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Research Article

RESEARCH ARTICLE

The recombinant LIC10508 is a plasma fibronectin, plasminogen, fibrinogen and C4BP-binding protein of *Leptospira interrogans*

Gabriela H. Siqueira[1,†], Aline F. Teixeira[1,2,†], Luis G. Fernandes[1,2], Gisele O. de Souza[3], Karin Kirchgatter[4], Eliete C. Romero[5], Silvio A. Vasconcellos[3], Monica L. Vieira[1] and Ana Lucia T. O. Nascimento[1,2,*,‡]

[1]Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503–900, Sao Paulo, SP, Brazil, [2]Programa de Pós-Graduação Interunidades em Biotecnologia, ICB, USP, Avenida Prof. Lineu Prestes, 1730, 05508–900, Sao Paulo, SP, Brazil, [3]Laboratório de Zoonoses Bacterianas do VPS, Faculdade de MedicinaVeterinária e Zootecnia, USP, Avenida Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, Sao Paulo, SP, Brazil, [4]Nucleo de Estudos em Malária, Superintendência de Controle de Endemias-SUCEN/IMT-SP, USP, Av. Dr. Enéas de Carvalho Aguiar 470, CEP 05403-000, Sao Paulo, SP, Brazil and [5]Centro de Bacteriologia, Instituto Adolfo Lutz, Avenida Dr. Arnaldo, 355, CEP 01246–902, Sao Paulo, Brazil

[*]**Corresponding author:** Centro de Biotecnologia, Instituto Butantan, Avenida Vital Brazil, 1500, 05503–900, Sao Paulo, SP, Brazil. Tel: (5511) 26279829; E-mail: ana.nascimento@butantan.gov.br
[†]These authors have contributed equally to this work.
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[‡]Ana Lucia T. O. Nascimento, http://orcid.org/0000-0003-4851-0870

ABSTRACT

Leptospirosis is a zoonosis caused by pathogenic *Leptospira spp*. In this study, we report that the recombinant proteins LIC10507, LIC10508 and LIC10509 are recognized by confirmed leptospirosis serum samples at both phases of the disease. The recombinant rLIC10508 and rLIC10507 are plasminogen (PLG)-binding proteins, capable of generating plasmin in the presence of a PLG activator. The proteins bind to PLG in a dose-dependent and saturable manner, fulfilling host–ligand interaction. Furthermore, rLIC10508 interacts with fibrinogen (Fg), plasma fibronectin and C4b binding protein (C4BP). The binding of rLIC10508 to Fg decreases the fibrin clotting in a thrombin-catalyzed reaction. The incubation with 4 μM of protein promoted 40% inhibition upon clotting formation. C4BP bound to rLIC10508 retained its cofactor activity for factor I promoting the cleavage of C4b protein, which may reduce the membrane attack complex formation. Although these proteins have high amino acid sequence similarity, rLIC10508 is the most talented of the three, a behavior that might be explained by its unique putative 3D structure, whereas structures of rLIC10507 and rLIC10509 are very similar. Plasmin generation (rLIC10507 and rLIC10508), together with decreasing fibrin clot formation (rLIC10508) and impairment of the complement system (rLIC10508) may help the bacteria to overcome host defense, facilitating the infection process.

Keywords: *Leptospira*; leptospirosis; plasmin; fibrinogen; C4BP; host interactions

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INTRODUCTION

Considered as a neglected infectious disease of human and veterinary concern, leptospirosis is caused by pathogenic species of the genus *Leptospira* (Levett 2001; Bharti *et al.* 2003). Human infection occurs mainly through contact with wild or domestic infected animals, or exposure to contaminated soil or water (Faine *et al.* 1999; Plank and Dean 2000). It is estimated that more than 500 000 cases of leptospirosis occur annually, and some studies indicate that the disease can represent 20–40% of febrile illnesses of unknown cause (Ko, Goarant and Picardeau 2009). Human leptospirosis incidence is low in developed countries and is mostly associated with water-related recreational or occupational activities (Haake *et al.* 2002; Hartskeerl, Collares-Pereira and Ellis 2011). In developing countries, the high incidence is associated with the lack of adequate sanitation. Additionally, leptospirosis has a great economic impact in the agricultural industry since the disease affects livestock inducing abortions, stillbirths, infertility, reduced milk production and death (Plank and Dean 2000).

The leptospires enter the host mainly via intact or damaged skin or mucosa, followed by rapid dissemination through the bloodstream. Soon after that, bacterial clearance from blood and migration into host tissues occurs, the kidneys and liver being the preferential sites for colonization. Infection produces a wide spectrum of clinical symptoms, varying from subclinical to severe manifestations. The most severe conditions are known as Weil's syndrome and leptospirosis pulmonary hemorrhage syndrome (LPHS), reaching up to 15% and 70% mortality rates, respectively (de Carvalho *et al.* 1992; Nicodemo *et al.* 1997; Segura *et al.* 2005).

The molecular mechanisms of pathogenicity and virulence of leptospires are still to be elucidated. Although the leptospiral genome, functional genomics and host–pathogen interactions are being actively investigated, to date only a few virulence factors have been demonstrated, and the origin of pathophysiological leptospirosis symptoms and severity of disease remain unknown (Ren *et al.* 2003; Nascimento *et al.* 2004b; Bulach *et al.* 2006; Picardeau *et al.* 2008; Cinco 2010; Evangelista and Coburn 2010; Adler *et al.* 2011).

We have previously shown that the proteins LIC10507, LIC10508 and LIC10509 are found on the outer membrane of pathogenic leptospires, expressed during infection of experimentally infected guinea pigs, and they promoted the upregulation of the adhesion molecules ICAM-1 and E-selectin in human umbilical vein endothelial cells (HUVECS) (Gomez *et al.* 2008). The recombinant protein LIC10508 (rLIC10508) was also shown to be recognized by antibodies present in the serum of leptospirosis patients, suggesting its expression during human infection (Gamberini *et al.* 2005, Evangelista *et al.* 2014b). In a PLG-binding assay, the rLIC10509 showed no interaction with human PLG (Vieira *et al.* 2010). In addition, by phage display screening, rLIC10508 has been found to bind endothelial and epithelial cells *in vitro* (Evangelista *et al.* 2014b).

In the present work, we aimed to further investigate the possible roles of the leptospiral proteins LIC10507, LIC10508 and LIC10509 in the host–pathogen interplay. We show that the proteins rLIC10507 and rLIC10508 bind to human plasminogen, generating protein-bound plasmin activity after addition of exogenous activator. Additionally, the protein rLIC10508 interacts with the serum components plasma fibronectin, C4BP and fibrinogen, showing the ability to interfere with fibrin clot formation and complement system regulation. Our data provide further support for the possible involvement of LIC10507 and, especially, LIC10508 proteins in pathogenesis.

