiting are manifestations commonly observed for some viruses. About 5 to 15% of infected individuals progress into a severe form of the disease, known as Weil’s syndrome, which is characterized by multiple organ complications, including renal and hepatic dysfunction, and cardiovascular damage, with a mortality rate of 15% [4]. Haemorrhagic pulmonary syndrome has been reported, which is characterized by massive bleeding in the lung. This form of the disease is associated with higher mortality rate, up to 70% of cases [5–10]. Animal vaccines against leptospirosis are available and are based on inactivated whole cell or membrane preparations. These
vaccines are lipopolysaccharide dependent, do not promote cross-reactivity against serovars that are not included in the preparation and require booster injections every year [11, 12]. Since there are approximately 250 pathogenic serovars of *Leptospira*, a cost-effective vaccine has long been pursued [13]. Only a few countries have licensed vaccines for humans that are similar to the veterinarian ones [11, 14].

Outer membrane proteins are the main focus of many research groups, because due to their location they can elicit immune response in hosts [15]. Reverse vaccinology, an approach that uses genome sequencing and bioinformatics technologies, allows the identification of membrane proteins that could act as putative targets for vaccine development, independently of their copy number in the bacterial cell [16–18].

In the present study, we report the characterization of two novel leptospiral proteins. The selected genes, LIC11122 and LIC12287, were identified by reverse vaccinology strategy in the genome sequences of *L. interrogans* serovar Copenhageni using the LipP webserver [19]. The CDSs LIC11122 and LIC12287 are predicted to encode a lipoprotein and a protein with signal peptide tag, respectively [20, 21]. We show that the proteins are located at the bacterial cell surface and the corresponding recombinant proteins bind laminin, and therefore are named Leptospiral surface adhesins, Lsa19 and Lsa14, with molecular mass of 20 and 14 kDa, respectively. We show that these are plasminogen (PLG)-binding proteins capable of generating plasmin, and that Lsa19 is probably expressed during infection.

**METHODS**

**Biological components**

Collagen, laminin, plasma and cellular fibronectin, elastin, vitronectin, PLG, fibrinogen and the control protein BSA were purchased from Sigma-Aldrich. 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 PLG was purified from human plasma. C4BP and factor H, isolated from normal human plasma. C4BP and factor H, isolated from normal human serum, and normal human sera were obtained from Complement Technology.

**Bacterial strains and serum samples**

The pathogenic attenuated *L. interrogans* serovar Copenhageni strain M20 was cultured at 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) [22]. *Escherichia coli* DH5α, *E. coli* BL21 Star(DE3)pLysS [23] and BL21-SI (salt-induced) [24] were used for the cloning steps and for recombinant protein expression, respectively. Human serum samples from patients with confirmed leptospirosis were from the Serum Collection of *Gerencia do Centro de Controle de Zoonoses, Coordenação de Vigilância em Saúde - COVISA, São Paulo, Brazil*, and were donated for research purposes only.

**In silico analysis of the CDSs LIC11122 and LIC12287**

Predicted CDSs LIC11122 (LIC_RS05785) and LIC12287 (LIC_RS11675) were selected from *L. interrogans* serovar Copenhageni genome sequences [25, 26] based on their cellular localization prediction by PSORT, http://psort.hgc.jp/form. html [27], and CELLO, http://cello.life.nctu.edu.tw/ [28, 29]. The signal peptide sequence was assessed by SignalP, www. cbs.dtu.dk/services/SignalP-3.0/ [20], and LipoP, www.cbs.dtu. dk/services/LipoP/ [19]. The Smart, http://smart.embl-heidelberg. de/ [30, 31], and Pfam, http://pfam.xfam.org/ [32], web servers were used to search for predicted functional and structural domains within the amino acid sequence. CLUSTALW2 multiple sequence alignment at www.ebi.ac.uk/Tools/msa/ clustalw2/ [33] was employed to generate the phylogram, based on the sequences available at GenBank.

