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In silico analysis and functional characterization of a leucine-rich repeat protein of *Leptospira interrogans*

João P. Gaspar [a,b], Maria B. Takahashi [a], Aline F. Teixeira [a], Ana L.T.O. Nascimento [a,*]

[a] Laboratório de Desenvolvimento de Vacinas, Instituto Butantan, Avenida Vital Brazil, São Paulo, SP, Brazil
[b] Programa de Pós-Graduação Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil

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ABSTRACT

Pathogenic spirochetes of the genus *Leptospira* are the causative agent of leptospirosis, a widely disseminated zoonosis that affects humans and animals. The ability of leptospires to quickly cross host barriers causing infection is not yet fully understood. Thus, understanding the mechanisms of pathogenicity is important to combat leptospiral infection. Outer membrane proteins are interesting targets to study as they are able to interact with host molecules. Proteins containing leucine-rich repeat (LRR) domains are characterized by the presence of multiple regions containing leucine residues and they have putative functions related to host-pathogen interactions. Hence, the present study aimed to clone and express the recombinant protein encoded by the LIC11098 gene, an LRR protein of *L. interrogans* serovar Copenhageni. *In silico* analyses predicted that the target protein is conserved among pathogenic strains of *Leptospira*, having a signal peptide and multiple LRR domains. The DNA sequence encoding the LRR protein was cloned in frame into the pAE vector, expressed without mutations in *Escherichia coli* and purified by His-tag chromatography. Circular dichroism (CD) spectrum showed that the recombinant protein was predominantly composed of β-sheets. A dose-dependent interaction was observed with cellular and plasma fibronectins, laminin and the complement system component C9, suggesting a possible role of the protein encoded by LIC11098 gene at the initial stages of infection.

1. Introduction

L. interrogans is the main causative agent of human leptospirosis, a zoonosis of worldwide distribution and global importance. Humans are the accidental and final host of leptospires (Bharti et al., 2003). *Rattus norvegicus*, which lives in a condition of sinanthropism with humans are considered the main vector of human leptospirosis (Martins and Spink, 2020). The infection may occur through direct contact with the urine of these infected animals or by indirect contact, i.e., with contaminated water and/or soil (Bharti et al., 2003). Contact of the pathogen with human mucosa or broken skin characterize the first stage of infection. The incubation period of the disease has an average of 5–14 days but can range from 2 to 30 days (Haake and Levett, 2015). Infected individuals often show a diverse range of clinical manifestations, from asymptomatic to severe conditions. Severe forms are known as Weil's syndrome (Vinetz, 1996) and pulmonary hemorrhagic syndrome (McBride et al., 2005) and account for about 10–50 % of leptospirosis mortality cases.

During the pathophysiology of the disease, leptospires interact with host molecules, such as extracellular matrix (ECM) components and cell receptors. Leptospires then rapidly disseminate through the blood stream, evade the complement attack and migrate into host tissues, where the kidneys and liver are the preferential sites for colonization (Daroz et al., 2021). Thus, external membrane proteins are essential for the success of infection and disease establishment and are therefore interesting targets to fight the disease.

Leucine-rich repeat (LRR) proteins are characterized by the presence of multiple regions containing several leucine residues. These repeated sequences form a classical horse-shoe shaped solenoid structure, in which the outer surface is formed by α-helices, while the interior consists of β-sheets (Kobe and Kajava, 2001). This structure favors the interaction with multiple substrates, mainly proteins (Bella et al., 2008). *L. interrogans* encodes at least 20 LRR-containing proteins, while *L. borgpetersenii* has 5, and the saprophyte *L. biflexa* genome contains only one annotated LRR protein-encoding gene (Eshghi et al., 2019; Picardeau, 2017; Picardeau et al., 2008). The fact that a high number of LRR proteins are concentrated among the pathogenic species suggests that these proteins may participate in the pathogenesis of *Leptospira*.

The crystal structures of 4 LRR proteins of *L. interrogans* have been

* Corresponding author.
E-mail address: ana.nascimento@butantan.gov.br (A.L.T.O. Nascimento).

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solved: LIC12234, LIC10831, LIC11098 and LIC12759. Like the majority of other *L. interrogans* LRR proteins, they possess no other domains than LRR. More precisely, LIC11098 and LIC12759 have 17 LRR domains each, while LIC10831 possesses 13 and LIC12234 only 8 of these domains. All of them have the characteristic α/β-solenoid structure, where each LRR unit interacts with the adjacent repeat by hydrogen bonding, thereby stabilizing the solenoid structure. The leucine residues are present in conserved positions on the protein structure and their side chains, buried in the interior of the structure, also play a role in maintaining protein topology (Miras et al., 2015). Additionally, it is well known that all LRR proteins have special N- and C- terminal regions that protect the hydrophobic interior of the solenoid (Bella et al., 2008). Recently, one of them, LIC10831 was characterized as a human VE- and E-cadherin binding protein (Eshghi et al., 2019).

Our aim in the present study was to further examine the LRR protein of *L. interrogans* encoded by the LIC11098 gene, by producing its recombinant protein in *E. coli* and assessing its putative role in leptospiral host-pathogen interaction.

2. Material and methods

2.1. Biological components

Laminin (human placenta, L6274), cellular fibronectin (human foreskin fibroblasts, 2518), collagen IV (human placenta, C5533), collagen I from rat tail and calf skin (rat tail, C3867, and calf skin, C8919) were used to represent the ECM components, and E-cadherin (human recombinant, 5085) was used as cell receptor. The plasma components tested were plasma fibronectin (human plasma, F2006), vitronectin (human plasma, V8379), fibrinogen and plasminogen (PLG, human plasma, P7999) (Sigma-Aldrich). Components of the complement system C3b, C4b, C4-binding protein, C6, C7, C8 and C9 (Complement Technology, USA) and factor H purified from human plasma (341274, EMD Chemicals, USA) were also used.

2.2. Leptospira strain and serum samples

The pathogenic *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 was cultured at 30 °C under aerobic conditions in liquid EMJH media (BD Difco, USA) supplemented with 10 % *Leptospira* enrichment EMJH medium (BD Difco, USA). Human serum samples from patients diagnosed with leptospirosis (at both onset and convalescent phase) or other febrile diseases (dengue, malaria, Chagas disease and HIV) were provided from the serum collection of of FIOCRUZ/RJ/Brazil and – Instituto Pasteur, SP/IMT-SP, USP, Brazil. These samples were for research purposes only.

2.3. In silico analysis

The coding sequence of LIC11098 was obtained from *L. interrogans* serovar Copenhageni NCBI databank (Protein Data Bank (PDB) - ID: 4U08). LIC11098 gene was analyzed *in silico* for prediction of its conformation, domains, cellular localization, signal peptide and cleavage site. CELLO, PSORTb and LipoP were used for prediction of cellular localization (Juncker et al., 2003; Yu et al., 2004, 2010). For comparative purposes, the coding sequences from LRR proteins LIC11098 (present work) and LIC10831 (Eshghi et al., 2019; Miras et al., 2015) were used to perform an alignment by Clustal Omega (PDB: 4U08 and 4U06, respectively). The aligned sequences were then utilized in the ESPript 3.0 program (Robert and Gouet, 2014) and combined with PDB information of the LRRs' secondary structure. An alignment figure of these two *L. interrogans* LRRs was created. Complementarily, LIC11098 and LIC10831 data obtained from PDB were used to acquire a tridimensional structure and LRRs alignment using the CE algorithm of PyMOL 2.5.4 (Educational License).

2.4. Cloning and recombinant protein expression

A pair of oligonucleotides was designed based on the genome of *L. interrogans* serovar Copenhageni. The sequence from signal peptide was not considered and restriction sites were added to the 5′ ends of the oligonucleotides for cloning into the pAE vector (Table 1).