MATERIALS AND METHODS

Biological components

Plasma fibronectin, fibrinogen, plasminogen and the control protein fetuin were purchased from Sigma-Aldrich (St Louis, MO, USA). Plasma fibronectin, plasminogen and fibrinogen were isolated from human plasma. C4BP and factor H, purified from normal human serum, were purchased from Complement Technology, Inc. (Tyler, TX, USA).

In silico sequence analysis

Predicted coding sequences (CDSs) LIC10507, LIC10508 and LIC10509 were identified on the *L. interrogans* serovar Copenhageni database http://bioinfo03.ibi.unicamp.br/leptospira/ (Nascimento *et al.* 2004b). The SMART, http://smart.embl-heidelbergde/ (Letunic, Doerks and Bork 2015), PFAM, http://www.sanger.ac.uk/Software/Pfam (Finn *et al.* 2006) and LipoP, http://www.cbs.dtu.dk/services/LipoP/ (Juncker *et al.* 2003) 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/ (Sievers *et al.* 2011). For the 3D structural analysis of proteins, LIC10507, LIC10508 and LIC10509 amino acid sequences of each protein were submitted to the online tool Phyre v.2 (Protein Homology/analogY Recognition Engine, http://www.sbg.bio.ic.ac.uk/phyre2) and the highest score alignments were selected for further studies. 3D structures of the outputs were obtained at Protein Data Bank (http://www.rcsb.org/pdb/), imported to Pymol software (The PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC.), a tool for molecular visualization, in which protein structures were spatially aligned.

DNA recombinant techniques

Predicted CDSs were amplified from *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 genomic DNA, without signal peptides, using the PCR technique and complementary primer pairs. Gel-purified PCR fragments were cloned into the pAE (Ramos *et al.* 2004) expression vector, which allows the expression of recombinant proteins with a minimal 6xHis-tag at the N-terminus. Detailed cloning, expression and purification of the recombinant proteins have been previously described (Gomez *et al.* 2008).

Microscopic agglutination test

The microscopic agglutination test (MAT) was performed according to Faine *et al.* (1999). In brief, an array of serovars of *Leptospira* spp. as antigens were employed: Australis, Autumnalis, Bataviae, Canicola, Castellonis, Celledoni, Copenhageni, Cynopteri, Djasiman, Grippotyphosa, Hardjo, Hebdomadis, Icterohaemorrhagiae, Javanica, Panama, Patoc, Pomona, Pyrogenes, Sejroe, Shermani, Tarassovi and Wolfii. All the strains were maintained in EMJH liquid medium (Difco, Detroit, MI, USA) at 29°C. A laboratory-confirmed case of leptospirosis was defined by demonstration of a fourfold micro-agglutination titer rise between paired serum samples. The probable predominant

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.

Reactivity of recombinant proteins with serum samples of human leptospirosis and of unrelated febrile diseases

Human IgG antibodies against rLIC10507, rLIC10508 and rLIC10509 were evaluated by enzyme-linked immunosorbent assay (ELISA). Serum samples of negative and positive MAT from confirmed leptospirosis patients and of febrile unrelated diseases, were diluted 1:100 and evaluated for total IgG using peroxidase-conjugated anti-human IgG antibodies (1:5000, Sigma-Aldrich, USA). Commercial healthy human sera were used as control and cutoff values were set at three standard deviations above the mean optical density at 492 nm wavelength (OD_{492nm}).

Antiserum production against recombinant proteins

BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg of the recombinant proteins mixed with 10% (v/v) Alhydrogel (2% $Al(OH)_3$, Brenntag Biosector, Frederikssund, Denmark) as an adjuvant. Negative control mice were injected with phosphate-buffered saline (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 titres.

Attachment of recombinant proteins to plasma components

In order to access the protein binding to individual macromolecules of plasma components, plasma fibronectin, fibrinogen, human PLG, factor H, C4BP and bovine serum albumin (BSA), gelatin or fetuin, as negative control proteins, 1 μg each in 100 μL PBS, were individually allowed to adhere in the wells for 3 h at 37°C. After this incubation period, the wells were washed three times and blocked with 200 μL of PBS containing 0.05% Tween (PBS-T) and 10% non-fat dry milk for 1 h at 37°C, followed by an overnight incubation at 4°C. The blocking solution was discarded and 1 μg of each recombinant protein in PBS was allowed to attach to the different substrates for 90 min at 37°C. After extensive washing, bound recombinant proteins were detected using polyclonal mouse antiserum produced against recombinant proteins. The appropriate serum dilution was determined by titration curve with the corresponding recombinant protein and the value of 1.0 at OD_{492nm} was employed. Incubation proceeded for 1 h at 37°C. After three washings with PBS-T, 100 μL of PBS containing horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000) was added per well for 1 h at 37°C. The wells were washed three times and o-phenylenediamine dihydrochloride (OPD; 1 mg mL^{-1}) in citrate phosphate buffer (pH 5.0) plus 1 μL mL^{-1} H_2O_2 was added (100 μL per well). The reaction was allowed to proceed for 15 min and interrupted by the addition of 50 μL of 4 N H_2SO_4. Readings were taken at 492 nm in a microplate reader (Multiskan EX; Thermo Fisher Scientific, Rockford, IL, USA). In another assay, we employed HRP-conjugated anti-His tag monoclonal (Sigma-Aldrich) antibodies, to confirm the bindings with His-tag proteins.

Dose-dependent interaction

Dose–response curves were performed to evaluate the attachment of recombinant proteins to plasma components. For this, microplates were coated overnight at 4°C with 100 μl of 10 μg mL^{-1} of each plasma component. Plates were washed and blocked for 2 h at 37°C with PBS-T containing 10% (w/v) non-fat dry milk. Increasing concentrations of the purified recombinant proteins in PBS (100 μL per well) were added and incubated for 90 min at 37°C. The assessment of bound proteins was performed using antiserum raised against the recombinant proteins, followed by HRP-conjugated goat anti-mouse IgG, as described above. The ELISA data were used to calculate the equilibrium dissociation constant (K_D) according to the method previously described (Lin *et al.* 2009) based on the equation: $K_D = (A_{max} \times [protein])/A) - [protein]$, where A is the absorbance at a given protein concentration, A_{max} 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).