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

Predicted CDSs LIC11122 and LIC12287 were amplified, without signal peptides, by PCR using *L. interrogans* serovar Copenhageni strain M20 genomic DNA, as template, and specific, complementary primer pairs: LIC11122, F: 5¢-CTGAGCTTAAGACCGCAGAAGTTGAG-3¢ and R: 5¢-GG TACCTCATTTTCATTGTGCAATCTGCTC-3¢; LIC12287, F: 5¢-CTCGAGTAGACATTAGGCACCTGCC-3¢ and R: 5¢- AAAGCTTTATTTTTCTTCGGTAGCA-3¢. Gel-purified PCR fragments (Illuistra GFX PCR DNA and Gel Band Purification kit; GE Healthcare Bio-Sciences) were sub-cloned into the pGEM-T Easy vector (Promega, Prodilmol) and cloned into the pAE expression vector at *XhoI* and *HindIII* (LIC12287) or *XhoI* and *KpnI* restriction sites (LIC11122). The pAE [34] vector enables expression of recombinant proteins with a minimal 6×His-tag at the N-terminus. All cloned sequences were confirmed by DNA sequencing with an ABI 3100 automatic sequencer (PE Applied Biosystems). The plasmids pAE-LIC12287 and pAE-LIC11122 were employed to transform BL21 Star(DE3)pLysS and BL21-SI expression host cells, respectively. *E. coli* BL21 Star(DE3)pLysS cells containing pAE-LIC12287 was grown at 37 °C in Luria–Bertani (LB) broth and *E. coli* BL21-SI containing pAE-LIC11122 were grown at 30 °C in 2× yeast-tryptone broth without NaCl (2YT/ON), both with 100 µg ampicillin ml⁻¹. The bacterial growth was achieved by continuous shaking until an optical density of 0.6 to 0.650 (OD600) was reached. Recombinant protein synthesis was induced by the addition of 0.1 mM IPTG for BL21 Star(DE3)pLysS and 75 mM NaCl for BL21-SI. After 3 h, the bacterial suspensions were centrifuged and re-suspended in lysis buffer (10 mM Tris/HCl, pH 8.0, 150 mM NaCl, 100 µg lysozyme ml⁻¹, 1 % Triton X-100, 2 mM PMSF). The bacterial cell pellets were lysed on ice with the aid of a sonicator tip (Digital Sonifier at 60 % intensity for 5 min). The soluble and insoluble fractions were separated by centrifugation at 12 857 g for 10 min at 4 °C. Soluble recombinant proteins...
were purified from the supernatants of the bacterial cell lysates using the AKTAprime Plus system (GE Healthcare). Insoluble recombinant proteins expressed in inclusion bodies were solubilized from bacterial pellets using a denaturant buffer (100 mM Tris/HCl, pH 8.0, 500 mM NaCl, 5 mM β-mercaptoethanol, 0.1% Triton X-100 and 8 M urea) and purified using metal chelating chromatography (Sepharose Fast Flow column; GE Healthcare). The rLIC12287 protein was refolded in a column by gradually removing urea (4 to 0 M). The efficiency of recombinant protein purification was evaluated by 12% SDS-PAGE. Fractions containing high concentrations of purified recombinant proteins were pooled and extensively dialysed against Tris/NaCl, pH 8.0 containing 0.1% glycine, then with PBS buffer containing 0.1% glycine. The concentrations of recombinant proteins were estimated by SDS-PAGE, by comparison with predetermined mass of BSA using densitometry analysis software GelQuant (DNR Bio-Imaging Systems).

Circular dichroism (CD) spectroscopy
Measurements were obtained by CD spectroscopy at room temperature in a Jasco J-810 spectropolarimeter (Japan Spectroscopic). CD spectroscopy of the far-UV spectrum for recombinant proteins in a 10 mM sodium phosphate buffer was performed. The spectra were measured and are presented as the averages of five scans recorded from 190 to 260 nm and the residual molar ellipticity was expressed in degree cm$^2$ dmol$^{-1}$. Spectral data were analysed with the software CAPITO [35] for estimation of the secondary structure content.

Microscopic agglutination test (MAT)
The MAT was performed as previously described [4] using a battery of 20 serovars of Leptospira spp. as antigens, as previously described [36]. The probable predominant serovar was considered to be the serovar with the highest dilution that could cause 50% agglutination. The MAT was considered negative when the titre was <100.

Production of polyclonal antiserum against Lsa19 and Lsa14 in mice
Female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 µg of the recombinant proteins adsorbed in 10% (v/v) Alhydrogel [2% Al(OH)$_3$; Brenntag Biosector], which was used as an adjuvant. Two subsequent booster injections were given at 2 week intervals with the same preparation described before. Negative control mice were injected with PBS plus Alhydrogel. Two weeks after each immunization, the mice were bled from retro-orbital plexus and pooled sera were analysed by ELISA for antibody titre determination.

Proteinase K (PK) accessibility assay
This assay was performed basically as the previously described assay [37]. In brief, suspensions of 5 ml live leptospires (L. interrogans serovar Copenhageni strain M20, $\sim 10^8$ cells ml$^{-1}$ per treatment) were harvested at 8000 g for 10 min at room temperature and washed once with PBS. Leptospires were re-suspended in 5 ml proteolysis buffer (10 mM Tris/HCl, pH 8.0, 5 mM CaCl$_2$) containing 25 µg PK ml$^{-1}$ (Sigma-Aldrich). Test tubes were incubated for 0 to 240 min, before the addition of 100 mM PMSF to stop PK activity. The suspensions were subsequently centrifuged at 11500 g for 5 min, washed twice with PBS and re-suspended in the same buffer for ELISA using antibodies against (1:50) Lsa19 (rLIC11122), Lsa14 (rLIC12287) and Dnak, as described below. Dnak is a cytoplasmic [38] leptomastigonal spiral, and was employed in our experiments as a negative control.

Immunoblotting assay
The purified recombinant proteins were loaded onto 15% SDS-PAGE and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare) in semi-dry equipment. Membranes were blocked with 10% non-fat dried milk and 1% BSA diluted in PBS containing 0.05% Tween 20 (PBS-T) and probed with anti-Lsa19 or anti-Lsa14 (1:1000) mouse polyclonal serum for 2 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000; Sigma) in PBS for 1 h. HRP-conjugated anti-His-tag mAbs (1:5000; Sigma) were also used. The protein reactivity was revealed by ECL reagent kit (GE Healthcare).

