Molecular Microbiology

WILEY

RESEARCH ARTICLE OPEN ACCESS

Leptospira Leptolysin Contributes to Serum Resistance but Is Not Essential for Acute Infection

Daniella dos Santos Courrol[1,2] | Cassia Moreira Santos[1,3] | Rosa Maria Chura-Chambi[1,4] | Lígia Morganti[4] | Kátia Eliane Santos Avelar[5] | Fernanda de Moraes Maia[6] | Rodrigo Nunes Rodrigues-da-Silva[6] | Elsio Augusto Wunder Jr[7,8,9] | Angela Silva Barbosa[1]

[1]Laboratory of Bacteriology, Butantan Institute, São Paulo, Brazil | [2]Department of Microbiology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil | [3]Laboratory of Bacterial Zoonosis, Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, São Paulo, Brazil | [4]Center of Biotechnology, Energy and Nuclear Research Institute (IPEN)-CNEN/SP, São Paulo, Brazil | [5]Laboratório de Referência Nacional para Leptospirose, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil | [6]Laboratório de Hantaviroses e Rickettsioses, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil | [7]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut, USA | [8]Department of Epidemiology of Microbial Diseases, Yale School of Public Health, New Haven, Connecticut, USA | [9]Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Ministério da Saúde, Salvador, Brazil

Correspondence: Angela Silva Barbosa (angela.barbosa@butantan.gov.br)

Received: 27 March 2024 | **Revised:** 16 October 2024 | **Accepted:** 18 October 2024

Funding: This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grants 2018/12896-2, 2019/22706-9 and 2017/12924-3, NIH grant R21AI163663 (EAWJ), and Fundação Butantan.

Keywords: complement system | leptolysin | *Leptospira* | serum resistance

ABSTRACT

Previous in vitro works focusing on virulence determinants of the spirochete *Leptospira* implicated metalloproteinases as putative contributing factors to the pathogenicity of these bacteria. Those proteins have the capacity to degrade extracellular matrix components (ECM) and proteins of host's innate immunity, notably effectors of the complement system. In this study, we gained further knowledge on the role of leptolysin, one of the leptospiral-secreted metalloproteinases, previously described as having a broad substrate specificity. We demonstrated that a proportion of human patients with mild leptospirosis evaluated in the current study produced antibodies that recognize leptolysin, thus indicating that the protease is expressed during host infection. Using recombinant protein and a knockout mutant strain, Manilae *leptolysin*$^{-}$, we determined that leptolysin contributes to *Leptospira interrogans* serum resistance in vitro, likely by proteolysis of complement molecules of the alternative, the classical, the lectin, and the terminal pathways. Furthermore, in a hamster model of infection, the mutant strain retained virulence; however, infected animals had lower bacterial loads in their kidneys. Further studies are necessary to better understand the role and potential redundancy of metalloproteinases on the pathogenicity of this important neglected disease.

1 | Introduction

Proteases are among the virulence determinants of a number of medically important pathogens. During the infectious process, pathogens produce proteases that may cleave peptide bonds, leading to irreversible structural changes, in addition to causing tissue damage and excessive inflammation (Viana et al. 2021). The mechanisms underlying entry, dissemination,

Daniella dos Santos Courrol and Cassia Moreira Santos contributed equally to this article.

persistence, and tissue damage resulting from infection by the pathogenic spirochete *Leptospira*—the causative agent of leptospirosis—are still being elucidated, but recent studies are suggestive that proteases secreted by this bacterium may contribute to the various stages of host colonization (Barbosa and Isaac 2020).

Leptospires penetrate host's damaged skin or mucous membranes, and after entry they reach the bloodstream causing lesions in the vascular endothelium that facilitate migration to various organs and tissues, especially the kidneys and liver (Murray 2015). The dissemination of these spirochetes within the host is rapid and efficient, normally attributed to their high motility associated with the ability to degrade the extracellular matrix (ECM) and modulate the innate immune response through the action of extracellular proteases (Fraga et al. 2014; Wunder Jr, Figueira, Benaroudj, et al. 2016; Wunder Jr, Figueira, Santos, et al. 2016; da Silva et al. 2018; Gibson et al. 2020). This apparatus facilitates the pathogen's invasion process, helping it to cross natural barriers such as connective tissue and basement membranes of the epithelium and endothelium.

We recently characterized leptolysin, a pappalysin-1 domain secreted protein that belongs to the category of short pappalysins (Courrol et al. 2022; da Silva et al. 2018). Like ulilysin from *Methanosarcina acetivorans* and mirolysin from *Tannerella forsythia* (Tallant et al. 2007; Koneru et al. 2017), leptolysin is a metzincin that mediates hydrolysis of extracellular matrix components and plasma proteins (Courrol et al. 2022). Mirolysin was also shown to inhibit all complement pathways through hydrolysis of mannose-binding lectin, ficolin-2 and ficolin-3, C4, and C5. Degradation of C5 by mirolysin produced biologically active C5a that induced migration of neutrophils in the human plasma (Jusko et al. 2015).

Leptolysin, a M43 metalloprotease, is present in *Leptospira* species belonging to all subclades, but is more conserved among pathogenic (P1) species. The enzyme exhibits maximum activity at pH 8.0 and 37°C, and is active in the presence of different salts, showing a marked preference for arginine residues in the P1 position. *Leptospira interrogans* leptolysin induces morphological changes in Human Kidney-2 cells, probably due to disturbances on cell–cell adhesion molecules and on the surrounding ECM produced by the cells. Mice intradermally injected with leptolysin in the dorsal skin present local hemorrhagic lesions that might be secondary to ECM and vascular endothelium damage (Courrol et al. 2022).

Assuming that leptolysin exhibits a broad-spectrum proteolytic profile, in the current study, we generated a *leptolysin*$^-$ mutant of *L. interrogans* serovar Manilae to gain further knowledge on the role of leptolysin during infection. In this work, we explored the protease's function in serum resistance and we evaluated its immunogenicity in natural infections using serum samples from Brazilian patients with mild leptospirosis.

2 | Results

2.1 | Leptolysin Contributes to *L. interrogans* Serum Resistance

To explore the role of leptolysin in serum resistance, a homologous recombination approach was used to generate a *leptolysin*$^-$ mutant of *L. interrogans* serovar Manilae strain L495 (Figure S1). Complement-mediated killing of Manilae wildtype (WT), Manilae *leptolysin*$^-$, and the saprophyte Patoc was then assessed upon incubation in 40% normal human sera (NHS) for 2 h. The survival percentage was calculated by comparing the amount of surviving leptospires incubated in NHS with those incubated in heat-inactivated NHS (HI-NHS). Manilae *leptolysin*$^-$ survival rate was intermediate between that of the wildtype and the saprophytic strains (Figure 1A). To further confirm the role of leptolysin on serum resistance, Patoc and Manilae *leptolysin-* strains were then incubated in NHS pretreated with the recombinant protease. Leptolysin-treated NHS allowed Patoc and Manilae *leptolysin-* survival in a dose-dependent manner to the point where the survival rate in NHS preincubated with 1 μM of leptolysin was similar to that observed in HI-NHS for both strains (Figure 1B). Those results collectively suggest that the metalloprotease leptolysin might target proteins of the complement system thus interfering with the activation of its cascade.