Characterization of protein binding to plasminogen

To determine the role of lysine residues in plasminogen–recombinant protein interactions, an ELISA assay was performed as previously described (Vieira *et al.* 2009). Briefly, the lysine analog 6-aminocaproic acid (ACA) (Sigma-Aldrich) was added together with recombinant proteins at a final concentration of 2 mM or 20 mM to the plasminogen-coated wells. The detection of bound recombinant proteins was performed as described above.

Plasmin enzymatic activity assay

ELISA microplates were coated overnight with 10 μg mL^{-1} recombinant proteins or BSA (negative control) in 100 μL PBS at 4°C. After blocking (2 h at 37°C) with PBS containing 10% (w/v) of non-fat dry milk, 10 μg mL^{-1} of human plasminogen was added and incubation continued for 2 h at 37°C. Wells were washed three times with PBS-T, and then 3 U of human urokinase plasminogen activator (uPA) (Sigma-Aldrich) was added together with 0.4 mM of plasmin-specific substrate D-valyl-leucyl-lysine-*p*-nitroanilidedihydrochloride (Sigma-Aldrich). Plates were incubated overnight at 37°C and substrate degradation was measured by taking the readings in a microplate reader at 405 nm.

Fibrin clot formation inhibition assay

The reaction of thrombin-catalyzed fibrin clot was performed in the presence of recombinant protein to evaluate its inhibitory effect on fibrin clot formation. Different concentrations (0–4 μM) of recombinant protein were resuspended in 150 mM NaCl and incubated for 2 h at 37°C with 1 mg mL^{-1} of fibrinogen. ELISA plates were coated with 90 μL per well of recombinant protein plus fibrinogen and 10 μL per well of thrombin (5 U mL^{-1}). The fibrin clot formation was measured every 2 min for 30 min by an ELISA plate reader at OD_{595nm}. The positive control of the reaction employed thrombin plus fibrinogen, without recombinant protein, while in the negative control thrombin was omitted. Reduction of the fibrin clot formation was calculated by comparing the value of the last reading point, at 30 min, with the positive control (100%). Analysis was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons.

Cofactor assay

Cofactor activity of C4BP bound to immobilized rLIC10508 was evaluated through cleavage of C4b by factor I. Two micrograms of recombinant protein were coated on microplate wells overnight at 4°C. After three washes with PBS-T, plates were blocked with PBS/BSA 3% for 2 h at 37°C. Then 2 μg per well of C4BP were allowed to attach to recombinant protein for 90 min. After washing, 1 μg of C4b and 500 ng of factor I were added per well and incubation proceeded for 16 h at 37°C. Reaction mixtures were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane (Hybond ECL; GE Healthcare, UK) in semi-dry blotting equipment (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). Membrane was blocked overnight at 4°C with 10% non-fat dried milk in PBS-T and then incubated with a murine monoclonal antibody against human C4d diluted 1:1000 for 2 h at room temperature. After washing, the membranes were incubated with HRP-conjugated anti-mouse IgG (1:5000; Sigma) in PBS-T with non-fat milk for 1 h. The reactivity was revealed by SuperSignal West Dura Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA) using Carestream Molecular Imaging (Equilab, Whitestone, NY, USA) connected to Gel Logic 2200PRO.

Ethics statements

All animal studies were approved by the Ethics Committee of the Instituto Butantan, Sao Paulo, SP, Brazil, under protocol number 890/12. 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 the Instituto Adolfo Lutz collection, Sao Paulo, Brazil, and were donated for research purposes. Serum samples from patients with other infectious diseases were obtained from the collections of the Laboratorio de Imunoepidemiologia, SUCEN, Sao Paulo, Brazil; Laboratorio de Protozoologia, IMT/USP, Sao Paulo, Brazil (sera from patients with Chagas' disease); Laboratorio de Virologia, IMT/USP, Sao Paulo, Brazil (sera from patients with human immune deficiency virus (HIV) infection and dengue); and Nucleo de Estudos em Malária, SUCEN/IMT/USP, Sao Paulo, Brazil (sera from patients with malaria). The Ethics Committee for Research with Human Beings of ICB/University of Sao Paulo has considered that this project is exempt of ethics approval because it does not involve human manipulation.

Statistical analysis

All results are expressed as the 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. Where stated, analysis was performed using one-way ANOVA, followed by Tukey post-test for pairwise comparisons.

RESULTS

Conservation of coding sequences among *Leptospira* spp.

The sequences LIC10507, LIC10508 and LIC10509, previously identified in the genome of *L. interrogans* serovar Copenhageni, encode for putative outer membrane proteins, having a signal peptide type II (SPII) with a cleavage site between amino acids 24–25 for LIC10507 and 19–20 for LIC10508 and LIC10509 (Gomez *et al.* 2008). In 2008, with a limited number of sequences available, BLAST analysis of these coding sequences showed similarity only to a sequence present in the genome of *L. interrogans* serovar Lai and absence in the saprophyte strain *Leptospira biflexa* (Gomez *et al.* 2008). An up-to-date multiple sequence alignment by the Clustal Omega program was performed to compare the CDSs LIC10507 (Fig. 1A), LIC10508 (Fig. 1B) and LIC10509 (Fig. 1C) with the sequences available in GenBank (Sievers *et al.* 2011). The resulting phylograms show that the three coding sequences are well conserved among pathogenic strains of *Leptospira* and absent in intermediate and saprophyte strains (Fig. 1). The coding sequence LIC10509 (Fig. 1C) features more proximity among the sequences analyzed, when compared with the coding sequences LIC10507 and LIC10508, which showed sequences organized in a more distant branch (Fig. 1A and B).