ELISA for detection of human antibodies
Human IgG antibodies against the proteins corresponding to Lsa19 and Lsa14 were detected by ELISA, as previously described [36]. Cut-off values were set at 3 SD above the mean $A_{492}$ values obtained from commercial normal human sera against the recombinant proteins.

Binding of Lsa19 and Lsa14 to extracellular matrix (ECM) and plasma components
Attachment of recombinant proteins to individual ECM macromolecules and plasma components was analysed according to a previously published protocol [39], with some modifications. Briefly, 96-well plates were coated with 1 µg laminin, collagen type I, collagen type IV, cellular and plasma fibronectin, elastin, vitronectin, human PLG, factor H, fibrinogen, C4BP and BSA (negative controls) in 100 µl PBS for 16 h at 4°C. The wells were washed three times with PBS-T and then blocked with 200 µl PBS-T containing 10% (w/v) non-fat dried milk for 2 h at 37°C. One microgram of each recombinant protein in 100 µl PBS was added to each well, and protein was allowed to attach to different substrates for 2 h at 37°C. After washing six times with PBS-T, bound recombinant proteins were detected by adding an appropriate dilution of mouse antiserum in 100 µl PBS. Dilutions of mouse antiserum against each recombinant protein were equalized based on the $A_{492}$ value of 1.0 in titration experiments. After three washings with PBS-T, 100 µl of PBS containing 1:5000-diluted HRP-conjugated rabbit anti-mouse IgG (Sigma-Aldrich) was added per well for 1 h at 37°C. In addition, anti-His mAbs were employed as protein-binding probes at a 1:5000 dilution rate. The wells were washed three times and o-phenylenediamine (Sigma-Aldrich) (1 mg ml$^{-1}$) in citrate phosphate buffer...
(pH 5.0) plus 1 µl H2O2 ml−1 was added (100 µl well−1). The reaction was allowed to proceed for 15 min then interrupted by the addition of 50 µl, 2 M H2SO4. Readings were taken at 492 nm in a microplate reader (MULTISKAN EX; Thermo Fisher). For statistical analyses, the binding of recombinant protein to ECM macromolecules and plasma components was compared with its binding to BSA, using Student’s two-tailed t-test, and the P-value was given in comparison with BSA, which was used as a negative control.

**Dose–response curves and Kd values**

ELISA plates were coated overnight with 1 µg laminin or PLG. For laminin, plates were blocked and increasing concentrations of purified Lsa19 (0–19 µM) and Lsa14 (0–5.5 µM) were added (100 µl well−1, in PBS) followed by incubation for 2 h at 37°C; for PLG, the concentration range of the proteins was 0–19 µM (Lsa19) and 0–3.0 µM (Lsa14). The assessment of bound protein was performed with polyclonal antiserum raised in mice against each protein followed by assessment of bound protein was performed with polyclonal antiserum raised in mice against each protein followed by HRP-conjugated anti-mouse IgG. The ELISA data, when reactions reached saturation point, were used to calculate the equilibrium dissociation constant (Kd), according to the described method (40) following the equation $K_d = \frac{(A_{\text{max}} - [\text{protein}])\cdot[A]}{[\text{protein}]}$, where A is the absorbance at a given protein concentration, A_{\text{max}} is the maximum absorbance for the ELISA plate reader (equilibrium), [protein] is the protein concentration and Kd is the equilibrium dissociation constant for a given protein concentration (ELISA data point).

**Antibody inhibition assay**

Ninety-six-well plates were coated with 100 µl of 10 µg ml−1 laminin or PLG solution for 16 h at 4°C. Plates were then washed with PBS-T and blocked for 2 h at 37°C. Before the next step, recombinant proteins were incubated for 1 h at 37°C with the corresponding antiserum anti-Lsa19 or anti-Lsa14, at 1:100 dilution in 100 µl PBS. After incubation, 1 µg of antibody-blocked recombinant protein was added to each well to interact with components for 2 h at 37°C. Plates were washed six times with PBS-T, and the bound recombinant protein was detected using HRP-conjugated mouse anti-His mAb (Sigma) at 1:5000 dilution for 1 h at 37°C. The interaction was revealed with o-phenylenediamine, as previously described. For statistical analysis, the percentage of recombinant protein binding was compared with its binding with untreated protein (100% binding) and proteins treated with pre-immune serum using the Student’s paired two-tailed t-test. A value of P<0.05 was considered to be statistically significant.

**Effect of recombinant protein denaturation on interaction with ECM and human plasma components**

The evaluation of protein denaturation on interaction with ECM and plasma components was performed by ELISA exactly as described above, but before adding to the plate, the proteins were heated for 10 min at 96°C. BSA was used as a negative control. For statistical analysis, the interaction of denatured recombinant proteins was compared with interaction of recombinant proteins that were not previously heated using the Student’s paired two-tailed t-test. A value of P<0.05 was considered to be statistically significant.