2.2 | Recombinant Leptolysin Cleaves Complement Proteins

Degradation of soluble complement molecules contributes to bacterial persistence in the bloodstream and is one of the mechanisms employed by pathogenic microorganisms during infection. In this work, we evaluated leptolysin proteolytic activity against complement proteins from the alternative, the classical, the lectin, and the terminal pathways. Initially, leptolysin activity was assessed using 10–80 nM of the protease, and for most of the complement components, 40 nM (0.1 μg) was the minimum dose required for efficient hydrolysis (Figure S2). Then, time-dependent hydrolysis was evaluated for up to 24 h incubation at 37°C. We observed that C2, C5, C7, C8, and C9 were efficiently degraded after 1 h of incubation whereas the other complement components required 2–24 h to be hydrolyzed (Figure 2). The metal chelator 1,10-phenanthroline abolished leptolysin proteolytic activity (Figure S3). We then assessed deposition of C5 and C9 on WT and leptolysin mutant strains, and we observed there was more deposition of both molecules in the knockout strain (Figure S4). Next, we evaluated whether leptolysin would have proteolytic activity on complement molecules present in human serum. According to Figure 3A, there was a dose-dependent degradation of C3 and C4. Furthermore, we showed that the proteolytic effect on these molecules was enhanced in the presence of thermolysin, another protease secreted by leptospires, previously described as acting on C3 (Fraga et al. 2014; Chura-Chambi et al. 2018). Almost complete inactivation of C3 and partial inactivation of C4 was observed in the presence of both proteases (Figure 3B).

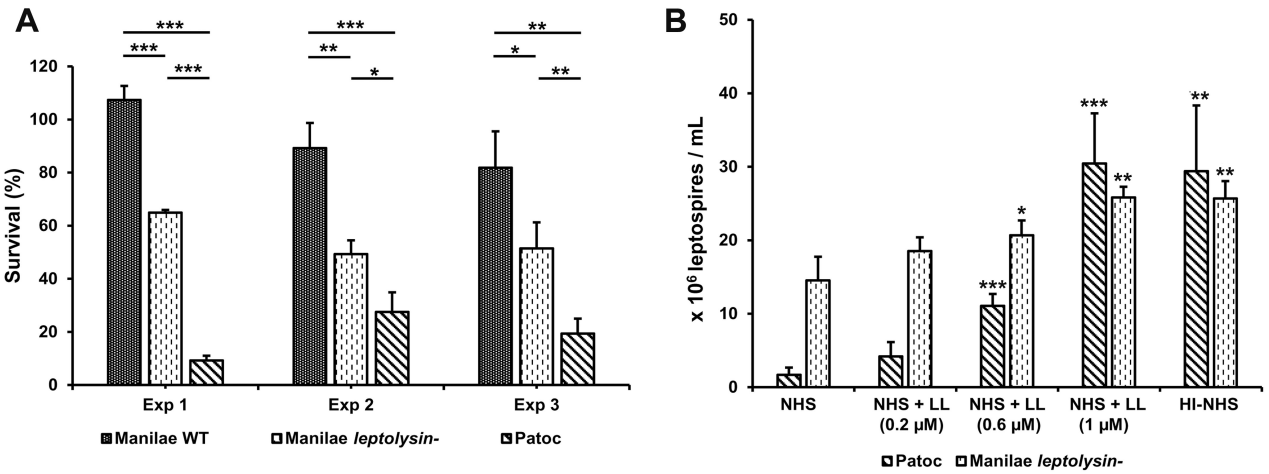

FIGURE 1 | Leptolysin contributes to *Leptospira interrogans* serum resistance. (A) Manilae WT, Manilae *leptolysin*$^-$ and *Leptospira biflexa* Patoc (5×10^7 bacteria) were incubated in 40% NHS or HI-NHS for 2 h. Bacteria were counted using dark field microscopy. Data of three independent experiments, each performed in triplicate, are presented as relative survival (NHS/HI-NHS). *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student *t*-test). (B) *Leptospira biflexa* Patoc and *L. interrogans* Manilae *leptolysin*- (5×10^7 bacteria) were incubated for 2 h in 40% HI-NHS, NHS or NHS pretreated with recombinant leptolysin (0.2–1 μM). Bacteria were counted using dark field microscopy. The results obtained from the incubations with NHS + leptolysin (0.2–1 μM) were compared with those obtained from incubations with NHS alone using the Student *t*-test. *$p<0.05$, *$p<0.01$, ***$p<0.001$. LL, leptolysin.

2.3 | Leptolysin Is Not Essential for Virulence of Pathogenic *Leptospira* During Acute Infection

Assuming that leptolysin degrades complement proteins and contributes to serum resistance, we then evaluated its role on virulence using a hamster model for acute leptospirosis infection. Hamsters were challenged by the "abraded skin" route with 10^3 leptospires. Time to death was slightly different for the two groups: animals inoculated with the WT strain died between Days 9 and 10 postinfection whereas those inoculated with the *leptolysin*$^-$ strain died between Days 10 and 11 postinfection ($p=0.031$) (Figure 4A). Despite the lack of significant difference regarding overall survival rate, renal colonization of infected animals, assessed by qPCR, indicated that *leptolysin*$^-$-infected hamsters had significant lower bacterial loads in the kidneys ($p<0.0001$) (Figure 4B).

2.4 | Leptolysin Is Recognized by Sera From Individuals With Laboratory-Confirmed Leptospirosis

Production and immunogenicity of leptolysin in natural infections were investigated on a cohort of 20 Brazilian patients with leptospirosis. Confirmed leptospirosis cases (Leptospirosis Group [LG]) and individuals presenting with other febrile illnesses, serving as a negative control group (NC), had a similar age profile (means of 34.4 ± 16.1 and 46.9 ± 23.8, respectively). Patients in the LG group presented symptoms at 12.25 ± 7.28 days (ranging from 4 to 29) before blood collection, with fever, myalgia, and headache being the most common symptoms reported by 95%, 75%, and 65% of patients, respectively. Complications, such as pulmonary hemorrhage, hemorrhagic manifestations, or renal insufficiency, were not reported by the patients. The symptomatology of LG individuals is summarized in Table 1. Regarding MAT results, titers ranged from 1:800 to 1:12,800 with 40% of patients' sera reacting to serogroup Icterohaemorrhagiae, 50% reacting to serogroup Tarassovi, of which 10% reacting to both, and 10% of patients were exclusively reactive to serogroups Sejroe or Grippotyphosa. Regarding the reactivity against leptolysin, 20% of patients presented IgM antibodies (Figure 5A) and 35% of patients presented IgG antibodies (Figure 5B) against the recombinant protein. A heatmap of IgM and IgG reactivity indexes against leptolysin is shown in Figure 5C. These data provide evidence that leptolysin is expressed during host infection. Positivity to leptolysin by ELISA was independent of the putative infecting serovar (Table S1).