Reactivity of rLIC10507, rLIC10508 and rLIC10509 with serum samples of individuals with leptospirosis and unrelated febrile diseases

To determine whether the *L. interrogans* proteins encoded by genes LIC10507, LIC10508 and LIC10509 are recognized by sera of infected individuals, we assessed the reactivity of the recombinant proteins measuring IgG antibodies present in paired serum

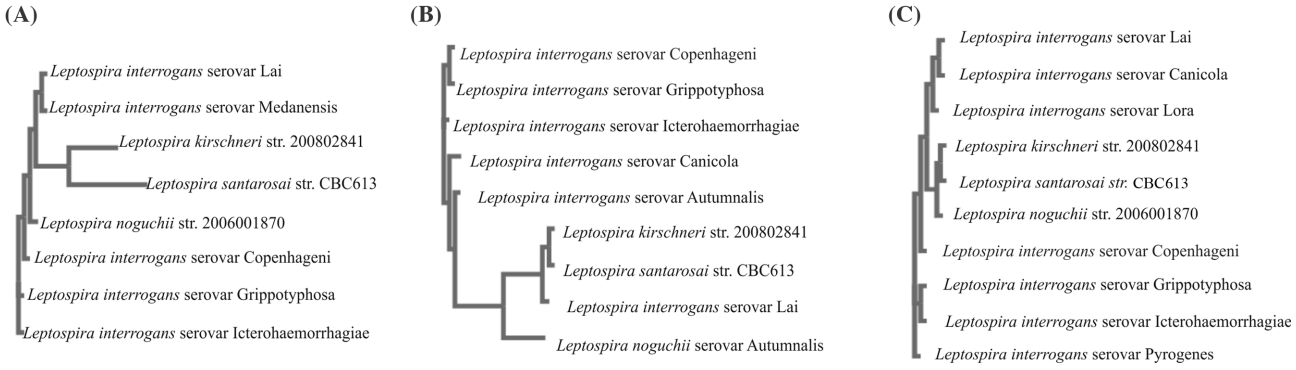

Figure 1. Analyses of LIC10507, LIC10508 and LIC10509 conservation among strains of *Leptospira* spp. Blast analyses were assessed among sequences of amino acids available in GeneBank database and leptospiral sequences were employed to perform Clustal Omega sequence alignments. The resulting phylograms show the proximity of LIC10507 (**A**), LIC10508 (**B**) and LIC10509 (**C**) sequences among pathogenic strains of *Leptospira*.

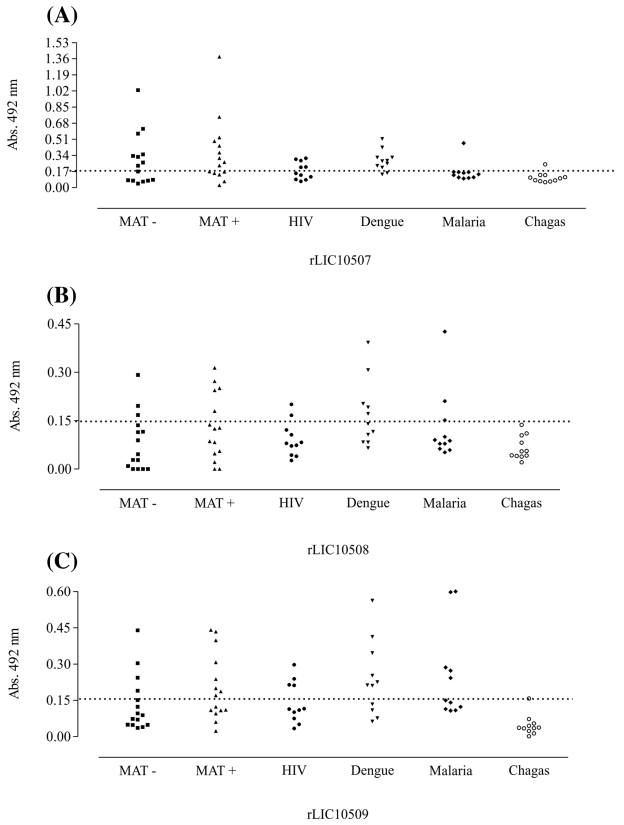

Figure 2. Reactivity of recombinant proteins with human serum samples. The reactivity of the recombinant proteins rLIC10507 (**A**), rLIC10508 (**B**) and rLIC10509 (**C**) was evaluated by measuring IgG antibodies present in serum samples of individuals diagnosed with leptospirosis, in both phases of the disease (at the onset (MAT−) and at the convalescent (MAT+) phase),with human serum samples from patients diagnosed with unrelated febrile diseases, and with normal serum. The cutoff values (dashed lines) are defined as the mean + 3 SD obtained with normal human sera.

samples at the onset (MAT−) and at the convalescent (MAT+) phase of disease. We employed a total of 15 samples of MAT-negative and of MAT-positive patients sera. The cutoff value was calculated with healthy human serum samples. Our data show that specific human IgG antibodies recognized all recombinant proteins in both phases of the disease (Fig. 2). Responders, 53% and 60%, presented IgG antibodies against rLIC10507 in the early and convalescent phase of the disease, respectively (Fig. 2A). The recombinant protein rLIC10508 had a lower reactivity, presenting only 20% and 33% of responders for MAT− and MAT+, respectively (Fig. 2B), while a total of 33% and 53% of responders were found to the protein rLIC10509 corresponding to the early and convalescent phase of the disease, respectively (Fig. 2C). These data confirmed previous results showing the reactivity of rLIC10508 with confirmed leptospirosis serum samples (Gamberini *et al.* 2005) and expand the results to rLIC10507 and rLIC10509, suggesting the expression of the three proteins during leptospirosis. We also analyzed the reactivity of recombinant proteins with serum samples of patients with unrelated infectious diseases, due to the non-specific clinical symptoms of leptospirosis. Thus, 11 serum samples of dengue, malaria, Chagas' disease and HIV were evaluated. The results obtained show that all proteins showed cross-reactivity with serum samples of the febrile diseases tested, especially with dengue fever (Fig. 2). Therefore, the rLIC10507, rLIC10508 and rLIC10509 proteins exhibited low specificity for leptospirosis infection and are not protein candidates for diagnosing the disease.

Binding of recombinant proteins to human plasma components

The interaction of rLIC10507, rLIC10508 and rLIC10509 with extracellular matrix molecules was evaluated previously and did not show consistent results, as similar binding intensity of the components was also observed with the control proteins (Gomez *et al.* 2008). Since our group has shown that several proteins interact with human plasma components, including plasma fibronectin, PLG, fibrinogen and the complement regulators C4BP and factor H (Vieira *et al.* 2010; Domingos *et al.* 2012; Fernandes *et al.* 2012; Souza *et al.* 2012; Oliveira *et al.* 2013; Siqueira *et al.* 2013), we evaluated whether the recombinant proteins could also interact with these components *in vitro*. The protein rLIC10509 was not evaluated for PLG binding, since negative data were previously described (Vieira *et al.* 2010). A screening-binding test of the components with the proteins showed that rLIC10507 and rLIC10508 bind to human PLG when compared with the proteins used as control (BSA, gelatin and fetuin) (Fig. 3A and 3B, respectively). In addition, rLIC10508 interacted with fibrinogen, plasma fibronectin and C4BP (Fig. 3B). In these assays, the reaction was probed with polyclonal serum against each protein. Further confirmation of these interactions was obtained with anti-His monoclonal antibodies (data not shown). A dose–response assay was also performed to quantify the binding of recombinant proteins to PLG. The results showed that rLIC10507 (Fig. 4A) and rLIC10508 (Fig. 4B) bind to PLG in a dose-dependent and saturable manner when increasing concentrations of the recombinant proteins were allowed to react with a fixed amount of PLG (1 μg). The estimated dissociation equilibrium constants (K_D) are 54.3 ± 30.5 nM and 38.3 ± 50.1 nM for rLIC10507 and rLIC10508, respectively.