**Effect of ionic strength on the binding of recombinant proteins with PLG**

Microplates were coated with 1 µg well−1 recombinant proteins Lsa14, Lsa19 or BSA (negative control), in 100 µl PBS for 16 h at 4°C. Plates were washed three times with PBS-T and blocked for 2 h at 37°C with PBS-T containing 10% (w/v) skimmed milk powder. To evaluate the effect of ionic strength on the interaction of recombinant proteins with human PLG, increasing concentrations of NaCl (50–550 mM) were added together with 1 µg PLG, followed by 90 min incubation at 37°C. As a blank control, proteins were incubated without PLG. After three washes with PBS-T, the plates were incubated with anti-PLG (1:5000) for 1 h. After this time, plates were washed three times with PBS-T, followed by incubation with anti-mouse IgG antibody conjugated to peroxidase, for 1 h. For statistical analysis, the interaction of PLG with Lsa14 and Lsa19 was compared with PLG without addition of NaCl (only 50 mM low salt PBS, IsPBS) by Student’s paired t-test. P<0.05 was considered statistically significant.

**Binding characterization of Lsa14 and Lsa19 with PLG and laminin**

To determine the role of lysine residues of the proteins in the binding to PLG, each protein was added to the component well coated with increasing concentrations (0 to 20 mM) of the lysine analogue 6-aminocaproic acid (ACA; Sigma-Aldrich). In order to evaluate the role of the carbohydrate moiety of laminin on interaction with recombinant proteins, laminin was coated and treated with increasing concentrations of sodium metaperiodate (0–100 mM) followed by incubation with 10 µg ml−1 of Lsa19 or Lsa14. Binding was detected as described above.

**Plasmin enzymatic activity assay**

ELISA plates (96-well) were coated overnight with 10 µg ml−1 of each recombinant protein or BSA (negative control) in 100 µl PBS at 4°C. Plates were washed with PBS-T and blocked for 2 h at 37°C with PBS containing 10% (w/v) skimmed dried milk. The blocking solution was discarded, 10 µg human PLG ml−1 was added per well (100 µl), and incubation proceeded for 2 h at 37°C. Wells were washed three times with PBS-T, and 4 ng human urokinase-type PLG activator (uPA; Sigma-Aldrich) was added per well together with 0.4 mM of specific substrate d-valyl-leucyl-lysine-p-nitroanilide dihydrochloride (Sigma-Aldrich). Plates were incubated overnight, and substrate degradation was measured by taking readings at 405 nm.

**Statistical analysis**

Results are expressed as mean±SD. Student’s paired t-test was used to determine the significance of differences between means and P<0.05 was considered statistically
significant. Three or two independent experiments were performed, each one in triplicate.

RESULTS

In silico analysis of coding sequences

Two CDSs were selected from chromosome I of *L. interrogans* serovar Copenhageni genome sequences based on bioinformatics analyses. According to the LipoP program [19], the protein encoded by the gene LIC11122 has a lipoprotein signal peptide SpII (signal peptidase II) between amino acids 20 and 21, while LIC12287 has a signal peptide SpI (signal peptidase I) between amino acids 21 and 22. The signals are recognized by the enzymes SpI and SpII, which are responsible for cleavage and cleavage/lipidation, respectively. The Cello program [28, 29] predicts LIC11122 and LIC12287 to be located at cytoplasm/periplasm and periplasm/extracellular, respectively. According to the PSORT program [27], LIC11122 and LIC12287 are predicted to be located at the inner and periplasmic/outer membrane, respectively. Conserved protein domain family FecR has been detected in LIC11122 by SMART/PFAM programs [31, 32, 44]. SMART/PFAM programs predict TRL-like protein family for LIC12287; this family includes the TRL protein of unknown function. The TRL protein domain is found in a locus that includes several tRNAs [45]. Multiple sequence alignment was performed with the CLUSTAL W2 program [33], comparing LIC11122 (Fig. 1a) and LIC12287 (Fig. 1b) with the leptospiral sequences available in GenBank. The resulting phylograms show the high level of sequence similarity among serovars of *L. interrogans* and other pathogenic species for both LIC11122 (Fig. 1a) and LIC12287 (Fig. 1b). The coding sequence LIC11122 was not identified in saprophyte strains, whereas LIC12287 was found in the non-pathogenic *Leptospira biflexa* serovar Patoc, which is organized in a distant subdivision (Fig. 1b). Quantitative proteomic studies have determined that LIC11122 has 47 copies per cell, while the protein encoded by LIC12287 presented a copy number below the threshold, but is upregulated upon treatment with ciprofloxacin, reaching 5165 copies per cell [46]. These data suggest a possible role of this protein in resistance/maintenance of leptospirosis.