3 | Discussion

Proteases play a crucial role in the survival and dissemination of extracellular pathogens by modulating or interfering with host's immune response, disrupting the mesh that makes up the extracellular matrix and basement membranes, and blocking fibrin network formation. These processes allow pathogenic microorganisms to migrate and proliferate far beyond the infection site (Robin and Westblade 2015).

The spirochete *Leptospira* is no exception to this rule and as a classical extracellular successful bacterium displays strategies that target ECM components and plasma proteins through own and host-acquired proteases such as plasminogen (revised in Barbosa and Isaac 2020). During leptospiral infection, secreted metalloproteases are one of the main actors of this process. The gelatinase ColA was shown to hydrolyze different types of collagens and facilitate transcytosis through endothelial and renal cell monolayers (Kassegne et al. 2014). The thermolysin encoded by *lic13322* promotes degradation of complement C3 and C6, and binds with high affinity to the terminal complement components C6, C7, C8,

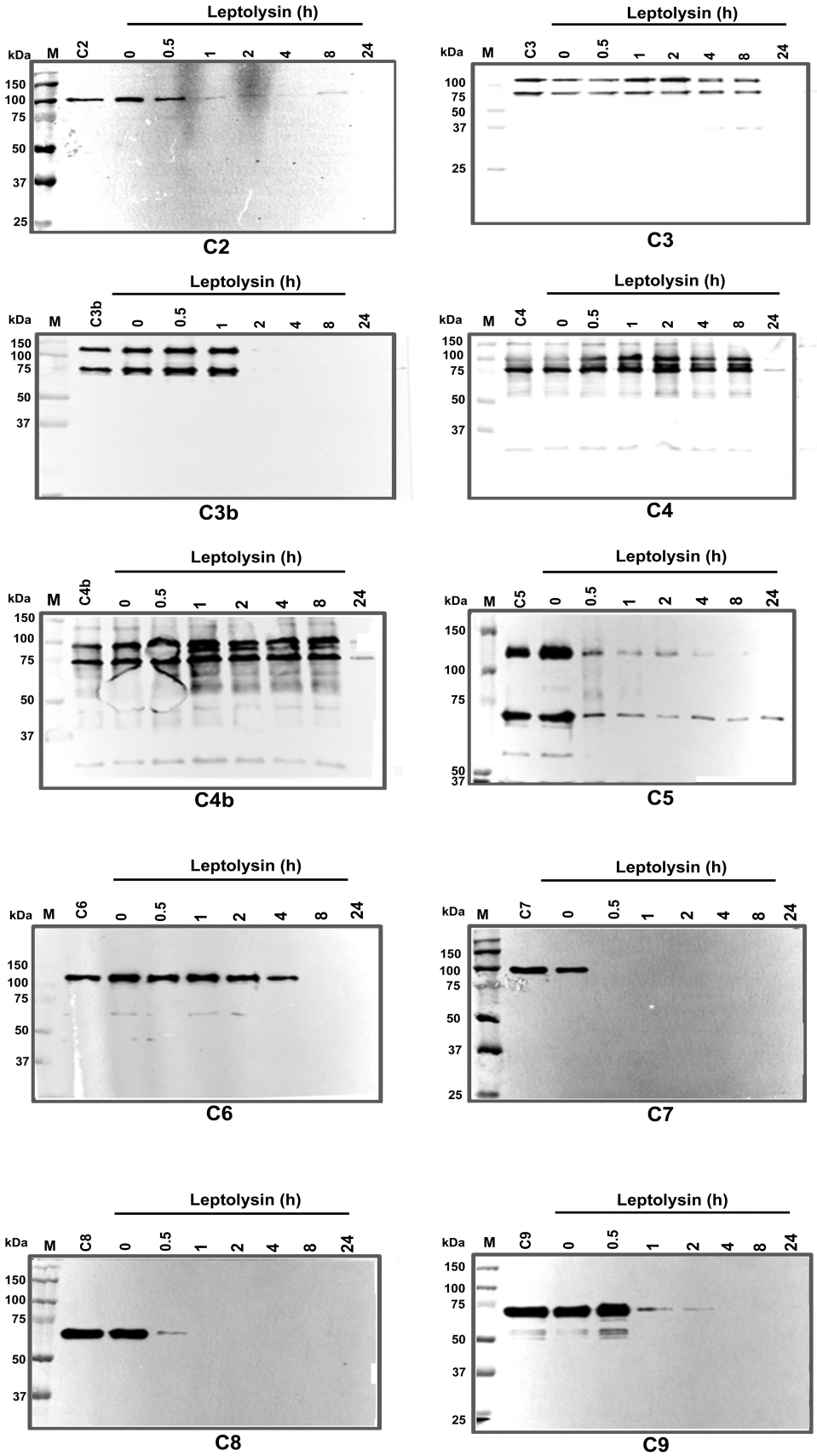

FIGURE 2 | Proteolytic activity of *Leptospira interrogans* leptolysin on complement proteins. 0.1 μg of recombinant *L. interrogans* leptolysin (40 nM) was incubated with 0.5 μg of complement proteins (50–140 nM) for up to 24 h at 37°C. Cleavage products were subjected to 10% SDS-polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes, and probed with specific antibodies as described in the Section 4.

