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Inactivation of the antimicrobial peptide LL-37 by pathogenic *Leptospira*

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A R T I C L E I N F O

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A B S T R A C T

Leptospires are aerobic, Gram-negative spirochetes with a high invasive capacity. Pathogenic leptospires secrete proteases that inactivate a variety of host's proteins including molecules of the extracellular matrix and of the human complement system. This strategy, used by several pathogens of medical importance, contributes to bacterial invasion and immune evasion. In the current work we present evidence that *Leptospira* proteases also target human cathelicidin (LL-37), an antimicrobial peptide that plays an important role in the innate immune response. By using six *Leptospira* strains, four pathogenic and two saprophytic, we demonstrated that proteases present in the supernatants of pathogenic strains were capable of degrading LL-37 in a time-dependent manner, whereas proteolytic degradation was not observed with the supernatants of the two saprophytic strains. Inactivation of LL-37 was prevented by using the 1,10-phenanthroline inhibitor, thus suggesting the involvement of metalloproteinases in this process. In addition, the antibacterial activity of LL-37 against two *Leptospira* strains was evaluated. Compared to the saprophytic strain, a greater resistance of the pathogenic strain to the action of the peptide was observed. Our data suggest that the capacity to inactivate the host defense peptide LL-37 may be part of the virulence arsenal of pathogenic *Leptospira*, and we hypothesize that its inactivation by the bacteria may influence the outcome of the disease.

1. Introduction

Bacteria of the genus *Leptospira* are thin, elongated, and helical shaped Gram-negative spirochetes that may cause disease in humans and animals. Hitherto, 64 species of *Leptospira* classified into two major clades have been described. The P clade comprises pathogenic species, and the S clade is composed of free-living, saprophytic species [1]. More than 1 million cases of human leptospirosis are reported each year worldwide, with approximately 60 thousand deaths [2].

Once in the host, pathogenic leptospires spread very rapidly, and the great dissemination capacity of these spirochetes is attributed, among other factors, to their efficiency in moving through viscous media [3]. They also employ strategies to modulate host's microenvironment and innate immune responses, such as the ability to resist serum bactericidal activity [4,5].

The secretion of proteases with the potential to inactivate essential host proteins is an important tool used by several microorganisms during the colonization process. Pathogenic leptospires are no exception to this, and have been shown to secrete proteases capable of degrading a range of host molecules, including human complement system components as well as extracellular matrix molecules [6–8]. Certain pathogens of medical significance also display mechanisms of resistance to antimicrobial peptides, which help them to cause serious infections. Although rather resistant to proteolytic degradation [9], antimicrobial peptides can be efficiently cleaved and inactivated by bacterial proteases with a broad spectrum of activity [10]. These soluble, low molecular weight molecules play an important role in the innate immune response of humans, animals, and plants. By inhibiting the growth of microorganisms in the skin and mucous surfaces and their subsequent dissemination they contribute to natural immune responses in humans [11].

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Upon insertion into membrane bilayers of their targets, antimicrobial peptides facilitate the elimination of microorganisms causing deleterious damage to them [12].

Cathelicidins are a family of antimicrobial peptides found in vertebrates. They are produced and stored as inactive pro-peptides in neutrophil granules [13,14], but are also produced by other immune cells including Natural Killer (NK) and mast cells, and by barrier epithelia [15–17]. Cathelicidins are constitutively expressed at low levels, but their expression is enhanced upon exposure to infectious agents leading to a local increase at the infection site. Approximately 30 members of the cathelicidin family have been described in mammals [18]. They have a conserved N-terminal cathelin domain and differ in their C-terminal regions, which are responsible for their known biological functions among which cytotoxic and antibacterial activities [19]. The only human cathelicidin identified so far, human cationic antimicrobial protein (hCAP18), has a molecular weight of 18 kDa, and consists of a signal peptide (30 aa residues), a highly conserved N-terminal domain (103 aa residues) and a C-terminal active domain (37 aa residues). To become active, the C-terminus with a predicted molecular mass of ~4 kDa corresponding to the peptide LL-37 is released from its precursor [20].