Cloning, expression and purification of the recombinant proteins

The coding sequences LIC11122 and LIC12287 were amplified without the signal peptide and cloned into pAE vector [34]. Protein expression of the recombinant proteins Lsa19 (rLIC11122) and Lsa14 (rLIC12287) was performed in *E. coli* BL21-SI and BL21 Star(DE3)pLysS, respectively. The protein Lsa19 was expressed partially in its soluble and partially in its insoluble form (Fig. 2a). Analysis of the samples after Lsa14 expression by SDS-PAGE showed that Lsa14 was expressed in its soluble/insoluble form (Fig. 2b). However, when Lsa19 and Lsa14 were membrane-blotted, and the proteins probed with mAb anti-His, Lsa19 was detected in its soluble/insoluble form (Fig. 2c), while Lsa14 was detected only in its insoluble form (Fig. 2d). Proteins were successfully purified from the soluble form (Lsa19) or

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**Fig. 1.** Sequence conservation among *Leptospira* spp. by CLUSTAL alignments. BLAST analysis was performed among sequences of amino acids available in GenBank database and leptospiral sequences were employed to perform CLUSTAL W multiple sequences alignments. The resulting phylograms show the high level of sequence conservation for LIC11122 (a) and LIC12287 (b) among pathogenic strains of *Leptospira*. LIC12287-similar sequences were identified in saprophyte strains, which are organized in more distant branches, showing lower degree of sequence identity.
Fig. 2. Analysis of recombinant proteins by SDS-PAGE, Western blotting and CD spectroscopy. (a) Lsa19 and (b) Lsa14 protein expression by NaCl-induced (75, 150 and 300 mM) E. coli BL21-SI and by IPTG-induced (0.01, 0.1 and 1.0 mM) E. coli BL21 Star(DE3)pLysS, respectively. The arrows in (a) and (b) indicate the expected protein bands of approximately 20 and 14.2 kDa molecular masses for Lsa19 and Lsa14, respectively. M, molecular protein mass marker (kDa); NI, non-induced total bacterial extract; I, total bacterial cell lysates after induction; S, soluble fraction of the induced culture; In, insoluble fraction of the induced culture in the presence of 8 M urea. (c, d) Western blotting analyses of the expressed recombinant proteins probed with monoclonal anti-polyhistidine antibody (1:5000). M, Molecular mass marker (kDa); 1, E. coli wild-type extract; 2, non-induced total bacteria extract; 3, total bacterial cell lysates after induction; 4, soluble fraction of the induced culture; 5, solubilized inclusion body. (e) Purified recombinant proteins after dialysis. 1, Lsa19; 2, Lsa14. (f, g) Western blotting analyses of recombinant proteins using the corresponding antiserum produced in mice (1:3000). M, Molecular mass marker (kDa); 1, Lsa19; 3, Lsa14; 2 and 4, Lsa23 (control). (h, i) CD spectra of Lsa19 and Lsa14 recombinant proteins after protein refolding. Far-ultraviolet CD spectra are shown as an average of five scans from 190 to 260 nm.
recovered from the inclusion bodies after 8 M urea solubilization (Lsa14), through metal-chelating chromatography (Fig. 2e). The recombinant protein bands were confirmed by Western blotting when the membranes were probed with polyclonal antibodies raised against each protein (Fig. 2f for Lsa19 and Fig. 2g for Lsa14). In these blots, another unrelated recombinant protein (Lsa23) was employed as a control (Fig. 2f, lane 2 and Fig. 2g, lane 4). Structural integrity of each purified protein was evaluated by CD spectroscopy (Fig. 2h, i). Analysis of the experimental spectra data by the CAPITO program [35] showed a predominance of β-strand for both proteins (51 % for Lsa19 and 50 % for Lsa14), indicating their suitability for additional studies.

**PK assessment of LIC11122 and LIC12287 CDSs**

We measured accessibility to PK by using a previously described assay [37]. It is anticipated that when bacteria are intact, the enzyme PK accesses only the surface proteins. The accessibility assay was performed at time intervals of 0, 1, 2 and 4 h of PK treatment. To confirm the integrity of the bacterial cell during interaction with PK, antiserum against the previously described leptospiral cytoplasmic protein DnaK [38] was used as control. Blank controls lacking primary antibody were also performed. After 1 h of treatment with PK, the proteins encoded by genes LIC12287 and LIC11122 showed a decrease of reactivity with their respective antiserum. Around 10 and 20 % of Lsa19 and Lsa14, respectively, were detected after 4 h. Leptospira probed with anti-DnaK showed no significant reduction of signal after 4 h incubation with PK (Fig. 3). These results suggest that LIC11122 and LIC12287 are surface exposed proteins.

**Recognition of Lsa19 and Lsa14 by antibodies present in leptospirosis human serum samples**

To examine whether Lsa19 and Lsa14 are recognized by antibodies in leptospirosis human serum samples, we assessed the reactivity of the proteins by measuring IgG antibodies present in paired serum samples at the onset (MAT−) and the convalescent (MAT+) phases of the disease, in comparison with the reactivity of commercial normal human serum samples, employed for cut-off calculation (see Methods). Antibodies recognized the protein Lsa19 in both phases of the disease, 63 and 55 % for MAT− and MAT+, respectively (Fig. 4a, b), while very low reactivity was detected with Lsa14, 20 and 35 % for MAT− and MAT+, respectively. The data suggest that the corresponding Lsa19 protein is probably expressed during leptospirosis. The higher reactivity observed for Lsa19 at the onset (MAT−) compared with the convalescent phase (MAT+) of the disease is unknown and a higher sample number should be used before any conclusion is drawn. The observed low reactivity of Lsa14 with antibodies in leptospirosis serum samples may be due to its low immunogenicity and/or low protein expression; the latter was previously reported by Malmström et al. [46].