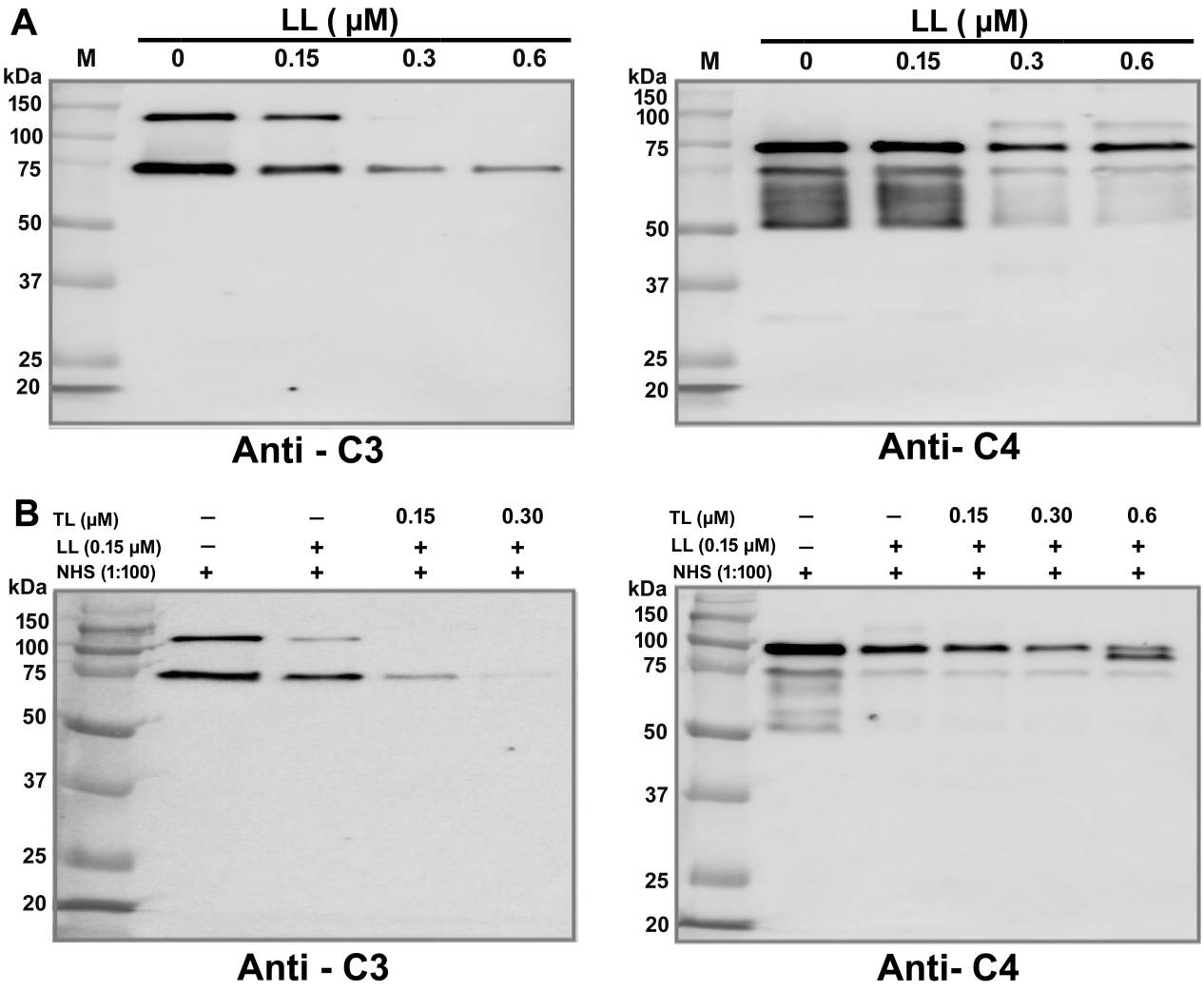

FIGURE 3 | Leptospiral proteases act synergistically to inactivate serum C3 and C4. (A) Recombinant *Leptospira interrogans* leptolysin (0.15–0.6 μM) or (B) recombinant leptolysin (0.15 μM) + recombinant thermolysin (0.15–0.6 μM) were incubated with NHS (diluted 1:100) for 24 h at 37°C. Cleavage products were subjected to 10% SDS- polyacrylamide gels under reducing conditions, transferred to nitrocellulose membranes, and probed with anti-C3 or anti-C4 as described in the Section 4. LL, leptolysin; TL, thermolysin.

and C9, preventing lysis of erythrocytes mediated by the membrane attack complex (Fraga et al. 2014; Amamura et al. 2017; Chura-Chambi et al. 2018). Furthermore, we recently characterized the metalloprotease leptolysin produced by *Leptospira* species belonging to the P and S clades. Leptolysin displays broad-spectrum activity and targets proteinaceous substrates including proteoglycans and human fibronectin. It also causes cytopathic effects on kidney cells, which may be partially attributed to ECM degradation (Courrol et al. 2022).

In the current study, we generated a *L. interrogans* leptolysin deletion mutant to explore the role of this protease. Our data collectively point to a role of leptolysin in helping bacteria to resist complement attack, as the knockout strain has a much lower capacity to survive in NHS compared with the parental strain Manilae L495. Cleavage assays indicate that leptolysin targets complement molecules of all three pathways, from C2 to C9, causing partial or complete degradation of those effector molecules, evidenced by *Leptospira biflexa* increased survival in NHS pretreated with the purified protease. However, the leptolysin mutant retained virulence in a hamster model of infection. As broken skin is the main and most physiological route by which leptospires cause infection (Gostic et al. 2019), 10^3 bacteria were administered on the animals´ shaved skin after a slight abrasion of the epidermis. All animals infected with either the wildtype or the mutant strain developed a clinically irreversible condition at 9–11 d.p.i., and histological studies revealed no substantial differences in the organs of wildtype and knockout-infected animals. Nevertheless, total bacterial loads in the kidneys were lower in the knockout-infected group, which could be possibly attributed to partial elimination of bacteria in the bloodstream by the action of the complement system. It is unlikely that the reduced number of spirochetes reaching the target organs stems from a lower penetration capacity of these bacteria in the initial stages of colonization, as the mutant strain did not present differences regarding the translocation ability through endothelial cell monolayers (Figure S5). It is worth mentioning that the strain used in this work, Manilae L495, is highly virulent ($LD_{50} < 10$, Zhu et al. 2023) and infection leads to animal death at Days 5–14 postinoculation depending on the route and infecting dose. Therefore, it is plausible to assume that complete—or

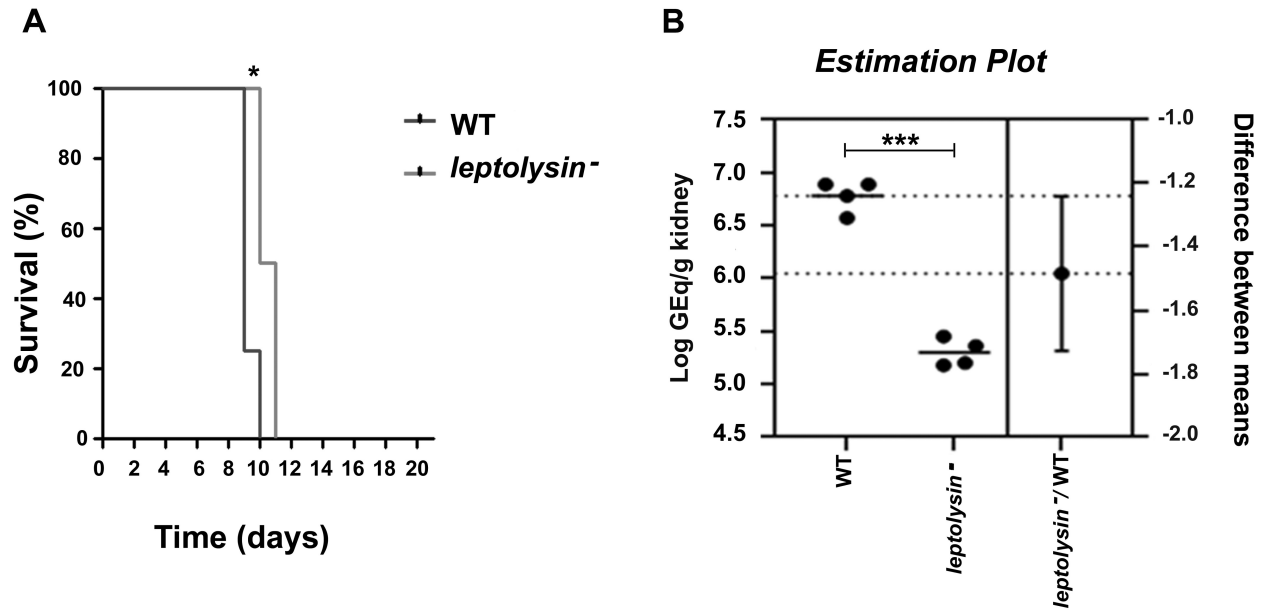

FIGURE 4 | Role of leptolysin in infection. (A) Survival curves of hamsters infected with Manilae WT and Manilae *leptolysin*⁻ strains. Four hamsters in each group were infected with 10^3 leptospires by the epicutaneous route. Survival curves were compared using the Log-rank (Mantel–Cox) test (*$p = 0.031$). (B) Quantification of bacterial loads in the kidneys. Genomic DNA isolated from the kidneys was subjected to qPCR with *lipL32* primers to quantify bacterial loads, expressed as log GEq/g of tissue. An unpaired *t*-test was used to compare bacterial loads in the kidneys (***$p < 0.0001$). The error bar on the right indicates the 95% confidence interval for the difference between means.