In the present study, we aimed to extend the range of investigation of possible targets for *Leptospira* proteases released to the extracellular milieu. We focused on antimicrobial peptides, notably on LL-37, which was shown to be particularly susceptible to degradation when in contact with culture supernatants of pathogenic *Leptospira* strains. Given that antimicrobial peptides are considered as potential therapeutic antibiotic candidates, increasing our current knowledge on the resistance mechanisms used by pathogenic *Leptospira* to counteract their effects may contribute to preventive or treatment approaches for leptospirosis.

2. Materials and Methods

2.1. Antimicrobial peptides

All antimicrobial peptides were chemically synthesized. LL-37 was purchased from Bachem (USA), and α-defensins HNP-1 and -2, and β-defensins 1 and 2 from Sigma-Aldrich™ (USA).

2.2. Leptospiral strains and growth conditions

The virulent strains *Leptospira interrogans* serovar Kennewick strain Fromm (LPF) and *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (L1-130), the culture-attenuated *Leptospira interrogans* serovar Copenhageni strain 10A (Cop 10A) and *Leptospira kirschneri* serovar Cynopteri strain 3522C (Cynopteri), and the saprophytes *Leptospira biflexa* serovar Patoc strain Patoc I (Patoc) and *Leptospira biflexa* serovar Andamana strain CH11 (Andamana) were provided by the Laboratory of Bacterial Zoonosis, School of Veterinary Medicine and Animal Sciences, University of São Paulo, Brazil. Virulence of LPF and L1-130 strains was maintained by iterative passages in hamsters. All strains were cultured at 28–30 °C under aerobic conditions in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco™ Laboratories, Sparks, Maryland, EUA) supplemented as previously described [21].

2.3. Collection of culture supernatants

Leptospira cultures grown for 7 days were harvested by centrifugation at 3200 x *g* for 25 min at room temperature. Bacterial pellets were washed twice in phosphate-buffered saline (PBS) pH 7.4, and after counting using a Petroff-Hausser chamber under dark-field microscopy, leptospires were suspended in PBS pH 7.4 (2×10^9/mL). Bacteria were incubated at 37 °C for 4 h to allow secretion of proteases because proteolytic activity against host's molecules is clearly observed under these conditions [6,8]. Supernatants were collected as previously described [6], and stored at −80 °C until use. Total proteins in the supernatants were estimated by using the BCA Protein Assay kit (Pierce) and were analyzed by 12% SDS-PAGE [22].

2.4. Gelatin zymography

Gelatinolytic enzymatic activity in *Leptospira* culture supernatants was assessed by zymography as described elsewhere [8].

2.5. Proteolytic degradation of antimicrobial peptides by Leptospira proteases

Proteolytic activity of proteases present in the *Leptospira* supernatants on antimicrobial peptides was assessed by incubating 5 μg of proteins from the culture supernatants with 4 μg of each antimicrobial peptide (LL-37, α-defensins HNP-1 and -2, and β-defensins 1 and 2) in a total volume of 25 μL. Samples were incubated for 1, 4, 7 or 20 h at 37 °C to simulate host's conditions. As negative controls, antimicrobial peptides or the bacterial culture supernatants diluted in PBS pH 7.4 were incubated at 37 °C for 20 h. The assays were performed in triplicate for all strains evaluated. Cleavage products were visualized on 15% silver-stained SDS-PAGE gels.