Amplicons for the gene LIC11098 were obtained by PCR on a Veriti™ 96-Well Fast Thermal Cycler (Applied Biosystems, USA). The process was performed using Q5® High-Fidelity DNA Polymerase (New England Biolabs, USA), and PCR conditions were according to the manufacturer's recommendations. Subsequently, the DNA sample was purified according to the descriptions of the IlustraTM GFXTM PCR DNA and Gel Band Purification Kit (GE Healthcare, UK)

LIC11098 DNA insert and pAE vector were digested and ligated by T4 DNA ligase (1 U/μL) (Invitrogen, USA). Samples of the insert and the vector were added in nuclease-free water for 10 min at 65 °C, followed by incubation at 25 ºC for 5 min. Then, the reactions were incubated in ice for 5 min. Finally, T4 ligase and its respective buffer were added. The reaction was performed for 2 h at 37 °C. *E. coli* strain DH5α was used for recombinant plasmid cloning. Positive clones were submitted to sequencing by the chain termination method using the primers T7 ("forward" TAATACGACTActATAGGG) and pAER ("reverse" CAGCAGCCAACTCAGTTCCT). The sequencing was performed by automatic sequencer ABI (PE Applied Biosystems, USA) (Sanger et al., 1977). The amplification reaction was performed according to the recommendations of Applied Biosystems. The chromatograms obtained were analyzed with the help of SnapGene Viewer Version 5.1.7 (GSL Biotech; available at www.snapgene.com).

E. coli strain BL21 Star (DE3) pLysS (Invitrogen) was used for expression of LIC11098 recombinant protein (rLIC11098). The bacteria were transformed with recombinant plasmids by thermal shock and incubated at 37 °C under agitation. The induction was performed after bacteria growth reached $OD_{600\ nm} = 0.6$ for 16 h at 18 °C, under agitation, in the presence of 1 mM IPTG. The *E. coli* cells were harvested by centrifugation at 4500 x *g*. The pellet was resuspended in lysis buffer (10 mM Tris pH 8.0, 150 mM NaCl, 200 μg/mL lysozyme, 2 mM phenylmethylsulfonyl fluoride and 1 % Triton X-100) and left on ice for 15 min. Samples were sonicated for 10 min using a Branson 450 Digital Sonifier (BransonS Corp., USA). The samples were centrifuged at 12,500 x *g* for 10 min, and the soluble fraction was used for protein purification.

2.5. Recombinant protein purification

The expression system used adds a sequence of 6 histidine residues (6XHis) in the N-terminal portion of the protein (Ramos et al., 2004). This enabled purification with a Sepharose chelating column (GE Healthcare) loaded with Ni^{2+} and detection of recombinant protein by anti-polyhistidine monoclonal antibodies (anti-His). After loading the soluble fraction on the column, contaminant proteins were washed away by addition of buffers containing low concentrations of imidazole (5, 20, 40, 60 and 100 mM), and the protein was eluted with a buffer containing 500 mM imidazole. Protein was dialyzed against 20 mM Tris pH 8.17 and 500 mM NaCl to remove imidazole concentrations in the samples. Fisherbrand™ Regenerated Cellulose Dialysis Tubing membrane (Fisherbrand, USA) was used for this step. The estimated concentration of the

Table 1
Oligonucleotides used to amplify LIC11098 gene from the genome of *L. interrogans* serovar Copenhageni.

Primer (5' - 3')	Restriction enzymes*	Nucleotide sequence	Amplicon
Forward	*Bam*HI	AAT**GGATCC**CAATCTAACGAAGCGCAA	1194 pb
Reverse	*Nco*I	GGCGG**CCATGG**TCATTCAAAATAAATAAT	

* Restriction enzymes sites are highlighted in bold and underlined in nucleotide sequence above.

recombinant protein was determined with the Bradford reagent (Sigma-Aldrich, USA), and samples were analyzed by SDS-12 % polyacrylamide gel electrophoresis (SDS-PAGE).

2.6. Structural evaluation of rLIC11098 by circular dichroism (CD)

Circular dichroism spectra were measured using a 1-mm optical path cell at intervals of 0.5 nm/s and were captured on a Jasco J-810 spectropolarimeter (Japan Spectroscopic, Japan). To perform the technique, the sample was dialyzed against sodium-phosphate buffer (0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4), pH 7.5, at 4 °C for 16 h. The spectra were expressed in terms of residual molar ellipticity and were taken between 185 and 250 nm. The experimental data were analyzed by the BeStSel program (Micsonai et al., 2022), which calculates the secondary structure content of the protein.

2.7. Antiserum production in mice against the rLIC11098 recombinant protein

Four female BALB/c mice (4–6 weeks old) were immunized subcutaneously with 10 μg of recombinant protein adsorbed in 10 % (vol/vol) of Alhydrogel [2 % $Al(OH)_3$; Brenntag Biosector], used as adjuvant. Two subsequent booster injections were given at 2-week intervals with the same preparation. Before and two weeks after each immunization, the mice were bled from the retro-orbital plexus, and pooled sera were analyzed by ELISA for determination of antibody titers. Prior to experiments, anti-recombinant protein sera were adsorbed with a suspension of *E. coli* to suppress the reactivity of anti-*E. coli* antibodies. All animal studies were approved by the Ethical Committee for Animal Research of the Instituto Butantan, Brazil, under CEUA protocol No. 5940180422. The Committee for Animal Research in Instituto Butantan adopts the guidelines of the Brazilian College of Animal Experimentation (COBEA).

2.8. Immunological detection of rLIC11098 by Western blotting

The recombinant protein was analyzed by SDS-PAGE and transferred to a nitrocellulose membrane "Hybond-ECL" (GE Healthcare) in a semi-wet system for 17 min and 30 s at 1.3 A/cm^2 in Tris-glycine plus 1.85 % SDS (transfer buffer). The membrane was then incubated with the blocking solution (PBS-T-10 % milk) and kept at 4 ºC for 16 h. The membrane was washed 3 times with PBS-T and incubated with the primary antibodies produced in mouse against LIC11098 (1:20,000). After 1 h of incubation at room temperature, the membrane was again washed 3 times for 5 min with PBS-T and incubated with mouse anti-IgG antibody conjugated with peroxidase for 1 h at room temperature. Six washes were performed for 5 min to remove excess secondary antibody. Reactivity was detected using the Amersham™ ECL Select™ Western Blotting Detection Reagent (GE Healthcare) kit.

2.9. rLIC11098 reactivity with serum samples from leptospirosis and other febrile diseases by ELISA

rLIC11098 was immobilized at a concentration of 500 ng/well in PBS in ELISA plates (High Binding, Costar, Corning Incorporated, USA). After incubation for 16 h, excess protein was removed by 3 washes with PBS-T. The plates were then blocked with PBS-T-10 % milk and incubated for 1 h at 37 °C. Leptospirosis, malaria, dengue, Chagas disease and HIV serum samples were used as primary antibody, and in the case of leptospirosis samples, two phases of the disease were examined: at the onset (MAT-) and convalescent (MAT+). The detection of the interaction between recombinant protein and disease serum samples was performed by using IgG anti-human produced in mice (1:5000) conjugated with peroxidase. The assay was revealed as described before. As a comparison, the cutoff value was used, and it was calculated using the mean (m) and the standard deviation (SD) of commercial normal human serum (Sigma-Aldrich), according to the following equation: $c = m + (3x\ SD)$, in which all samples that achieved a value above the cutoff were considered reactive.