**Adhesion of the recombinant proteins to ECM components**

We set out to evaluate whether the recombinant proteins Lsa19 and Lsa14 have the ability to interact with ECM components. Thus, laminin, elastin, vitronectin, collagen type I, collagen type IV, cellular fibronectin and the control proteins BSA and fetuin were immobilized on 96-well microtitration plates, and recombinant protein attachment to the components was assessed by ELISA, as previously described [47], using antiserum against each recombinant protein. Statistically significant interactions were observed with Lsa19, Lsa14 and laminin, when values were compared to the negative controls BSA and fetuin (Fig. 5a). These interactions were further confirmed using anti-His mAb (Fig. 5b) (*P<0.05). A dose-dependent response curve was observed for the binding of Lsa19 and laminin, reaching saturation level at 10 µM (Fig. 5c). Likewise, Lsa14 and laminin produced a dose--response curve when increasing concentrations of protein were added, and saturation was reached at 1.0 µM (Fig. 5d). The calculated dissociation equilibrium constants (K_D) for the recombinant proteins Lsa19 and Lsa14 are K_D=812.43±372.67 nM and K_D=51.71±44.11 nM, respectively. The affinity of Lsa14 for laminin is similar to the ones previously described for Lsa66 [48] and Lsa77 [39]. Lsa19 has an affinity to laminin similar to the adhesin described for Lsa26 which has a K_D=952.2±418.9 nM [49]. Treatment of recombinant proteins with the respective antiserum, prior to binding assays, produced a significant reduction in the binding of the proteins with laminin, 82 and 67 % for Lsa19 and Lsa14, respectively, at 1: 50 serum dilution (Fig. 6a, b). Pre-immune serum caused a modest, not statistically
significant, or no reduction in the binding. The data suggest that immunogenic epitopes are located close to the binding domains (Fig. 6a, b). The effect of protein structure on the binding with laminin was evaluated by heat denaturing the protein prior to the interaction assays. Untreated protein was employed as a control. The data show that a modest reduction, statistically not significant, was observed in the binding of Lsa19, while approximately 40 % reduction was detected with Lsa14 (Fig. 6c). The results indicate that Lsa19 protein structure is not relevant for the interaction with laminin, while Lsa14 structure seems to be important for binding to this ECM (Fig. 6c). Sodium metaperiodate oxidation of laminin sugar residues caused significant reduction only in the binding of Lsa19 (74, 60 and 62 % for 25, 50 and 100 mM of sodium metaperiodate, respectively), suggesting that laminin carbohydrate moieties are critical for the interaction with this protein (Fig. 6d).

**Binding of Lsa19 and Lsa14 to human plasma components**

Our group has reported that the interaction of *Leptospira* with PLG-generating plasmin degrades laminin and fibronectin, facilitating bacterial invasion [50, 51]. Moreover, we have shown that several proteins interact with human plasma components, including plasma fibronectin, PLG, fibrinogen and the complement regulators C4BP and factor H [37, 39, 49, 52–55]. We set out to evaluate whether the recombinant proteins could interact with the plasma molecules. Components (1 µg each) were coated onto ELISA plates and allowed to interact with recombinant proteins. The results showed that both Lsa19 and Lsa14 interact with PLG (P<0.05), when the reaction was probed with the corresponding polyclonal antibodies (Fig. 7a). The data were confirmed when the reactions were probed with anti-His antibodies (Fig. 7b). No binding was detected with the other tested molecules. The interaction of Lsa19 and Lsa14 with PLG was dependent on protein concentration, and saturation was reached with 10 µM (Fig. 7c) and 1.50 µM (Fig. 7d) for Lsa19 and Lsa14, respectively. The calculated $K_D$ for the reactions are 1465.20±1051.01 and 83.10±27.69 nM for Lsa19 and Lsa14, respectively. The affinity of the protein Lsa14 to PLG is the same order of magnitude as for the adhesin Lsa32, which presented a $K_D$ of 81.5±31.1 nM [56]. Incubating the proteins with their respective antisera, prior to the binding reactions, allowed us to assess the participation of immune epitopes of the proteins in the binding with PLG. The results show that at 1 : 50 serum dilution the interaction was reduced by 90 and 62 % for Lsa19 and Lsa14, respectively, suggesting that immunogenic epitopes of both proteins participate in their binding with PLG (Fig. 8a, b). Pre-immune serum had only a modest effect on the binding for both proteins. The influence of protein structure on the binding was evaluated by heat-denaturing the proteins prior to the attachment reaction. The data show a strong reduction in the binding of both proteins with PLG, suggesting that conformational epitopes are important for these interactions (Fig. 8c).