TABLE 1 | Symptoms reported by patients with leptospirosis included in this study.

Symptoms	Frequency—*n* (%)
Fever	19 (95)
Myalgia	15 (75)
Headache	13 (65)
Calf pain	12 (60)
Vomit	10 (50)
Jaundice	10 (50)
Prostration	9 (45)
Diarrhea	8 (40)
Respiratory changes	5 (25)
Conjunctival congestion	4 (20)
Abdominal pain	1 (5)
Queasiness	0 (0)

at least effective—inactivation of the complement system in vivo requires a complex arsenal in which multiple bacterial escape mechanisms come into play to subvert the action of the host's innate immune system. Interestingly, in a short-term murine model of *Leptospira* hematogenous dissemination, nonpathogenic *L. biflexa* serovar Patoc could survive in the host for up to 6 h, but bacterial burdens in the liver, kidney, and bladder were lower than those observed with the virulent strain Manilae (Surdel et al. 2022). Thus, in this infection model nonpathogenic *Leptospira* were not fully cleared from the bloodstream by the complement system, but their reduced ability to circumvent complement attack combined with the fact that they are devoid of other virulence mechanisms leads to a less effective capacity to colonize target organs and to inability to cause disease.

Furthermore, a number of mutants retaining virulence have been described in *Leptospira* (revised in Bulach and Adler 2018). Inactivation of genes encoding for proteins potentially associated with virulence—possessing adhesion, invasion or evasion properties, in vivo expression, or relatedness to other known virulence factors—may not always result in an attenuated strain, which is strongly suggestive of a high level of functional redundancy in this spirochete (Bulach and Adler 2018). As already mentioned, leptospires possess collagen- and elastin-degrading enzymes, as well as four thermolysins and M16-type metallopeptidases (Kassegne et al. 2014; Hashimoto et al. 2013; Fraga et al. 2014; Ge et al. 2020). These proteases seem to display overlapping, redundant, or synergistic functions, as shown in Figure 3.

Regarding leptolysin expression and immunogenicity, our data support that this protein is expressed in human cases of leptospirosis and is immunogenic in natural infections. However, in this cohort of patients with mild symptoms of leptospirosis, we did not observe serological evidence of leptolysin in all patients. This lack of reactivity may be related to the limited number of patients included in the study and to the expression level of leptolysin in those patients. Regulation of protein expression by factors such as osmolarity and temperature has been demonstrated for several leptospiral proteins (Lo et al. 2009) but remains unexplored for leptolysin and other proteases. In this context, we highlight the need for new studies to explore the association of leptolysin with severity or

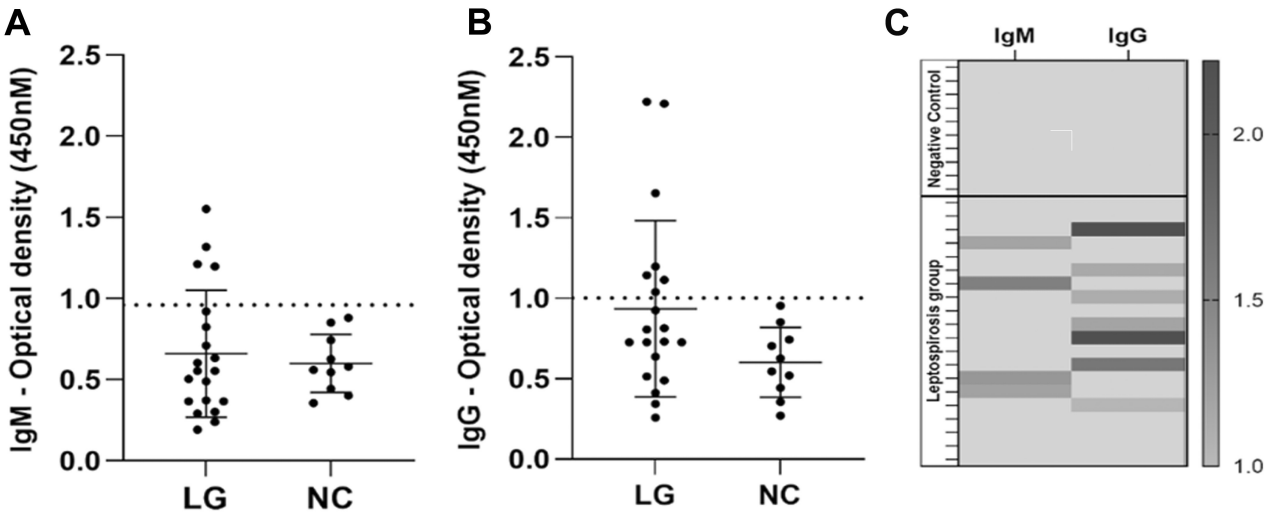

FIGURE 5 | Evaluation of seroreactivity of Brazilian patients with leptospirosis against leptolysin. (A) IgM and (B) IgG reactivity against leptolysin. Black dots indicate optical density values of each sample. The traced line indicates the threshold of IgM (0.95) and IgG (1.03) reactivity. (C) Heatmap of IgM and IgG reactivity indexes against leptolysin. Values higher than 1 represent responder individuals and were indicated in the red color scale, and nonresponders were indicated by a gray color. LG, leptospirosis Group; NC, negative control group.

symptoms, and to understand the factors associated with its expression.

In this study, we delved into the in vivo role of the metalloprotease leptolysin from *L. interrogans*. Despite clear in vitro evidence showcasing the proteolytic activity of recombinant leptolysin on various proteinaceous substrates, such as ECM and complement molecules (Courrol et al. 2022; this study), the leptolysin$^-$ mutant strain retained the ability to cause lethal infection in the hamster model, albeit with reduced bacterial loads in the kidneys. Therefore, although leptolysin may contribute to *Leptospira* survival in the bloodstream by cleaving complement components, it alone may not suffice to fully counteract the effects of the complement cascade. The findings underscore the complexity of the virulence arsenal employed by leptospires for survival in the host, relying on a multifaceted interplay of factors, including diverse mechanisms ensuring their persistence in the circulation.