2.10. LIC11098 coding sequence (CDS) detection from secreted protein fraction (SPF)

SPF were obtained according to the protocol described by Teixeira and colleagues (Teixeira et al., 2022). A culture of *L. interrogans* serovar Copenhageni strain M20, 100 mL, was harvested by centrifugation at 3000 x g for 30 min. The supernatant was discarded, and the pellet was washed with 10 mL of 1x PBS; the cells were centrifuged again, resuspended in 10 mL of 1x PBS and kept at 30 °C for 4 h for secretion of residual proteins. The supernatant was recovered by centrifugation at 3000 x g for 30 min at RT. Samples were concentrated 10x by using Amicon ultrafiltration with a molecular mass cutoff of 10 kDa (Millipore, USA) and dialyzed in 10 mM ammonium bicarbonate. SPF was quantified by the Bradford assay. The proteins were detected by SDS-PAGE and Western blotting, using mouse anti-rLIC11098 (1:10,000) or a mix of anti-rLIC11098 (1:10,000) and anti-rLipL32 (1:1000) antibodies for the primary detection diluted in PBS-T-10 %milk blocking solution; final detection was performed with anti-mouse antibodies conjugated with peroxidase (1:5000). Protein bands were developed as described above.

2.11. Evaluation of the interaction between recombinant protein and host components

rLIC11098, 1 μg, was immobilized in 96-well ELISA plates and incubated for 16 h at 4 °C in PBS. After the wells were washed 3 times with PBS-T, PBS-T-10 % milk blocking solution was added for 2 h at 37 °C. Each component (1 μg/well) was added in PBS-T-1 % milk blocking solution mixture, followed by incubation for 2 h at 37 °C. The analysis of the interaction between the recombinant protein and the components was performed by using antibodies against the host components, produced in rabbit, mouse or goat, diluted in PBS-T-1 % milk blocking solution. The negative controls consisted of PBS-T-10 % milk and anti-host component antibodies. Detection of the reaction occurred by adding anti-rabbit/mouse/goat antibodies conjugated with peroxidase diluted in PBS-T-1 % milk blocking solution. Dose-response assays between the recombinant protein and host components were performed following the same procedure.

2.12. Evaluation of the effect of heparin on the interaction between rLIC11098 and C9

rLIC11098, 1 μg, was immobilized in a 96-well ELISA plate, followed by incubation with 10 % PBS-T-milk for 2 h at 37 °C. Subsequently, 1 μg/well of C9 component was added in a solution containing an increasing concentration of heparin, followed by 2 h incubation. The detection proceeded with the addition of antibody against C9 (1:10,000) and peroxidase-conjugated anti-goat IgG (1:50,000). Incubation of antibodies was carried out at 37 °C for 1 h. The interaction was detected as described previously.

2.13. Effect of rLIC11098 on the polymerization of C9 assay

The effect of rLIC11098 on C9 polymerization was assessed according to a previously published protocol (Zhang et al., 2011). Negative control protein, fetuin (2.5 μg) and different concentrations of rLIC11098 (1.25, 2.5 and 5 μg) were pre-incubated with 3 μg C9 in 20 mM Tris-HCl, pH 7.4, at 37 °C for 40 min. After incubation, 50 μM $ZnCl_2$ in 20 mM Tris-HCl, pH 7.2, was added. This mixture was incubated for 2 h at 37 °C. The samples were analyzed in a 4–20 % gradient polyacrylamide gel (Bio-Rad, CA), and C9 polymerization was detected by Coomassie blue staining.

2.14. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 (GraphPad Prism, USA). For all experiments performed, analysis was performed using the two-tailed t-test. For dose-response analysis, the curves were fitted by nonlinear regression analysis, considering reaction saturation with "*one site-specific binding*" option. Values lower than baseline detection of the equipment (that is, an OPD solution in an ELISA-well with an approximate absorbance of 0.05) are not reliable for statistical analysis and were therefore considered non-reactive.

3. Results

3.1. Bioinformatical analysis: modular architecture and conservation of LIC11098 among Leptospira spp

In silico analysis performed by the CELLO and PSORTb programs indicated that the LIC11098 LRR protein is extracellular. LipoP predicted the signal peptide sequence with a higher probability of being recognized by the enzyme signal peptidase I (SpI), with a cleavage site located between amino acids 29 and 30 of the polypeptide sequence (Juncker et al., 2003).

To evaluate LIC11098 conservation among *Leptospira* genera, its amino acid sequence was searched in the NCBI databank, and the alignment performed by BLASTp showed information about the target protein cover query and the percentage of identity between LIC11098 and its analogues within *Leptospira* spp. At first, we aimed to highlight the strains and species that show higher conservation of this protein. Our second analysis consisted in comparing the sequence of LIC11098 of *L. interrogans* serovar Copenhageni with LIC11098 analogue sequences from different *Leptospira* species, considering the new taxonomic division established by Vincent and colleagues (Vincent et al., 2019). On the basis of these data, Clustal Omega (Madeira et al., 2022) was used to construct two phylograms. The results show that the protein sequence is well conserved among pathogenic strains of the genus (Subclade P1), especially in the different strains of *L. interrogans*, showing a high rate of coverage and identity (Fig. 1A). Conversely, species considered saprophytic (Subclades S1 and S2) and intermediate (Subclade P2), despite showing a high coverage, have a low identity rate relative to the protein (Fig. 1B).

Clustal Omega and ESPript programs were also used to align the amino acid sequence of LIC11098 and LIC10831 (Fig. 2). Both proteins have had their crystal structures solved (Miras et al., 2015). However, only LIC10831 has had its function validated as a probable virulence factor by binding human E- and VE-cadherins (Eshghi et al., 2019). As demonstrated in Fig. 2A, the two proteins appear to have a very similar secondary structure. According to the data obtained with ESPript and PDB, LIC11098 has 17 helices and 18 β-sheets, while LIC10831 has 16 helices and 15 β-sheets, and the amino acid alignment exhibits a conservation of the residues responsible for the secondary structure composition. BLASTp complementary analysis exhibited an 82 % coverage and 59.63 % of identity between LIC11098 mature sequence