**Effect of NaCl concentration on the binding of Lsa14 and Lsa19 with PLG**

In order to assess whether ionic strength was important for the interaction of recombinant proteins with PLG, increasing NaCl concentrations, ranging from 50 to 550 mM in lSpBS, were employed and the bindings were evaluated. The
data demonstrate that 56% reduction in the binding of Lsa14 to PLG was detected with 150 mM NaCl, close to the physiological concentration, reaching a 69% decrease at the highest saturation NaCl concentration (550 mM) (Fig. 8d). Increasing NaCl concentrations had no effect on the binding of Lsa19 to PLG (Fig. 8d). The fact that the binding of Lsa14 to PLG decreased significantly at physiological concentration (120 mM) [57] suggests that this interaction may not be relevant in vivo.

**Binding of recombinant proteins to PLG via kringle domains**

The PLG kringle domains participate in the interaction with the lysine residues of the bacterial protein receptors. We have shown that these domains participate in the binding of PLG with intact live *L. interrogans* serovar Copenhageni strain M20 because the derivative and analogue of lysine, ACA, almost totally inhibited the binding. We evaluated the participation of lysine residues in binding of the recombinant proteins to PLG by the addition of ACA to the reaction mixtures. A strong, statistically significant inhibition of the interaction of Lsa19 and Lsa14 with PLG was observed when 2 mM ACA was added to the reaction (Fig. 8e). The results strongly suggest the participation of the kringle domains in the interaction of Lsa19 and Lsa14 proteins with PLG (*P<0.05*).

**Generation of plasmin from PLG-bound recombinant proteins**

PLG bound to leptospiral proteins can be activated to plasmin by host activators [50]. We have, thus, evaluated whether PLG bound to Lsa19 or Lsa14 could generate plasmin in the presence of a PLG activator. ELISA plates were coated with the recombinant proteins followed by incubation with PLG. Unbound PLG was removed and uPA was added. The reaction was incubated overnight, and the plasmin activity was...
evaluated by measuring the cleavage of the plasmin-specific chromogenic substrate at 405 nm. The PLG captured by the two recombinant proteins could be converted into plasmin, as indirectly demonstrated by specific proteolytic activity (Fig. 8f). Negative controls without PLG, uPA or chromogenic substrate showed no enzymatic activity.

**DISCUSSION**

Exploring genome sequences using the reverse vaccinology approach has identified several virulence factors of important pathogens, uncovering new antigen targets that are under preclinical and clinical investigation [58]. These include the factor H binding protein of *Neisseria meningitidis*, which evades complement-mediated killing in the presence of human serum [59], and putative virulence factors of *Streptococcus pneumoniae* [60]. The genome annotation of *L. interrogans* serovar Copenhageni predicted 184 CDSs to be exported to the leptospiral surface as lipoproteins [25]. Currently, 20 species of *Leptospira* have had their genomes sequenced between pathogenic, intermediate and saprophytic strains [61].

We have mined the genome of *L. interrogans*, searching for CDSs that are predicted to be located at the outer membrane or have signal peptide annotated as hypothetical or of...
unknown function. We believe that due to their location they have the ability to induce immune response in hosts. This strategy leads us to assign several proteins as putative leptospiral adhesins [39, 47, 49, 53, 62–65] and PLG-binding proteins, capable of generating plasmin, helping the bacterium to evade the immune system and to overcome tissue barriers [50–52, 66]. Furthermore, proteins that interact with fibrinogen inhibiting clot formation [52, 53] and proteins that bind complement regulators, helping leptospires to escape the immune system, have also been identified and characterized [37, 49, 54, 67].

In this study, we report two novel CDSs LIC11122 and LIC12287 annotated as hypothetical proteins, one of them as a probable lipoprotein (LIC11122). More recently, the CDS LIC11122 has been re-annotated as a sigma factor. Cellular localization experiments consistently indicate that both proteins are surface exposed and could mediate *Leptospira*–host interactions.

A dose-dependent, specific and saturable binding of Lsa14 and Lsa19 to immobilized laminin was observed, fulfilling the properties of a typical receptor–ligand interaction. Metaperiodate oxidation of laminin caused significant reduction in the binding activity of Lsa19, strongly suggesting the involvement of laminin carbohydrate moieties in the interaction. This result is in agreement with the previously published data pointing to an important role of