Although the interaction of rLIC10508 with plasma fibronectin (Fig. 4C), fibrinogen (Fig. 4D) and C4BP (Fig. 4E) is dose-dependent, saturation was not reached up to the 4500 nM recombinant protein concentration (maximum protein concentration achieved), suggesting low binding affinity of rLIC10508 with these components.

Plasmin generation from PLG-bound recombinant proteins

Previous works have reported that enzymatically active plasmin is generated by PLG bound to leptospiral protein receptors when its activator is present (Vieira *et al.* 2010; Souza *et al.* 2012; Siqueira *et al.* 2013; Fernandes *et al.* 2014; Teixeira *et al.* 2015). To assess whether PLG bound to rLIC10507 and rLIC10508 proteins generates proteolytic activity, a microplate was coated with each recombinant protein and incubated with PLG. Following this, uPA-type PLG activator was added, together with a plasmin-specific chromogenic substrate, and PLA activity was indirectly evaluated by measuring the cleavage of the PLA-specific chromogenic substrate at 405 nm. The data show that PLG bound to both recombinant proteins, in the presence of activator, could be converted into active plasmin (Fig. 5A). BSA, which does not bind PLG, control reactions which lacked one of the components, employed as a negative control of the reaction, did not show enzymatic activity.

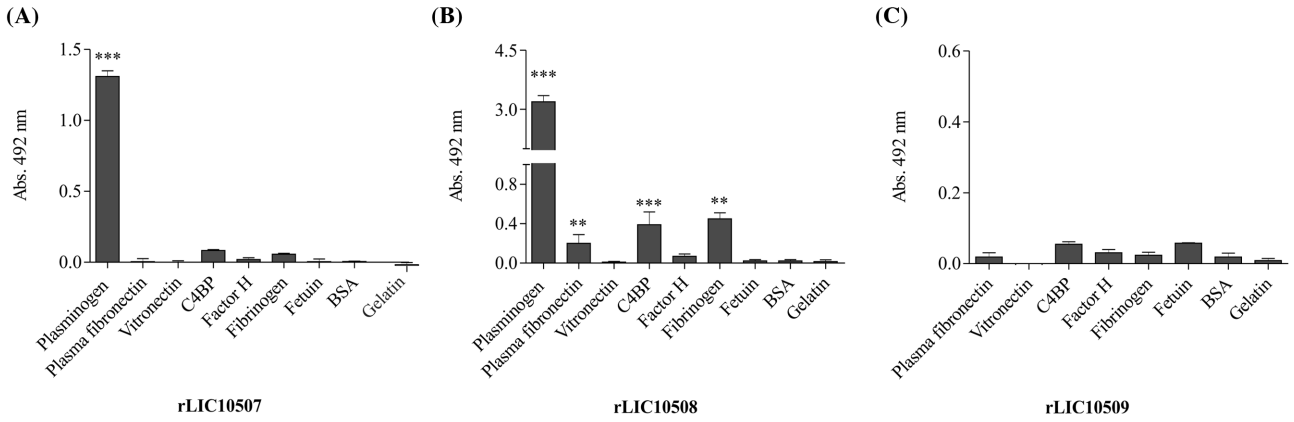

Figure 3. Recombinant proteins binding to plasma components. Human purified plasminogen (PLG) (in **A** and **B**), plasma fibronectin, vitronectin, C4BP, factor H and fibrinogen (10 μg mL^{-1}) were individually coated onto ELISA plates. Bovine serum albumin (BSA), fetuin and gelatin were used as negative controls for non-specific binding. The recombinant proteins rLIC10507 (A), rLIC10508 (B) and rLIC10509 (**C**) were added to each well (10 μg mL^{-1}) and antibodies against recombinant protein detected binding. All data represent mean ± the standard deviation from three replicates and are representative of three independent experiments. For statistical analyses, the binding of recombinant proteins to the plasma components was compared with their binding to fetuin and BSA by the two-tailed *t*-test (**$P < 0.01$ and ***$P < 0.001$).