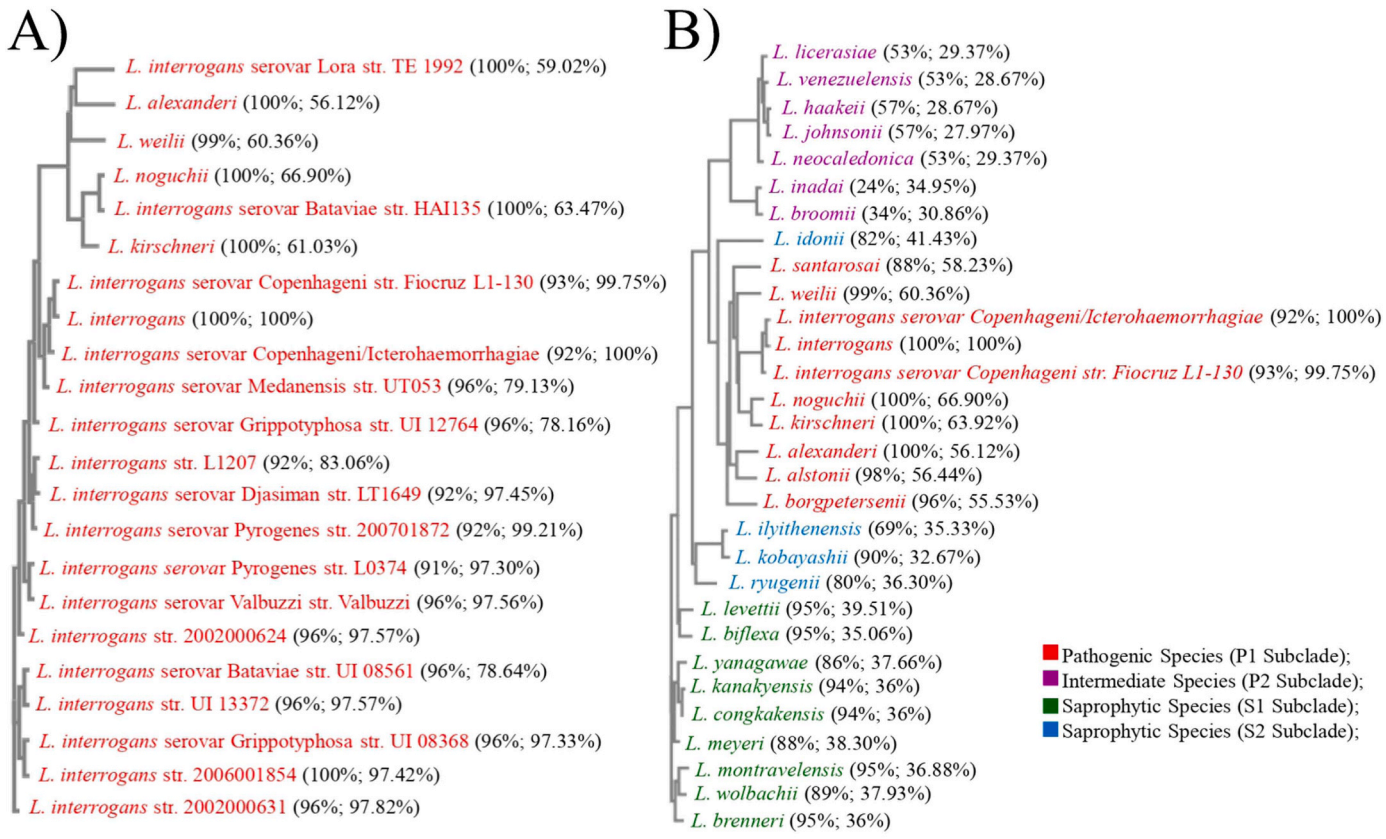

Fig. 1. Bioinformatic analysis of *L. interrogans* serovar Copenhageni LIC11098 LRR CDS (A and B) Conservation analysis of LIC11098 CDS among *Leptospira* species. Fig. 1 A and B show the conservation of LIC11098 among different strains and species of *Leptospira*. In phylogram (A) the general proteome of *Leptospira* genus was used as a parameter, in order to align the target protein with the strains and species that express the target protein with higher similarity. In phylogram (B) the proteome of different species of *Leptospira* genus was used as a parameter. Based on the raw data provided by BLASTp, multiple alignments were performed through Clustal Omega program, which provided the above phylograms. In parentheses, there are, respectively, the percentage of coverage of the protein database sequences compared to the LIC11098 amino acid sequence and the percentage of identity between the database sequences with LIC11098 sequence per length of coverage. The colored subtitles represent, in red, the pathogenic species of the genus *Leptospira*, in purple, the intermediate species, while in green and in blue exemplify saprophytic species.

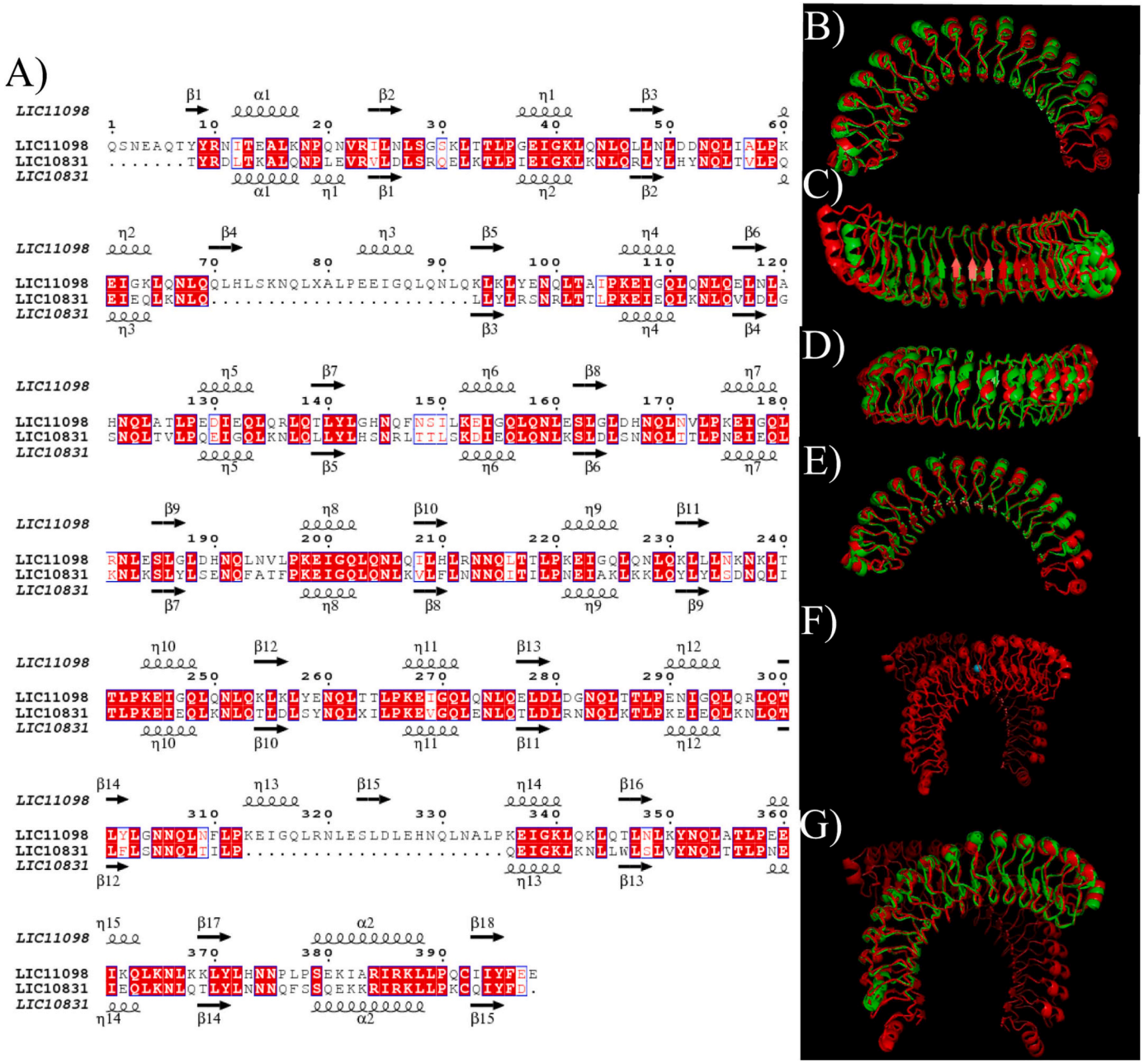

Fig. 2. Bioinformatic alignment of *L. interrogans* LRR proteins, LIC11098 and LIC10831. (A) ESPript 3.0 program alignment of LIC11098 and LIC10831 peptide sequence, highlighting their conserved residues in red and secondary structure similarity, represented by α- and η-helix and β-sheets. (B to G) LIC11098 and LIC10831 tertiary structure analyzed by PyMOL 2.5.4 program. Different angle images of LIC11098 (colored in red) and LIC10831 (colored in green) tertiary structure alignment are shown in images B, C, D and E. Image F shows LIC11098 dimeric form, which is related to the presence of calcium ion (represented as the blue sphere) between each monomer. The last image (G) represents the alignment of LIC11098 dimer and LIC10831 monomer.

and PDB LIC10831 sequence, respectively, corroborating with ESPript's alignment analysis. For comparative purposes, LIC10831 (Eshghi et al., 2019; Miras et al., 2015) and LIC11098 tertiary structures were superimposed using CE alignment algorithm, which compares the proteins based on their structure. For this comparison, the r.m.s. (root mean square deviation) value obtained was 1.322294. This measure evaluates the mean distance between corresponding atoms on the aligned protein structure. A r.m.s value below 2 demonstrates that LIC11098 and LIC10831 have a high structural similarity but also might have significant localized differences. The results are shown in Fig. 2B-E. Different angles of LIC11098 (red) and LIC10831 (green) overlapped structures are exhibited, highlighting their similarity. Additionally, PyMOL was used to generate LIC11098 quaternary structure, a homodimer, which is formed in the presence of divalent ions such as calcium (Fig. 2F), as described before (Miras et al., 2015), then LIC10831 was superimposed against LIC11098 dimer (Fig. 2G).