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Fig. 7. Interaction of Lsa19 and Lsa14 with plasma components. (a, b) Wells were coated with 1 µg of each plasma component or control proteins BSA and fetuin followed by incubation with 1 µg of recombinant protein Lsa19 or Lsa14 and per well. Binding was evaluated using polyclonal anti-Lsa19 or anti-Lsa14 (a) or anti-His mAb (b). Data represent the mean±SD for absorbance at 492 nm of three replicates for each protein and are representative of two independent experiments. For statistical analysis, the attachment of recombinant proteins to plasma components was compared with their binding to BSA by Student’s two-tailed t-test (*P<0.05). (c, d) Dose-dependent binding experiments with PLG. One microgram of PLG was incubated with increasing concentrations of each recombinant protein. Binding was detected using antiserum raised in mice against each recombinant protein at an appropriate dilution. BSA was included as a negative control. Each experiment was performed in triplicate and the results are expressed as the mean±SD absorbance at 492 nm for each point and are representative of two independent experiments.
Fig. 8. Characterization of interaction between recombinant proteins and PLG and plasmin generation. One microgram of Lsa19 (a) or Lsa14 (b) recombinant protein was incubated for 1 h at 37 °C with different dilutions of the corresponding antiserum (1 : 50 and 1 : 100); pre-immune serum or absence of serum was employed as negative control. The reaction mixture was added to the microplate wells previously immobilized with 1 µg of PLG. In (c), the proteins were heat-denatured at 96 °C for 10 min before being incubated with PLG. Detection of binding was carried out with anti-His mAb. Bars represent the mean of percentage of protein binding to immobilized PLG ± SD of three replicates and are representative of two independent experiments. (d) Effect of ionic strength on the binding of recombinant proteins with PLG. One microgram of Lsa19 or Lsa14 was coated on microplate wells and incubated with 1 µg of PLG and increasing concentrations of NaCl (50 to 550 mM) were added. As blank control, proteins were incubated without PLG. For statistical analysis, the interaction of PLG with Lsa14 and with Lsa19 was compared in presence and absence of NaCl (only 50 mM isPBS) by the Student’s paired t-test (*P<0.05). (e) Role of protein lysine residues in the protein–PLG interaction. Binding of Lsa19 and Lsa14 to
detected in the presence of PLG activator. The interaction was detected using antiserum produced in mice against each recombinant protein. Bars represent the mean ± SD of absorbance at 492 nm for three replicates and are representative of two independent experiments. Attachment of recombinant protein in the presence of ACA was compared with its production in mice against each recombinant protein. Bars represent the mean ± SD of absorbance degradation of three replicates for each condition and are representative of two independent experiments. Statistically significant binding was calculated in comparison with the negative control (BSA) (*P<0.05).

laminin carbohydrate moieties in the interaction with pathogens [68, 69]. The low dissociation constant observed for Lsa14 is at the same order of magnitude as the constant described for Lsa66 and Lsa77 with the same ligand [39, 48]. The Lsa19 has an affinity for laminin similar to Lsa26 with a K_D of 952±418 nM [49]. As reported for numerous pathogens, the presence of many adhesins in *Leptospira* is probably related to the bacterial invasion tactics, and we may anticipate the need of a multi-component vaccine against leptospirosis.

The fibrinolytic system PLG/plasmin has been considered to have a critical function during invasion and establishment of the infection for several micro-organisms such as *Streptococcus* spp., *Leishmania mexicana* and *Staphylococcus aureus* [70–75]. In the case of spirochaetes, the PLG/plasmin was studied with several species of *Borrelia* and with *Treponema denticola* and suggested to have an important role during infectiveness [76–80]. To date, several spirochaetal PLG-binding proteins have been described, and at the expense of host PLG-activator, resulted in plasmin generation. Plasmin is a serine protease that has the ability to degrade a large spectrum of substrates, including fibrin clots, connective tissue and components of ECM [81, 82]. Indeed, we have reported that plasmin generation triggers the upregulation of matrix metalloproteinase, increasing the proteolytic power of *Leptospira*, facilitating bacterial penetration through human umbilical vein endothelial cells [52]. The recombinant proteins Lsa19 and Lsa14 interact with PLG, dose-dependently on protein concentration, with saturable binding and at high affinity. Plasmin generation was detected in the presence of PLG activator. In *Leptospira*, plasmin generation can degrade immune mediators, such as C3b and IgG, and this property might contribute to bacterial attachment and tissue invasion. The protein corresponding to LIC11122 (Lsa19) is annotated as a sigma factor. Moreover, this protein is predicted to be partially cytoplasmic in its location. Together, these findings suggest that Lsa19 may be a novel moonlighting protein of *Leptospira*, acting intracellularly as a sigma factor and functioning on the cell surface as an adhesin and a PLG-binding protein.

In summary, we report the characterization of two novel leptosporal proteins selected from genome sequences of *L. interrogans* serovar Copenhageni, encoded by LIC11122 and LIC12287 genes. The protein corresponding to LIC12287 (Lsa14) was previously genome-annotated as a hypothetical protein of unknown function, and we may now tentatively assign this protein as a putative leptospiral adhesin and PLG-binding protein, having the potential to contribute to bacterial attachment and tissue invasion. The protein corresponding to LIC11122 (Lsa19) is annotated as a sigma factor. Moreover, this protein is predicted to be partially cytoplasmic in its location. Together, these findings suggest that Lsa19 may be a novel moonlighting protein of *Leptospira*, acting intracellularly as a sigma factor and functioning on the cell surface as an adhesin and a PLG-binding protein.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Ethical statement

All animal studies were approved by the Ethical Committee for Animal Research of the Instituto Butantan, Sao Paulo, SP, Brazil, under protocol no. 1192/11. The Committee for Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation (COBEA). This work was evaluated by the Ethics Committee on Human Research (CEPSH) of the Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, SP, Brazil, which certified that the work does not involve human manipulation to warrant approval, as the ethical principles required by the Committee under the 658/14 protocol.

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