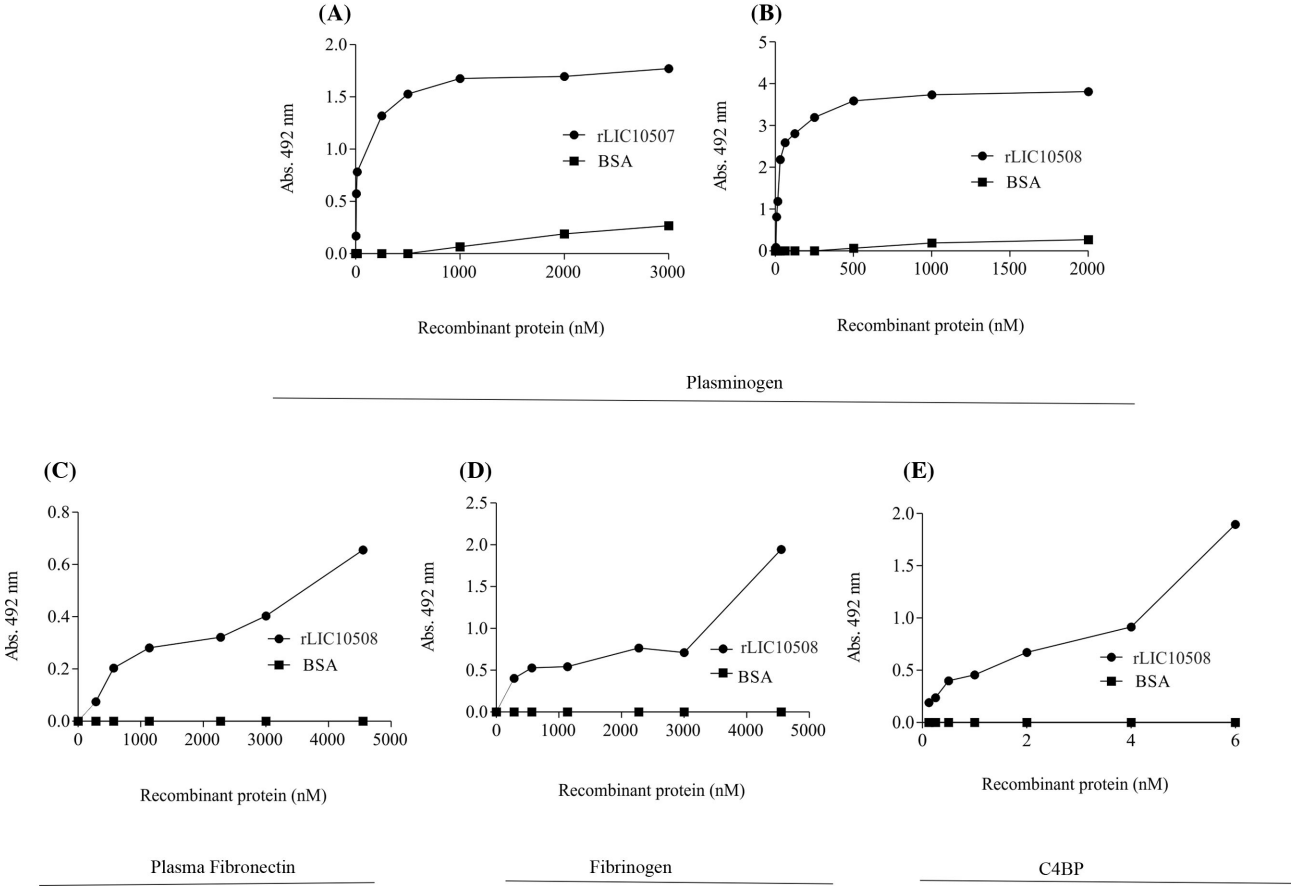

Figure 4. Evaluation of recombinant proteins' dose-dependent interactions to plasma components. Dose–response assay was performed with plasminogen and recombinant proteins rLIC10507 (**A**) and rLIC10508 (**B**). The recombinant protein rLIC10508 was evaluated with plasma fibronectin (**C**), fibrinogen (**D**) and C4BP (**E**). One microgram of these components was immobilized onto ELISA plates and increasing concentrations of each recombinant protein were added. Binding was detected by using antiserum specific for each protein. BSA was included as a negative control. Each experiment was performed in triplicate and expressed as the mean of absorbance at 492 nm.

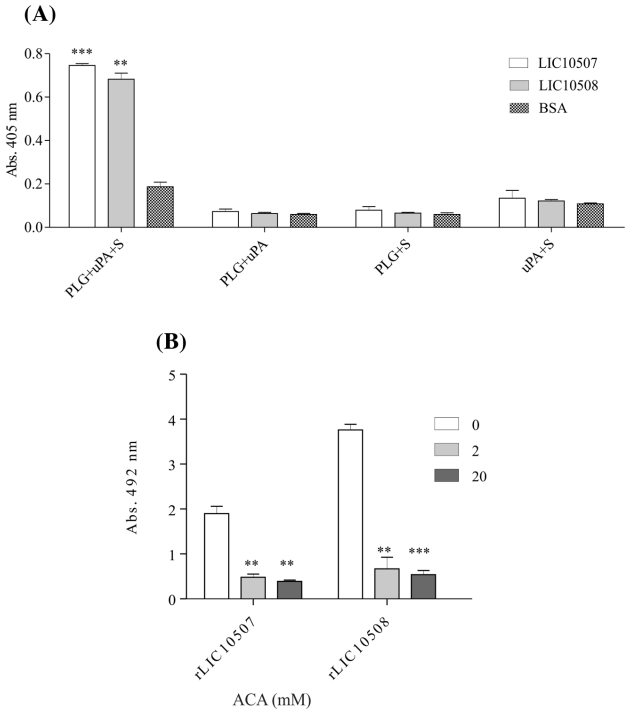

Figure 5. Plasmin (PLA) generation and the role of lysine residues in the interaction between the recombinant proteins and PLG. **(A)** PLA generation by activation of PLG bound to recombinant proteins was measured indirectly by the cleavage of the specific PLA colorimetric substrate using a modified ELISA. The immobilized recombinant proteins received the following treatment: PLG, uPA and the specific PLA substrate (PLG + uPA + S) or controls lacking one of the three components of the reaction. BSA was employed as negative control. Bars represent mean ± SD as a measure of the relative substrate cleavage from three replicates for each experimental group and are representative of two independent experiments. Statistically significant differences in comparison with BSA are shown (**$P < 0.01$ and ***$P < 0.001$). In **(B)**, binding of recombinant proteins to PLG was carried out in the presence or absence (no inhibition) of the lysine analogue ACA. Bound proteins were detected by antiserum raised in mice. Bars represent mean ± SD of triplicate determinations and are representative of two independent experiments. For statistical analyses, the attachment of the recombinant protein in the presence of ACA was compared with the binding to PLG without ACA (no inhibition) by the two-tailed *t*-test (**$P < 0.01$ and ***$P < 0.001$).

The recombinant proteins bind to PLG via kringle domains

It is well known that PLG kringle domains mediate interactions with lysine residues of the bacterial receptors (Lahteenmaki, Kuusela and Korhonen 2001). Vieira and collaborators (2009) have shown that these domains participate in the binding of PLG with intact live *L. interrogans* serovar Copenhageni L1-130 cells, since the analog of lysine, ACA, almost totally inhibited binding. On this basis, the participation of lysine residues in the binding of PLG to recombinant proteins was evaluated by the addition of ACA to the reaction mixture. The obtained results show that when 2 mM or 20 mM ACA was added to the reaction, the binding of the rLIC10507 and rLIC10508 proteins to PLG (Fig. 5B) was almost completely abolished. These results strongly suggest the participation of the kringle domains in the interaction of rLIC10507 and rLIC10508 proteins to PLG.