3.2. Cloning, expression, purification, and CD analysis of rLIC11098

The LIC11098 gene was amplified from *L. interrogans* serovar Copenhageni genome and cloned without signal peptide sequence into the pAE vector. The positive clone was used to transform the *E. coli* Star (DE3) pLysS strain, and bacteria were induced with IPTG. The rLIC11098 was purified from soluble *E. coli* lysate fraction successfully. After purification, the target protein was dialyzed to remove imidazole and analyzed by SDS-PAGE (Fig. 3A). A major protein band is observed,

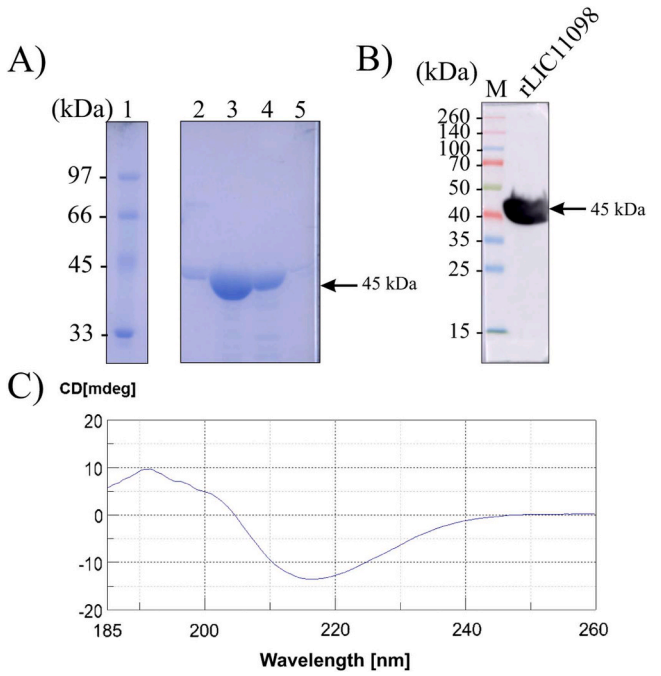

Fig. 3. rLIC11098 protein purification, detection by polyclonal antibodies, and secondary structure evaluation by CD. (A) SDS-PAGE of purified rLIC11098 after dialysis in 20 mM Tris pH 8.17 and 500 mM NaCl buffer. The lanes showed (1) molecular mass marker; (2-5) different concentrations of rLIC11098 (0.17, 0.93, 0.35 and 0.01 mg/mL, lanes 2, 3, 4 and 5, respectively), quantified after dialysis. Lane 4 shows the protein on its highest concentration, of 0.93 mg/mL. (B) Western Blotting of 15 μg of recombinant protein LIC11098 probed with polyclonal antibodies raised by immunization of BALB/c mice with the recombinant protein (1:20,000). The lanes showed (M) Molecular mass marker, followed by rLIC11098 detection with polyclonal antibodies produced in mice. (C) Evaluation of rLIC11098 secondary structure by CD. The spectrum was measured using a 1 mm optical path cell with 0.5 nm intervals by a spectropolarimeter.

with the expected molecular mass of 45 kDa, corroborating the predicted size by ProtParam of 46.89 kDa. rLIC11098 was quantified by the Bradford assay. The determined protein concentration was of 0.17, 0.93, 0.35 and 0.01 mg/mL of samples shown in Fig. 3A (lanes 2, 3, 4 and 5, respectively). Western blotting showed that rLIC11098 is recognized by anti-rLIC11098 polyclonal antibodies raised in mice, with the expected molecular mass (Fig. 3B).

The recombinant protein was submitted to CD analysis of its secondary structure. The α-helix-rich structures show a positive signal peak at the wavelength close to 190 nm, at the same time that it displays two peaks of negative signals close to wavelengths 208 and 220 nm, while the structures rich in the conformation of β-sheets shows a positive peak between wavelengths 190 and 200 nm and a single negative signal peak between wavelengths 210 and 220 nm (Holzwarth and Doty, 1965). On the basis of the data obtained experimentally and BeStSel software, it was possible to predict the percentage of secondary structures present in the protein (Fig. 3C). The presence of two signal peaks, one positive around 191 nm and the other negative close to 215 nm, is a characteristic pattern of structures rich in β-sheets. Table 2 shows the prediction of the secondary structure performed by the BeStSel program, highlighting that 31.3 % of its structure corresponds to α-helix conformations, while β-sheets comprise 54.5 % of its structure (antiparallel and parallel).

3.3. Reactivity of rLIC11098 with leptospirosis, dengue, malaria, chagas disease and HIV serum samples

Aiming to determine whether the recombinant protein is expressed during the leptospirosis infection process and its immunogenicity, reactivity with 20 pairs of leptospirosis serum samples was analyzed. The serum samples were organized in negative MAT, which represents the onset of disease, before the serum conversion into agglutinating antibodies against leptospires, and in positive MAT, when there is a seroconversion (Haake and Levett, 2015). The results obtained show that 20 and 45 % of serum samples were reactive at the initial phase of the disease (MAT-) and at the convalescent phase (MAT+), respectively (data not shown).

Recombinant protein reactivity against antibodies existing in other disease serum samples, including dengue, malaria, Chagas disease and HIV, was evaluated (data not shown). We noted that there was a considerable reactivity of the serum samples with recombinant protein. Dengue, malaria, Chagas and HIV serum sample reactivity with the recombinant protein was 66.66, 55.55, 22.22 and 44.44 %, respectively. The data showed that rLIC11098 was recognized not only by antibodies present in leptospirosis samples, but that there was also antibody cross-reactivity in samples of the febrile diseases tested.

Table 2
Analysis of the secondary structure of rLIC11098 by BeStSel.

Helix	31.3 %	Helix 1 (regular)	31.3 %
		Helix 2 (distorced)	0 %
Antiparallel	16.5 %	**Antiparallel 1** (left-twisted)	12.2 %
		Antiparallel 2 (relaxed)	0 %
		Antiparallel 3 (right-twisted)	4.3 %
Parallel	38 %		
Turn	0 %		
Others	14.2 %		

3.4. Analysis of protein localization by SDS-PAGE and Western blotting

To determine whether LIC11098 would be detected within the pool of secreted proteins of *L. interrogans,* SPF were analyzed by SDS-PAGE (Fig. 4A) and Western blotting (Fig. 4B) by using antibodies against rLIC11098. LipL32 was used as positive control, since it was detected in leptospiral exoproteome (Teixeira et al., 2022), and rLIC1150, another *L. interrogans* LRR protein under study in our laboratory (FOLTRAN *et al.,* unpublished results), was used to verify if there is a cross-reaction between anti-LIC11098 antibodies and other LRR proteins. As observed in Fig. 4A (lanes 1 and 2), several protein bands could be detected in SPF, indicating a significant diversity of proteins in the leptospiral secretome. In Fig. 4B, lanes 3 and 4 also represent SPF. Lanes 5, 6 and 7 show the recombinant proteins rLIC11098, rLipL32 and rLIC11505, respectively. In the Western blotting analysis, two major bands were detected at SPF samples (Fig. 4B, lanes 1 and 2). The lower band, with 32 kDa, corresponds to LipL32, while the higher band corresponds to a protein with a molecular mass around 60 kDa, similar to the size of rLIC11505. Between the 32 and 60 kDa bands, a weaker band of approximately 45 kDa can be observed, which possibly corresponds to LIC11098. This supposition is reinforced by the fact that when the Western blotting analysis was performed only with anti-rLIC11098 antibody (Fig. 4B, lanes 3 and 4), a stronger signal around 45 kDa was observed, suggesting that this band indeed corresponds to LIC11098. As expected, the recombinant proteins rLIC11098 and rLipL32, used as control, were successfully detected with a mix of anti-rLIC11098 and anti-rLipL32 antibodies (Fig. 4B, lanes 5 and 6, respectively) Interestingly, rLIC11505 was also detected, which demonstrates that anti-rLIC11098 antibodies are able to recognize other LRR proteins.