4 | Experimental Procedures

4.1 | Ethics Statement

Animal experimentation protocols were prepared and approved according to the guidelines of the Committee on Ethics of Instituto Butantan (protocol # 6453310521) and the Institutional Committee for the Use of Experimental Animals, Yale University (2023–11,424). Serum samples of Brazilian patients were sourced from the National Reference Laboratory for Leptospirosis at Fiocruz-RJ. The study was executed in stringent adherence to ethical standards, in compliance with the guidelines and protocols sanctioned by the Ethics Committee of the Oswaldo Cruz Foundation and the National Ethics Committee of Brazil. The ethics approval for this study was granted under the reference number: CAAE: 31405820.8.0000.5262.

4.2 | Leptospirosis Patients

The recognition of recombinant leptolysin by antibodies from Brazilian patients was assessed on a cohort of 20 individuals who had been diagnosed with leptospirosis, LG. These patients presented non-acute, mild disease. Additionally, 10 individuals presenting with other febrile illnesses, serving as a NC, were included in the study. The diagnosis of leptospirosis in these patients was done by the microscopic agglutination test (MAT).

4.3 | Bacterial Strains and Culture Conditions

Leptospira interrogans serovar Manilae strain L495 (Manilae WT), the Manilae *LMANv2_470060/lic13434* mutant (Manilae *leptolysin*$^-$) and the saprophyte *L. biflexa* serovar Patoc strain Patoc 1 (Patoc) were grown in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium at 29°C. Strains were cultured, and supernatants were collected and stored as previously described (Oliveira et al. 2021).

4.4 | Construction of *L. interrogans* Serovar Manilae *Leptolysin*$^-$ Mutant

The knockout mutant was obtained by allelic exchange, essentially as described by Wunder Jr, Figueira, Benaroudj, et al. (2016), Wunder Jr, Figueira, Santos, et al. (2016). Upstream and downstream regions of the *LMANv2_470060/lic13434* gene were amplified from the genomic DNA of *L. interrogans* serovar Manilae strain L495 using primers UF1 (GCTCTAGAtttgcgttcgtttgtatcgg) and UR1 (CCCAAGCTTttggaacttaaggattaagt) for the upstream region and DF2 (CGGGATCCctcaccctcttgtatattaa) and DR2 (GACTAGTcattgcgcatgtcttcatcc) for the downstream region. The PCR products were digested with XbaI and HindIII, and BamHI and SpeI, respectively. The spectinomycin resistance

(SpcR) cassette was amplified from *Staphylococcus aureus* using primers SPC_F (CCCAAGCTTaaagtaagcacctgttattgc) and SPC_R (CGGGATCCcccgagcttcaaggaagat) (Bauby, Saint Girons, and Picardeau 2003), and the PCR product was digested with HindIII and BamHI. The three digested PCR products were transformed into the non-replicative plasmid pSW29T (Picardeau 2008), previously digested with XbaI and SpeI. The final plasmid, containing the flanking regions of the *LMANv2_470060/lic13434* gene and SpcR cassette insertion, was transfected into the donor strain *E. coli* β2163 cells, and introduced into the Manilae L495 strain by conjugation, as previously described (Picardeau 2008). After 4–6 weeks of plate incubation at 30°C (1% agar plates of EMJH containing spectinomycin), antibiotic-resistant transformants were inoculated into liquid EMJH supplemented with 50 μg/mL of spectinomycin, and examined for allelic exchange in the target gene by PCR, using primers *lic13434*_F (aaatcctcggctttaatt) and *lic13434*_R (ctcaccctcttgtatattaa).

4.5 | Cloning, Expression and Purification of Recombinant Proteins

The recombinant leptolysin used in this study was previously produced by Courrol et al. (2022). Briefly, the sequence corresponding to full-length leptolysin (*LMANv2_470060 / lic13434*) but excluding the N-terminal signal peptide was amplified by PCR from genomic DNA of *L. interrogans* serovar Copenhageni strain 10A. The fragment was cloned into the pAE expression vector (Ramos et al. 2004). High hydrostatic pressure was used for the solubilization and refolding of leptolysin as described in Courrol et al. (2022). Recombinant thermolysin was previously obtained, as described by Chura-Chambi et al. (2018).

4.6 | Serum Resistance Assays

Manilae WT, Manilae *leptolysin*$^{-}$, and Patoc (5×10^7 leptospires) were incubated in 40% normal human serum (NHS) (Complement Technologies) or in 40% heat-inactivated normal human serum (HI-NHS) at 37°C for 2 h. Bacteria were counted under a dark field microscope using a Petroff-Hausser chamber. The Patoc strain was also incubated at 37°C for 2 h in 40% NHS pretreated for 30 min with 1, 3, or 5 μg of recombinant leptolysin. Three independent assays were performed and survival of strains was compared using unpaired *t*-test. A p value < 0.05 was considered statistically significant. The analyses were performed with the software GraphPad Prism 6.

4.7 | Degradation of Complement Proteins by Leptolysin

Recombinant leptolysin (40 nM or 0.1 μg) was incubated with 0.5 μg of complement C2 (# A112), C3 (# A113), C3b (# A114), C4 (# A105), C4b (# A108), C5 (# A120), C6 (# A123), C7 (# A124), C8 (# A125), and C9 (# A126) (Complement Technologies) for up to 24 h in 50 mM Tris HCl, 200 mM NaCl, 10 mM $CaCl_2$, and 0.05% CHAPS, pH 7.4, at 37°C. In control samples, each substrate was incubated in the assay buffer under identical conditions for 24 h. These assays were also performed in the presence of 5 mmol/L 1,10-phenanthroline by preincubating recombinant leptolysin with this metalloprotease inhibitor for 30 min before the addition of each complement protein. In addition, recombinant leptolysin/thermolysin (0.15–0.6 μM) were incubated with human serum (Complement Technologies) diluted 1:100 for 24 h. Following electrophoresis by 10% SDS-PAGE, the proteins were electroblotted onto nitrocellulose membranes. Degradation products were detected using goat anti-human IgG primary antibodies (Complement Technologies): anti-C2 (# A212, 1:5000), anti-C3 (# A213, 1:5000), anti-C4 (# A205, 1: 2500) anti-C5 (# A220, 1:2000), anti-C6 (# A223, 1:5000), anti-C7 (# A224, 1:5000), anti-C8 (# A225, 1:5000), and anti-C9 (# A226, 1:5000), followed by peroxidase-conjugated rabbit anti-goat antibodies (1:10,000) (Sigma-Aldrich, St. Louis, MO, USA). Positive signals were detected by chemiluminescence (West Pico, Pierce) using Alliance HD6, an Uvitec chemiluminescence Documentation System (Uvitec, Cambridge, UK).

4.8 | Deposition of Complement Proteins

Manilae WT and Manilae *leptolysin*- strains (10^9 bacteria) were harvested by centrifugation at 9000×*g* for 20 min and gently washed in PBS. Next, they were incubated with 2 μg of purified C5 and C9 at 37°C for 15 min. After five washes with PBS, they were incubated with anti-C5 and anti-C9 (1:5000), and bound antibodies were detected with secondary peroxidase-conjugated IgG antibodies (1:10,000). After three washes with PBS, pellets were suspended in 50 μL of citrate phosphate buffer (pH 5.0) and bacteria were transferred to ELISA plate wells. O-phenylenediamine (0.04%) in citrate phosphate buffer (pH 5.0) plus 0.01% H_2O_2 was added. The reaction proceeded for 20 min and was then interrupted by the addition of 50 μL of 8 M H_2SO_4. Absorbance was measured at 492 nm. Means ± the SD for three independent experiments, each performed in triplicate are shown. $^*p < 0.05$; $^{**}p < 0.001$ (Student *t*-test).