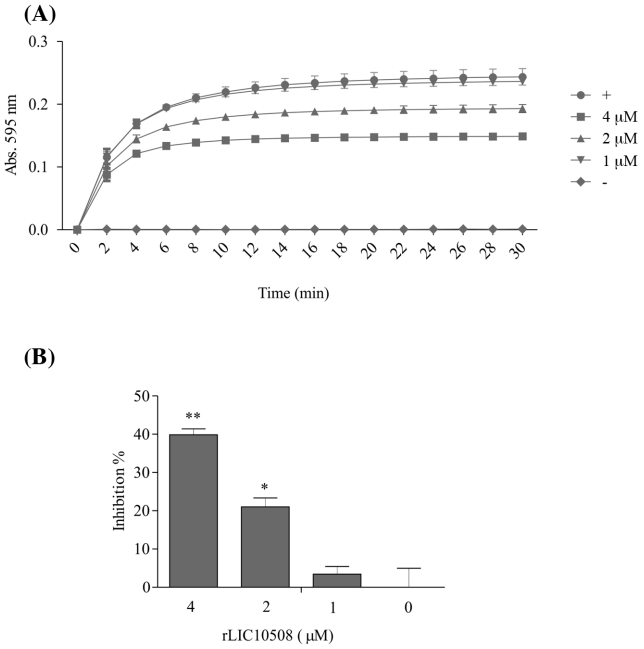

Figure 6. Inhibition of fibrin clot formation by rLIC10508. **(A)** Different concentrations (0–4 μM) of rLIC10508 were pre-incubated with fibrinogen (1 mg mL^{-1}) and 5 U mL^{-1} of thrombin were placed into ELISA plates. The positive control of the reaction employed fibrinogen plus 5 U mL^{-1} thrombin, while in the negative control thrombin was omitted. The fibrin clot formation was measured every 2 min. In **(B)** the percentage inhibition is shown. The measurements were performed in triplicate and curves are representative of two independent experiments. Significance was assessed by Students two-tailed *t* test *$P < 0.05$ and **$P < 0.01$.

Inhibition of fibrin formation by rLIC10508 bound to fibrinogen

As rLIC10508 showed an ability to bind fibrinogen (Fg), we decided to investigate whether this interaction was capable of interfering in fibrin clot formation in the thrombin-catalyzed reaction. A control lacking thrombin was used as a negative for clot formation and the treatment, in which no recombinant protein was added, used to infer the maximal clotting formation. Different recombinant protein concentrations (1–4 μM) were pre-incubated with 1 mg/mL of Fg at 37°C for 2 h. The reaction mixtures were placed onto ELISA plates and 5 U mL^{-1} of thrombin was added per well. The fibrin clot formation was evaluated every 2 min. The measurements were performed in triplicate and representative curves of two independent experiments are shown in Fig. 6A. The higher the protein concentration, the less fibrin clotting was observed. Incubation with 4 μM of rLIC10508 promoted 40% inhibition of clot formation ($P < 0.01$), followed by 21% inhibition after incubation of Fg with 2 μM of recombinant protein ($P < 0.05$) (Fig. 6B). No statistically significant inhibition was obtained when Fg was incubated with 1 μM rLIC10508.

Cofactor activity of C4BP bound to rLIC10508

It is well known that C4BP bound to live leptospires cells maintains its cofactor activity for factor I helping in the cleavage of C4b (Barbosa *et al.* 2009). Because rLIC10508 was able to interact with this complement regulator, we decided to evaluate if C4BP bound to rLIC10508 would keep its functionality as a cofactor. For this, rLIC10508 was coated onto a microplate well and incubated

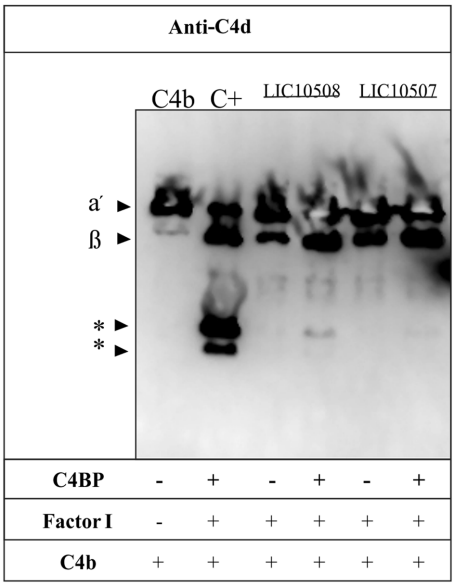

Figure 7. Cofactor activity of C4BP bound to recombinant protein. Recombinant LIC10508 was coated (2 μg per well) on microplates wells and allowed to interact with the C4BP. C4b (1 μg) and factor I (500 ng) were added and the reaction mixtures were incubated for 16 h at 37°C. The products of cleavage of C4b were detected by western blotting with anti-human C4d monoclonal antibodies. Recombinant LIC10507 was used as a negative control, and the reaction without recombinant protein was used as positive control (C+). The symbols represent: alpha and beta chain from C4b and * cleavage products of C4b.

with purified C4BP. The protein rLIC10507, which did not bind to C4BP, was used as negative control, while reaction lacking the recombinant protein was used as positive control (C+). Unbound C4BP was washed away, and C4b was added together with factor I. Reaction mixtures were separated by SDS-PAGE; proteins were blotted onto membrane and probed with anti-C4d. A 45 kDa C4d fragment resulting from C4b factor I-mediated cleavage was visualized in the positive control reaction (Fig. 7). A similar cleavage product, but less pronounced, was detected when rLIC10508 was added to the reaction mixture. No cleavage product was observed when C4BP was absent or when the rLIC10507 protein was used. This result shows that the cofactor activity of C4BP is retained when bound to rLIC10508.

In silico 3D structure prediction of the native proteins

In order to gain insights into the 3D structure of the predicted proteins, amino acids of each sequence were submitted to the online tool Phyre and the highest score alignments were selected for comparison of 3D structures. They were aligned, and as depicted in Fig. 8, it is possible to observe a spatial coincidence between the structures of LIC10507 and LIC10509. LIC10508 presented a discrepancy in structure compared with the other two proteins analyzed and might explain the distinct binding performance observed with this protein.

DISCUSSION

The analysis of *L. interrogans* serovar Copenhageni L1–130 genomic sequence revealed the presence of a genomic region in chromosome I (position 574512–619577) that has distinguishing features, some of them suggesting the acquisition through horizontal transfer, including higher G + C content, flanking by insertion sequences and predicted coding to several membrane-associated proteins with internal repeat sequences (Nascimento *et al.* 2004a,b; Gamberini *et al.* 2005). Positioned in this region are three putative lipoproteins encoded by the genes LIC10507, LIC10508 and LIC10509, described as hypothetical proteins of unknown function. The recombinant LIC10508 was recognized by confirmed leptospirosis serum sample (Gamberini *et al.* 2005). Moreover, rLIC10507, rLIC10508 and rLIC10509 were reported to stimulate the cell adhesion molecules ICAM and E-selectin in HUVECs, but adhesion of these proteins to extracellular matrixes was unspecific (Gomez *et al.* 2008). Indeed, low binding activity of LIC10508 to fibronectin was detected by protein microarray screening assay (Pinne, Matsunaga and Haake 2012). Several studies have comprehensively demonstrated that leptospires can adhere *in vitro* to some cell lines (Thomas and Higbie 1990; Liu *et al.* 2007; Figueira *et al.* 2011; Zhang *et al.* 2012; Evangelista *et al.* 2014a). More recently, the putative lipoprotein LIC10508 was identified by *in vitro* phage display for clones that interact with endothelial cells and may contribute to bacterial dissemination (Evangelista *et al.* 2014b).