3.5. Interaction between rLIC11098 and human components

Outer membrane and extracellular proteins are the key factor that allows *L. interrogans* to interact with host proteins and initiate the processes of adhesion, invasion and immune system evasion, aiming at the colonization of target tissues. Thus, the ability of rLIC11098 to adhere to

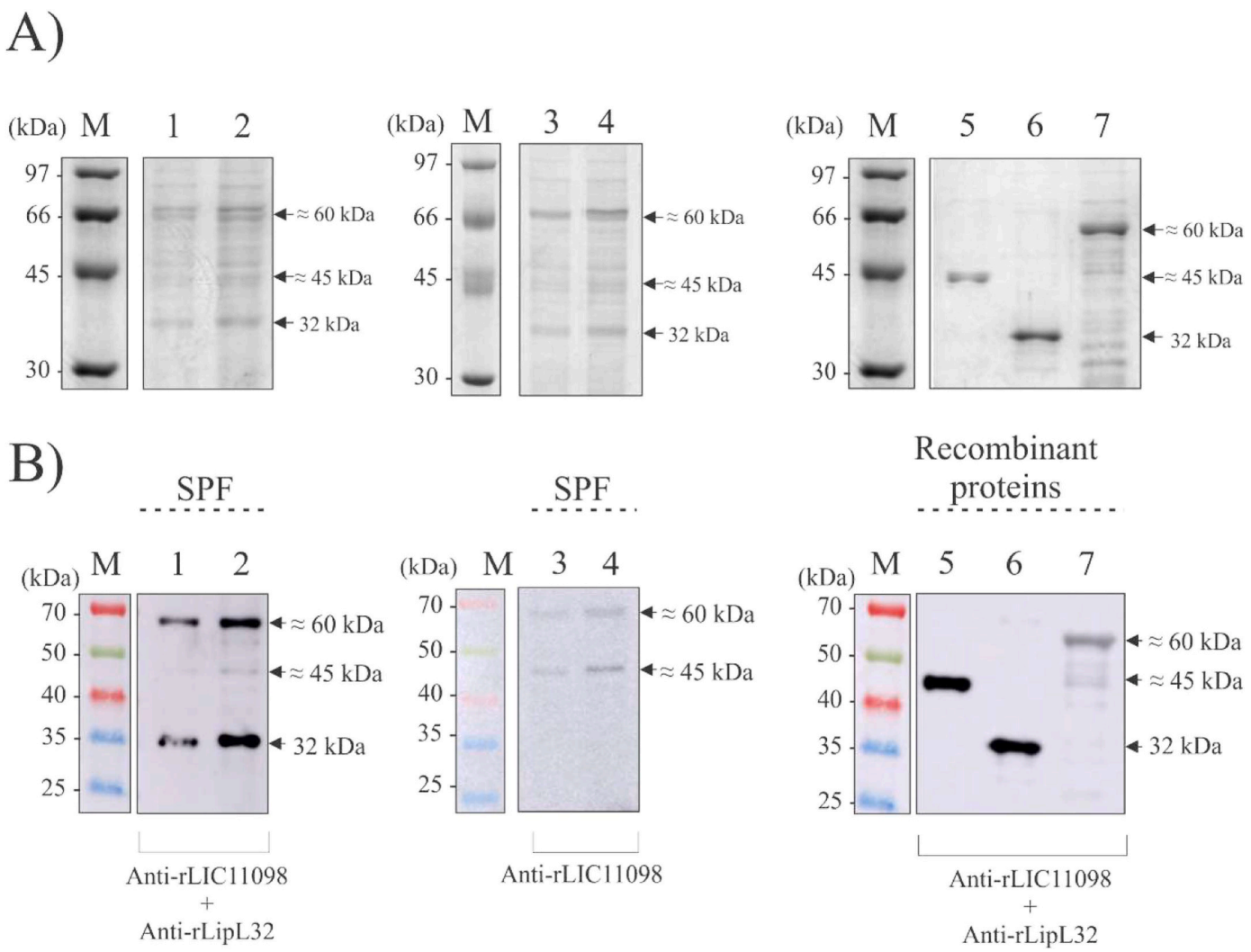

Fig. 4. Analysis of the presence of LIC11098 in *L. interrogans* serovar Copenhageni SPF. The experiment was performed in a SDS-PAGE (A) and in a Western blotting (B). In B: lanes (1) 1.2 μg of SPF; (2) 2.4 μg of SPF; (3) 2.4 μg of SPF; (4) 4.8 μg of SPF; (5) rLIC11098; (6) rLipL32; (7) rLIC11505; The detection of the proteins was performed with an anti-rLIC11098 (1:10,000) and anti-rLipL32 (1:1000) mix or anti-rLIC11098 alone and subsequent addition of anti-mice IgG conjugated with peroxidase (1:5000). rLipL32 was used as control protein.

host components was evaluated by ELISA. ECM components (collagen I and IV, cellular fibronectin and laminin), cell receptor (E-cadherin), plasma components (plasminogen, fibrinogen, vitronectin, plasma fibronectin and complement system components (C3b, C4b, C4-binding protein, C7, C8, C9 and factor H) were selected and their interactions with rLIC11098 were determined.

Target protein was able to interact with several host proteins, showing it to be a versatile, multifunctional *L. interrogans* protein. It interacted with cellular fibronectin and laminin of ECM (Fig. 5A), plasminogen, fibrinogen, and plasma fibronectin of plasma components (Fig. 5B), and C9 of the complement system (Fig. 5C). Plasminogen and fibrinogen, despite showing statistical difference in protein binding assay, did not show a dose-dependent response (data not shown). The binding of rLIC11098 with C9, cellular fibronectin and laminin was dose-dependent and saturable (Fig. 5D-G), and the calculated K_D values are shown in Table 3. Only binding with plasma fibronectin was shown to be non-saturable. The data from these experiments suggest that rLIC11098 may play an important role in different steps of *L. interrogans* infection.

3.6. Binding characterization of human C9 with rLIC11098

It is known that some complement system components, such as C9, have heparin-binding domains (YU et al. (2005)). Therefore, aiming to analyze a possible interference of heparin in C9-rLIC11098 binding, we designed an ELISA experiment to evaluate the effect of increasing heparin concentrations. As shown in Fig. 6A, when high concentrations of heparin were used, binding of C9 with rLIC11098 was significantly reduced, suggesting that heparin-binding sites are probably involved in the interaction.

Complementarily, as the target protein showed the ability to bind C9, we aimed to evaluate its capability to interfere with the C9 polymerization and, by extension, to inhibit membrane attack complex (MAC) formation, in the presence of zinc ions. To evaluate this hypothesis, rLIC11098 was incubated with C9 in the presence of $ZnCl_2$. The controls were as follows: C9 plus $ZnCl_2$, used as positive (lane 1), C9/$ZnCl_2$ plus fetuin, used as negative (lane 5) and C9 without $ZnCl_2$, used as non-polymerized marker (lane 6) (Fig. 6B). Increasing concentration of rLIC11098 had no impact on C9 polymerization (lanes 2–4) (Fig. 6B), suggesting that rLIC11098 binding does not interfere with this pathway.