4.9 | Evaluation of Virulence in the Hamster Model of Infection

Hamster scarification infection with leptospires was conducted essentially as described by Gostic et al. (2019) and Zhang et al. (2012). Three-week-old male Golden Syrian hamsters (*Mesocricetus auratus*) (four animals per group) were shaved over their flank 1 day before inoculation. On the day of challenge each animal was anesthetized with isoflurane and an area of approximately 3–4 cm^2 was abraded by gentle scraping with a surgical scalpel blade to slightly damage the outermost layer of the skin (stratum corneum). A volume of 50 μL of EMJH medium with 10^3 leptospires was inoculated onto the abraded area, followed by the application of a transparent film dressing to keep the inoculum in place for 5 min. After removal of the dressing, the area was gently washed with distilled water. Hamsters were monitored twice daily after infection. The humane endpoint adopted for hamsters included signs of illness such as difficulty in moving, breathing, and/or signs of bleeding or seizure. Kidneys were collected from all animals after euthanasia, and DNA was extracted for quantitative real-time PCR (qPCR) targeting *lipL32*, as previously described (Wunder Jr, Figueira, Benaroudj, et al. 2016; Wunder Jr, Figueira, Santos, et al. 2016).

4.10 | Evaluation of the Leptolysin Immunogenicity in Human Leptospirosis

Samples of confirmed LG and of the control group (NC) were screened for the presence of naturally acquired antibodies against the recombinant leptolysin by ELISA as previously described (Soares et al. 2020; Matos et al. 2019). Briefly, MaxiSorp 96-well plates (Nunc, Rochester, NY, USA) were coated with 5 μg/mL of recombinant leptolysin. After overnight incubation at 4°C, plates were washed with phosphate-buffered saline (PBS) and blocked with PBS-containing 5% nonfat dry milk (PBS-M) for 1 h at 37°C. Individual samples diluted 1:100 on PBS-M were added in duplicate wells, and the plates were incubated at 37°C for 1 h. After three washes with PBS-Tween20 (0.05%), bound antibodies were detected with peroxidase-conjugated goat anti-human IgG (SouthernBiotech, catalog number: 2048-05) or goat anti-human IgM (catalog number: 2020-05), diluted at 1:1000 (in PBS-M), and incubated for 1 h at 37°C, followed by TMB (3,3′,5,5′-tetramethylbenzidine). The reaction was stopped by the addition of HCl (1 N), and the absorbance was read at 450 nm using an xMark microplate absorbance spectrophotometer (Bio-Rad, Hercules, CA, USA). Thresholds for defining seroreactive for IgG or IgM were calculated using the mean optical density (OD) of 10 NC samples plus 2 standard deviations. Samples from LG were considered IgG/IgM responders if their OD was greater than the threshold (IgM threshold: 0.95, IgG threshold: 1.03).

Author Contributions

Daniella dos Santos Courrol: conceptualization, methodology, investigation, validation. **Cassia Moreira Santos:** methodology, validation, investigation, conceptualization. **Rosa Maria Chura-Chambi:** methodology, investigation. **Lígia Morganti:** funding acquisition, validation. **Kátia Eliane Santos Avelar:** methodology, investigation. **Fernanda de Moraes Maia:** methodology, investigation. **Rodrigo Nunes Rodrigues-da-Silva:** funding acquisition, methodology, validation, supervision. **Elsio Augusto Wunder Jr:** investigation, formal analysis, resources, methodology, writing – review and editing, validation. **Angela Silva Barbosa:** conceptualization, formal analysis, project administration, resources, supervision, writing – original draft, writing – review and editing, validation, funding acquisition.

Acknowledgments

We would like to thank Matilde Costa Lima de Souza (Laboratory of Bacteriology, Instituto Butantan, São Paulo, Brazil), for technical assistance. This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), grants 2018/12896-2, 2019/22706-9 and 2017/12924-3, NIH grant R21AI163663 (EAWJ), and Fundação Butantan.

Data Availability Statement

The data that support the findings of this study are openly available in Repositório do Instituto Butantan at https://repositorio.butantan.gov.br/.

References

Amamura, T. A., T. R. Fraga, S. A. Vasconcellos, A. S. Barbosa, and L. Isaac. 2017. "Pathogenic *Leptospira* Secreted Proteases Target the Membrane Attack Complex: A Potential Role for Thermolysin in Complement Inhibition." *Frontiers in Microbiology* 8: 958. https://doi.org/10.3389/fmicb.2017.00958.

Barbosa, A. S., and L. Isaac. 2020. "Strategies Used by *Leptospira* Spirochetes to Evade the Host Complement System." *FEBS Letters* 594: 2633–2644. https://doi.org/10.1002/1873-3468.13768.

Bauby, H., I. Saint Girons, and M. Picardeau. 2003. "Construction and Complementation of the First Auxotrophic Mutant in the Spirochaete *Leptospira meyeri*." *Microbiology (Reading, England)* 149, no. Pt 3: 689–693. https://doi.org/10.1099/mic.0.26065-0.

Bulach, D., and B. Adler. 2018. "Leptospiral Genomics and Pathogenesis." *Current Topics in Microbiology and Immunology* 415: 189–214. https://doi.org/10.1007/82_2018_87.

Chura-Chambi, R. M., T. R. Fraga, L. B. da Silva, et al. 2018. "*Leptospira interrogans* Thermolysin Refolded at High Pressure and Alkaline pH Displays Proteolytic Activity Against Complement C3." *Biotechnology Reports (Amsterdam, Netherlands)* 19: e00266. https://doi.org/10.1016/j.btre.2018.e00266.

Courrol, D. D. S., C. C. F. da Silva, L. G. Prado, et al. 2022. "Leptolysin, a *Leptospira* Secreted Metalloprotease of the Pappalysin Family With Broad-Spectrum Activity." *Frontiers in Cellular and Infection Microbiology* 12: 966370. https://doi.org/10.3389/fcimb.2022.966370.

da Silva, L. B., M. C. Menezes, E. S. Kitano, et al. 2018. "*Leptospira interrogans* Secreted Proteases Degrade Extracellular Matrix and Plasma Proteins From the Host." *Frontiers in Cellular and Infection Microbiology* 8: 92. https://doi.org/10.3389/fcimb.2018.00092.

Fraga, T. R., D.d S. Courrol, M. M. Castiblanco-Valencia, et al. 2014. "Immune Evasion by Pathogenic Leptospira Strains: The Secretion of Proteases That Directly Cleave Complement Proteins." *Journal of Infectious Diseases* 209: 876–886. https://doi.org/10.1093/infdis/jit569.