After adherence, the next step for the pathogens is to overcome the barriers imposed by epithelial tissues and extracellular matrixes. In this sense, the proteolytic activity achieved by subversion of host proteases by pathogens has been reported to be important during several bacterial infections (Lahteenmaki, Kuusela and Korhonen 2001). We have previously reported that *Leptospira* interact with PLG and, in the presence of uPA, generate plasmin, a broad-spectrum serine protease component of the fibrinolytic system (Vieira *et al.* 2009, 2012). To date, several leptospiral PLG-binding proteins have been identified, which may mediate the interaction of the bacteria with this proteolytic system (Vieira *et al.* 2010; Mendes *et al.* 2011; Domingos *et al.* 2012; Fernandes *et al.* 2012, 2014; Souza *et al.* 2012; Nogueira *et al.* 2013; Wolff *et al.* 2013; Siqueira *et al.* 2013; Teixeira *et al.* 2015; Vieira and Nascimento 2015; Domingos *et al.* 2015).

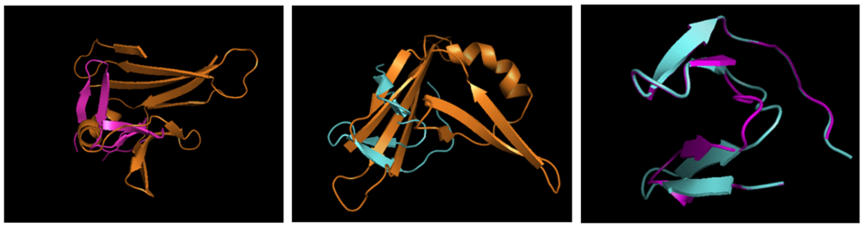

Figure 8. 3D structure predictions of the three proteins and analysis of their spatial configurations. Amino acids sequences of protein LIC10507, LIC10508 and LIC10509 were submitted to the online tool Phyre v.2 and the highest score alignments selected. 3D structures of the outputs, obtained at Protein Data Bank, were imported to Pymol software and structures were spatially aligned.

In our previous work, we show that rLIC10509 did not interact with PLG (Vieira *et al.* 2010). Here we report that proteins rLIC10507 and rLIC10508 are PLG-binding proteins. Plasmin bound to the leptospiral surface degrades IgG and C3b, probably decreasing opsonophagocytosis by immune competent cells (Vieira *et al.* 2011). C3b cleavage by the leptospiral moonlight protein factor Tu, previously identified as PLG-binding protein (Vieira *et al.* 2012), was recently reported (Wolff *et al.* 2013). The protein E (PE) of *Haemophilus influenza* (Barthel *et al.* 2012), Sbi and Efb of *Staphylococcus aureus* (Koch *et al.* 2012) and the surface protein BBA70 of *Borrelia burgdorferi* (Koenigs *et al.* 2013) are examples of PLG-binding proteins generating plasmin, which these pathogens employ to interfere with the alternative complement pathway. Leptospires endowed with plasmin activity penetrate HUVECs cells more efficiently than in its absence (Vieira *et al.* 2013). Thus, it is possible that LIC10508, together with LIC10507 may contribute to bacterial invasion and immune evasion through plasmin generation.

Furthermore, the protein rLIC10508 shows notable features. It interacts with Fg and with complement regulator of the classical pathway C4BP. The interaction of rLIC10508 with human Fg resulted in inhibition of fibrin clotting by thrombin-catalyzed reaction. We have demonstrated that virulent *L. interrogans* serovar Copenhageni Fiocruz L1-130, attenuated *L. interrogans* serovar Copenhageni M-20 and non-pathogenic saprophytic *L. biflexa* serovar Patoc Patoc 1 strains have the capacity to bind Fg and partially inhibit fibrin clot formation (Oliveira *et al.* 2013). Several leptospiral proteins including LigB (Lin *et al.* 2011) and OmpL1, among others (Oliveira *et al.* 2013) have been reported to interact with human Fg. Thus, it is possible that rLIC10508 may act in concert with other reported Fg-binding proteins to mediate the interaction of Fg to *Leptospira*. Decreasing fibrin clot formation would cause an imbalance of the coagulation cascade that may contribute to the bleeding process, helping the pathogen dissemination.

Pathogenic *Leptospira* showed various degrees of serum resistance while the saprophytic strain was serum sensitive (Cinco and Banfi 1983; Meri *et al.* 2005). Furthermore, it has been shown that serum resistance was correlated with the binding of FH, the alternative pathway main regulator of the complement system (Meri *et al.* 2005). Several leptospiral proteins have been reported to bind factor H (Verma *et al.* 2006; Siqueira *et al.* 2013). Moreover, studies have indicated that serum-resistance leptospires are capable of binding C4BP, the classical pathway complement regulator, while the saprophyte strain is not (Barbosa *et al.* 2009; Cinco 2010). The LIC10508 protein, together with the previously identified leptospiral C4BP-binding proteins (Barbosa *et al.* 2010; Domingos *et al.* 2012; Souza *et al.* 2012; Siqueira *et al.* 2013), can assist the bacteria to counteract the killing by the complement system, an important step for bacteria spreading.

Although these proteins have a high degree of amino acid sequence similarity (Gomez *et al.* 2008), rLIC10508 is the most versatile of the three in mediating interactions with host components. This unique behavior might be explained by its divergent 3D structure, the structures of LIC10507 and LIC10509 being very similar.

Altogether, our results support the presence of LIC10507, LIC10508 and LIC10509 during infection. The proteins LIC10507 and LIC10508 are most probably involved in the leptospiral pathogenesis. The increase of proteolytic power of *Leptospira* by plasmin generation may help the pathogen to overcome tissue penetration through extracellular matrix degradation and to escape the immune system by decreasing opsonophagocytosis. Moreover, the protein LIC10508 may promote bleeding and immune evasion by interacting with human Fg and C4BP, respectively. Thus, LIC10508 is a talented leptospiral protein capable of binding to several host components that may facilitate the establishment of a leptospirosis infection.

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