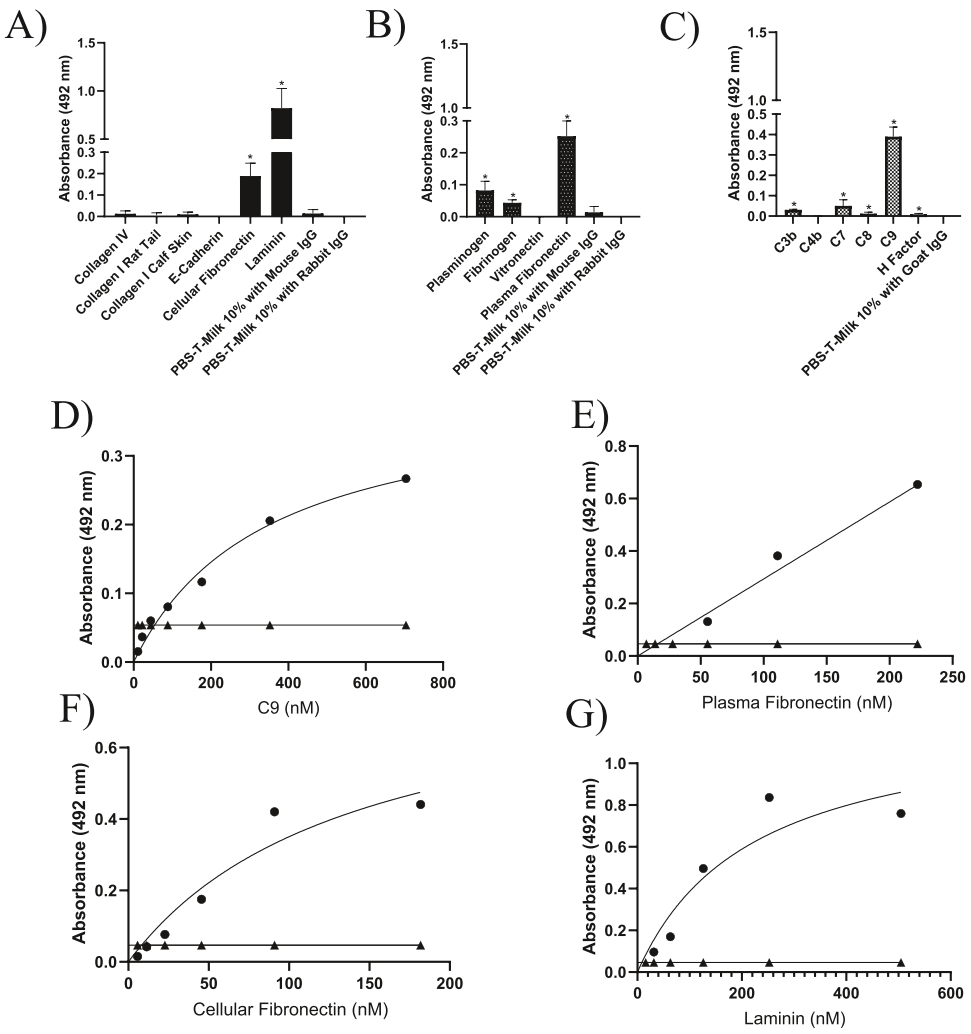

Fig. 5. Evaluation of the recombinant protein LIC11098 reactivity with host components. One μg of rLIC11098 was immobilized on 96-well ELISA plate. ECM (A), plasma components (B), and complement system components (C) were allowed to interact with recombinant protein for 2 h. The detection proceeded with addition of antibodies against the ECM, plasma components and complement system components (collagen IV: 1:400; collagen I: 1:200; E-cadherin: 1:200; cellular fibronectin: 1:1000; laminin: 1:200; plasminogen: 1:5000; fibrinogen: 1:4000; vitronectin: 1:2000; plasma fibronectin: 1:1000; C7, C8 and C9: 1:10,000; factor H, C3b and C4b: 1:5000), followed by addition of anti- IgG conjugated with peroxidase. Incubation of all antibodies used was at 37 °C for 1 h. The interaction between recombinant protein and host components was analyzed by comparison with negative controls (PBS-T-Milk 10 %/ anti-goat or anti-mouse or anti-rabbit) by two-tailed t-test. "*" represents statistical significance associated with PBS-T-Milk 10 %/ anti-goat or anti-mouse or anti-rabbit. The same protocol was performed for dose-response analysis using increasing concentrations of cellular and plasma fibronectin, C9, and laminin. (D to G) Dose-response graph of the interaction between recombinant protein and host-components. (D) C9, (E) Plasma fibronectin, (F) Cellular fibronectin, and (G) Laminin.

Table 3
Dissociation constants (K_D) values calculated from dose-response saturable binding between rLIC11098 and host proteins.

Component	K_D	Standard deviation
Cellular Fibronectin	139,5 nM	71,71 nM
Laminin	220,3 nM	112,8 nM
C9	345,4 nM	82,61 nM

4. Discussion

Pathogenic *Leptospira* need to cross the natural barriers of the host to cause leptospirosis, so that penetration into the body takes place through mucous membranes or wounds on the skin. This first barrier consists of a range of ECM components, such as collagen, elastin, laminin, cellular fibronectin, glycosaminoglycans such as heparin and chondroitin sulfates, and cellular-adhesion receptors, such as E-cadherin and integrins. Leptospires have the ability to interact with these components via their outer membrane and extracellular proteins, and this adhesion process is necessary for infection (Cosate et al., 2016; Evangelista et al., 2014). After the adhesion stage, the pathogenic leptospires must enter the bloodstream and disseminate to the target organs (Cinco, 2010). Once in the bloodstream, the pathogenic *Leptospira* have to evade the host defense mechanisms, to proliferate in multiple organs, reaching immunologically favored sites, such as proximal renal tubules (Haake and Levett, 2015). Several leptospiral external membrane proteins are able to interact with host molecules, many of which are characterized as multifunctional proteins, probably acting in more than one step of the infectious process. Among those proteins, rLIC11711, rLIC12587, rLIC13529, rLIC10774 and rLIC13086 are examples of characterized proteins capable of interacting with complement system proteins, ECM components and/or cellular-adhesion receptors (Cavenague et al., 2019; Daroz et al., 2021; Kochi et al., 2019)

In the present work, a hypothetical LRR protein of unknown function encoded by LIC11098 gene was studied. *In silico* analysis demonstrated that its localization is extracellular, which was experimentally confirmed, indicating the capability of this molecule to interact with

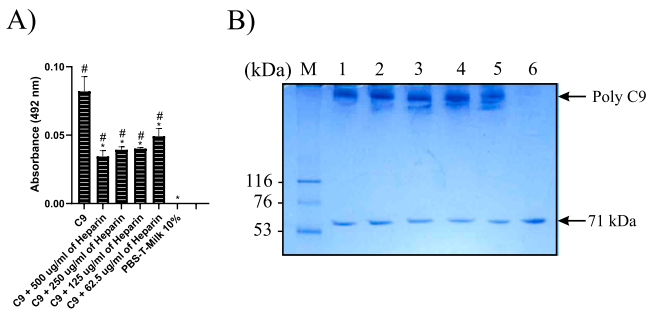

Fig. 6. Evaluation of the interaction between rLIC11098 and C9. (A) One μg of rLIC11098 was immobilized on 96-well ELISA plate. C9 complement system component in a solution without or with different concentrations of heparin was allowed to interact with recombinant protein for 2 h. The detection proceeded with addition of antibody against C9 each (1:10,000), with previous addition of anti-goat IgG (1:50,000) conjugated with peroxidase. Incubation of all antibodies used was performed at 37 °C for 1 h. The interaction between the recombinant protein and host components was analyzed by comparison with negative control by two-tailed t-test. "*" represents statistical significance associated with C9 without heparin solution and "#" represents statistical significance associated PBS-T-Milk 10 % solution. (B) One μg of C9 was incubated with different amounts of rLIC11098 or fetuin (negative control), for 40 minutes at 37 °C. Subsequently, there was an addition of $ZnCl_2$, which promotes C9 polymerization. This incubation occurred at 37 °C for 2 h. Purified C9 with and without zinc was also used as positive and negative control, respectively. These samples were subjected to a gradient SDS-PAGE (4–20 %). The bands were visualized by Coomassie blue staining. (M) high molecular mass marker; (1) purified C9 with $ZnCl_2$ (positive control); (2−4) incubation of C9/$ZnCl_2$ with 1.25, 2.5 and 5 μg of rLIC11098, respectively; (5) incubation of C9/$ZnCl_2$ with fetuin (negative control); (6) purified C9 without $ZnCl_2$. The arrow indicates non-polymerized C9 and polymerized C9, at a molecular mass of 71 kDa and at a higher molecular mass, respectively.

host molecules. *In silico* conservation analysis showed this LRR protein is well conserved among pathogenic species of genus *Leptospira*, suggesting a possible role of LIC11098 in the virulence processes.