Ge, Y. M., A. H. Sun, D. M. Ojcius, et al. 2020. "M16-Type Metallopeptidases Are Involved in Virulence for Invasiveness and Diffusion of *Leptospira interrogans* and Transmission of Leptospirosis." *Journal of Infectious Diseases* 222: 1008–1020. https://doi.org/10.1093/infdis/jiaa176.

Gibson, K. H., F. Trajtenberg, E. A. Wunder, et al. 2020. "An Asymmetric Sheath Controls Flagellar Supercoiling and Motility in the Leptospira Spirochete." *eLife* 9: e53672. https://doi.org/10.7554/eLife.53672.

Gostic, K. M., E. A. Wunder Jr., V. Bisht, et al. 2019. "Mechanistic Dose-Response Modelling of Animal Challenge Data Shows That Intact Skin Is a Crucial Barrier to Leptospiral Infection." *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 374: 20190367. https://doi.org/10.1098/rstb.2019.0367.

Hashimoto, V. L., P. A., Abreu, E., Carvalho, et al. 2013. "Evaluation of the Elastinolytic Activity and Protective Effect of Leptallo I, a Protein Composed by Metalloprotease and FA5/8C Domains, From Leptospira Interrogans Copenhageni." *Microbial Pathogenesis* 61–62: 29–36. https://doi.org/10.1016/j.micpath.2013.04.011.

Jusko, M., J. Potempa, D. Mizgalska, et al. 2015. "A Metalloproteinase Mirolysin of *Tannerella forsythia* Inhibits all Pathways of the Complement System." *Journal of Immunology (Baltimore, Md.: 1950)* 195: 2231–2240. https://doi.org/10.4049/jimmunol.1402892.

Kassegne, K., W. Hu, D. M. Ojcius, et al. 2014. "Identification of Collagenase as a Critical Virulence Factor for Invasiveness and Transmission of Pathogenic Leptospira Species." *Journal of Infectious Diseases* 209: 1105–1115. https://doi.org/10.1093/infdis/jit659.

Koneru, L., M. Ksiazek, I. Waligorska, et al. 2017. "Mirolysin, a LysargiNase From *Tannerella forsythia*, Proteolytically Inactivates the Human Cathelicidin, LL-37." *Biological Chemistry* 398: 395–409. https://doi.org/10.1515/hsz-2016-0267.

Lo, M., S. J. Cordwell, D. M. Bulach, and B. Adler. 2009. "Comparative Transcriptional and Translational Analysis of Leptospiral Outer Membrane Protein Expression in Response to Temperature." *PLoS Neglected Tropical Diseases* 3: e560. https://doi.org/10.1371/journal.pntd.0000560.

Matos, A. D. S., R. N. Rodrigues-da-Silva, I. F. Soares, et al. 2019. "Antibody Responses Against *Plasmodium vivax* TRAP Recombinant and Synthetic Antigens in Naturally Exposed Individuals From the Brazilian Amazon." *Frontiers in Immunology* 10: 2230. https://doi.org/10.3389/fimmu.2019.02230.

Murray, G. L. 2015. "The Molecular Basis of Leptospiral Pathogenesis." *Current Topics in Microbiology and Immunology* 387: 139–185. https://doi.org/10.1007/978-3-662-45059-8_7.

Oliveira, P. N., D. S. Courrol, R. M. Chura-Chambi, et al. 2021. "Inactivation of the Antimicrobial Peptide LL-37 by Pathogenic Leptospira." *Microbial Pathogenesis* 150: 104704. https://doi.org/10.1016/j.micpath.2020.104704.

Picardeau, M. 2008. "Conjugative Transfer Between *Escherichia coli* and *Leptospira* spp. as a New Genetic Tool." *Applied and Environmental Microbiology* 74: 319–322. https://doi.org/10.1128/AEM.02172-07.

Ramos, C. R., P. A. Abreu, A. L. Nascimento, and P. L. Ho. 2004. "A High-Copy T7 *Escherichia coli* Expression Vector for the Production of Recombinant Proteins With a Minimal N-Terminal his-Tagged Fusion Peptide." *Brazilian Journal of Medical and Biological Research = Revista Brasileira de Pesquisas Medicas e Biologicas* 37: 1103–1109. https://doi.org/10.1590/s0100-879x2004000800001.

Robin, C., and L. Westblade. 2015. *Survival Strategies of Extracellular Bacterial Pathogens in Molecular Medical Microbiology*. 2nd ed, 475–489. London, UK: Academic Press. https://doi.org/10.1016/B978-0-12-397169-2.00027-5.

Soares, I. F., C. López-Camacho, R. N. Rodrigues-da-Silva, et al. 2020. "Recombinant *Plasmodium vivax* Circumsporozoite Surface Protein Allelic Variants: Antibody Recognition by Individuals From Three Communities in the Brazilian Amazon." *Scientific Reports* 10: 14020. https://doi.org/10.1038/s41598-020-70893-3.

Surdel, M. C., P. N. Anderson, B. L. Hahn, and J. Coburn. 2022. "Hematogenous Dissemination of Pathogenic and Non-pathogenic *Leptospira* in a Short-Term Murine Model of Infection." *Frontiers in Cellular and Infection Microbiology* 12: 917962. https://doi.org/10.3389/fcimb.2022.917962.

Tallant, C., R. García-Castellanos, A. Marrero, et al. 2007. "Activity of Ulilysin, an Archaeal PAPP-A-Related Gelatinase and IGFBP Protease." *Biological Chemistry* 388: 1243–1253. https://doi.org/10.1515/BC.2007.143.

Viana, F., S. S. Peringathara, A. Rizvi, and G. N. Schroeder. 2021. "Host Manipulation by Bacterial Type III and Type IV Secretion System Effector Proteases." *Cellular Microbiology* 23: e13384. https://doi.org/10.1111/cmi.13384.

Wunder, E. A., Jr., C. P. Figueira, G. R. Santos, et al. 2016. "Real-Time PCR Reveals Rapid Dissemination of *Leptospira interrogans* After Intraperitoneal and Conjunctival Inoculation of Hamsters." *Infection and Immunity* 84: 2105–2115. https://doi.org/10.1128/IAI.00094-16.

Wunder, E. A., Jr., C. P. Figueira, N. Benaroudj, et al. 2016. "A Novel Flagellar Sheath Protein, FcpA, Determines Filament Coiling, Translational Motility and Virulence for the Leptospira Spirochete." *Molecular Microbiology* 101: 457–470. https://doi.org/10.1111/mmi.13403.

Zhang, Y., X. L. Lou, H. L. Yang, et al. 2012. "Establishment of a Leptospirosis Model in Guinea Pigs Using an Epicutaneous Inoculations Route." *BMC Infectious Diseases* 12: 20. https://doi.org/10.1186/1471-2334-12-20.

Zhu, W., F. J. Passalia, C. Hamond, et al. 2023. "MPL36, a Major Plasminogen (PLG) Receptor in Pathogenic *Leptospira*, Has an Essential Role During Infection." *PLoS Pathogens* 19: e1011313. https://doi.org/10.1371/journal.ppat.1011313.

Supporting Information

Additional supporting information can be found online in the Supporting Information section.