LRR proteins show a classical tridimensional structure, characterized by the form of a horseshoe, in which α-helices are localized in the convex side, while β-sheets are located in the concave side. In literature, it is described that this curved structure favors protein-protein interactions (Bella et al., 2008; Kobe and Kajava, 2001; Miras et al., 2015). We have demonstrated in our analysis that rLIC11098 showed a characteristic secondary structure conformation, which is extremely important for the development of its function. Many bacterial LRRs of pathogens are related to the invasion, adhesion and colonization processes of host tissues and immune system evasion. For example, the internalins from *Listeria monocytogenes*, which are associated with pathogen invasion into host cells (Ireton et al., 2021), the membrane proteins YopM and ipaH of gram-negative bacteria *Yersinia pestis* and *Shigella flexneri*, respectively, which are associated with immune system evasion (Fernandez-Prada et al., 2000; Leung et al., 1990); the bspA protein of the gram-negative *Bacteroides forsythus* and the Slr protein of *Streptococcus pyogenes*, proteins that interact with ECM components, are associated with the adhesion to and colonization of host tissues (Bober et al., 2011; Sharma et al., 1998).

Moreover, it has been experimentally demonstrated that another LRR of *L. interrogans*, encoded by the LIC10831 gene, behaves like an adhesin, binding with the same magnitude to human E- and VE-cadherins (Eshghi et al., 2019). The fact that LIC10831 is able to interact with both types of cadherins is an important strategy for target tissue adhering and colonization of the host (Barnett et al., 1999). Another LRR, called LRR20, encoded by the LSS11580 gene of *L. santarosai* (Hsu et al., 2020) was also able to bind to human E-cadherin (Hsu and Yang, 2022), evidencing once again the role of LRRs in leptospiral virulence, as well as their relevant role for the first steps of the pathogenesis of leptospirosis (Daroz et al., 2021).

Contrarily of the two characterized leptospiral LRR proteins, rLIC11098 did not interact with E-cadherin. Eshghi and collaborators (Eshghi et al., 2019) reported that LIC10831-E-cadherin interaction occurs via a binding pocket composed of two LIC10831 residues, F305 and W326. In the amino acid alignment analysis (see Fig. 2A), it can be seen that LIC11098 (PDB: 4U08) have the residues Y302 and T346 instead F305 and W326, and this could be the reason why rLIC11098 does not interact with E-cadherin. However, it seems that there are other binding mechanisms involved in this interaction, since it has been shown that LSS11580 of *L. santarosai* has the ability to interact with E-cadherin even without the same residues in the binding pocket (Hsu et al., 2020). The authors discuss that the interaction between LSS11580 and the host molecule is mediated by charge-charge interactions by negatively charged residues of this LRR concave face, highlighting the role of D56, E59 and E123 residues in this interaction. As none of these residues occurred in LIC11098 amino acid sequence, it is possible that this charge-charge interaction also does not happen with LIC11098. Another hypothesis comes from the fact that LIC11098 is able to dimerize in the presence of divalent ions, which could modify its structural conformation and/or blockage of binding sites, enabling LIC11098 to bind a broader spectrum of molecules compared to LIC10831 and LSS11580.

In agreement with this hypothesis, rLIC11098 was able to interact with ECM and plasma components. rLIC11098 binds cellular fibronectin and laminin in a dose-dependent manner. The resulting K_D values are not comparable with other studies of *L. interrogans* protein function characterization. However, it is known that functional important binding usually occurs in a millimolar to femtomolar range (Schreiber and Keating, 2011), suggesting that our data may have biological relevance. Laminin is a glycoprotein that composes the basal membrane of epithelial tissue (Durbeej, 2010), and cellular fibronectin is another glycoprotein that composes the ECM, related to the maintenance of the matrix architecture and tissue organization (To and Midwood, 2011). The interaction of rLIC11098 with these components probably facilitates the adhesion of the bacteria to the host. This interaction may be associated with tissue permeabilization and colonization (Sato and Coburn, 2017; Takahashi et al., 2021), to participate in the process of adhesion and to facilitate the arrival of the bacteria at the bloodstream.

The recombinant protein LIC11098 was demonstrated to interact with plasma fibronectin and C9 in a dose-response manner. Plasma fibronectin is related to the formation of fibrin clots in the course of tissue lesions and is also important for interaction with other components of the coagulation cascade (To and Midwood, 2011). The formation of the fibrin clot and the action of the other components involved in the coagulation cascade has as main function stopping bleeding, but also prevents the spread of pathogens out of the blood vessels (Wagenaar et al., 2010). The process to interact with proteins related to the coagulation cascade, therefore, facilitates leptospiral dissemination from the bloodstream to the target organs (Oliveira et al., 2013). C9, complement system component, and other proteins, such C3b, C4b, and C5–8, are necessary components for the activation of all pathways of the complement system. These proteins belong to a cascade of cleavages whose ultimate goal is the polymerization of C9 and the formation of MAC, responsible for pore formation in the membrane of the pathogen and consequently its lysis (Sarma and Ward, 2011). To survive the passage through the host bloodstream, virulent leptospires must evade the complement system (Cinco, 2010). Thus, cleavage or binding to any component of the complement system proteins means reducing the possibility of MAC formation, characterized as a way to evade the immune system (Cavenague et al., 2019). Since C9 has heparin-binding domains, the interference of heparin in the C9-rLIC11098 interaction was experimentally tested. The results showed an interesting pattern, similar to what was shown in assays using LIC13259 (Cavenague et al., 2019), in which increasing concentrations of heparin reduce recombinant protein binding to C9. This suggests that interaction occurs via C9 heparin binding domains. However, in contrast to LIC13259 and

LIC12587, the LRR protein was not able to inhibit C9 polymerization at the concentrations tested, indicating that it does not contribute directly or effectively to preventing MAC formation (Cavenague et al., 2019; Kochi et al., 2019).

We observed that rLIC11098 was recognized by antibodies present in leptospirosis serum samples, suggesting its expression during infection. However, the reactivity of rLIC11098 against antibodies present in serum samples from diseases such as dengue, malaria, Chagas disease and HIV was elevated, pointing to a lack of specificity for leptospirosis and it could be related to its LRR structure, which is shared among proteins of several pathogens. We have shown cross-reactivity when anti-rLIC11098 is used to recognize rLIC11505, another LRR protein, corroborating the data presented by Eshghi and collaborators (Eshghi et al., 2019), who showed antibody cross-reactivity between leptospiral LRR proteins.

5. Conclusion

In conclusion, recombinant LRR protein, encoded by the gene LIC11098 of *L. interrogans* was characterized here as an extracellular protein that is most probably expressed during infection. The recombinant protein was able to bind to laminin and cellular fibronectin, demonstrating a significant role in the adhesion step of the infection. Among plasma proteins, LIC11098 could bind to plasma fibronectin, indicating a potential role of interfering with the fibrinolytic system. Thus, it is possible that this recombinant LRR protein may play an important role at different steps of leptospiral infection.

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