2.6. Identification of protease classes involved in the degradation of LL-37 by specific protease inhibition

Culture supernatants (5 μg of total proteins) were preincubated with inhibitors of serine (5 mmol/L phenylmethylsulphonyl fluoride), metallo- (5 mmol/L 1,10-phenanthroline), cysteine (28 μmol/L E−64) or aspartyl (5 μmol/L pepstatin) proteases for 30 min at 37 °C before the addition of LL-37 (4 μg). Except for E64, which was dissolved in phosphate buffered saline, PMSF, 1,10 phenanthroline, and pepstatin were dissolved in ethanol or in ethanol-water (1:1) following manufacturer's instructions. Reactions were incubated at 37 °C for additional 20 h. In the positive controls, protease inhibitors were omitted, and in the negative controls LL-37 or the bacterial culture supernatants diluted in PBS pH 7.4 were incubated at 37 °C for 20 h. The incubation products were visualized on 15% silver-stained SDS-PAGE gels.

2.7. Cloning, expression, and purification of the metalloproteases thermolysin and pappalysin-1 domain protein

Thermolysin encoded by *LIC13322* and pappalysin-1 domain protein encoded by *LIC13434* were produced as recombinant proteins in *Escherichia coli*. A fragment corresponding to the prothermolysin (74 kDa) was amplified by polymerase chain reaction (PCR) from genomic DNA of *L. interrogans* serovar Copenhageni strain 10A using the following primers: F – 5′ CTCGAGCAAGTTCCAGAGAA 3′, and R – 5′CGCAAGCTTCTAAATACTG 3'. The sequence corresponding to full-length pappalysin-1 domain protein (50 kDa) was also amplified by PCR using the same *Leptospira* strain and the following primers: F – 5′ GGATCCAAAGGCAAAGACGATAATTCTAAAAAC 3′ and R - 5′ CCATGGTTAATATACAAGAGGGTGAGAACGG 3'. *E. coli* DH5α was the cloning host strain, and *E. coli* BL21 (SI) and *E. coli* BL21 (DE3) were used for expression of thermolysin and of pappalysin-1 domain protein, respectively, using a T7 promoter-based expression plasmid pAE [23]. Thermolysin and pappalysin-1 domain protein inclusion bodies were solubilized and refolded using high hydrostatic pressure and alkaline pH, as previously described [24]. Refolded proteases were purified by high-performance size-exclusion chromatography (HPSEC) on a Tosohaas (Montgomeryville, PA, USA) G2000 SW column (60 cm 7.5 mm i.d., particle size 10 mm, pore size 125 Å) coupled to a 7.5 cm 7.5 mm i.d. SW guard column. The mobile phase used was 0.025 M sodium phosphate, pH 8.0, at a flow rate of 1.0 mL/min. Sample elution was detected by UV absorbance at a wavelength of 220 nm.

2.8. *Leptospira* viability quantification upon incubation with LL-37

The anti-leptospiral activity of LL-37 was assessed by the resazurin reduction test (Alamar Blue assay). Briefly, resazurin (blue and nonfluorescent) becomes pink and highly fluorescent when is reduced to resorufin. The reduction of resazurin correlates with the number of live cells [25]. The virulent *L. interrogans* strain LPF and the saprophytic *L. biflexa* strain Patoc I were grown for 7 days at 28–30 °C in modified EMJH as previously described. Then, 6×10^6 bacteria were incubated with different concentrations of LL-37 (3, 6, 12, 25, 37.5 and 50 μM). The reactions were incubated for 2 h at 37 °C, and 20 μL of Resazurin (Sigma-Aldrich™) at 0.02% w/v was added to each tube. Incubations proceeded for 2 additional hours in the dark. The reaction products (100 μL) were transferred to 96-well culture microplates, and the absorbance was obtained in a microplate reader (Multiskan®EX (Thermo Fisher Scientific, USA)) at 550 nm (reduced) and 595 nm (oxidized). Controls included medium alone and medium with resazurin. The level of reduction of resazurin was calculated according to the manufacturer's formula of Alamar Blue assay. *Leptospira* viability was also assessed in the presence of LL-37 (25 μM) preincubated with LPF or Patoc I supernatant (5 μg total protein) for 20 h, essentially as described above. LL-37 in PBS (also preincubated for 20 h) was used as a control.

2.9. Statistical analysis

The independent samples Student's *t*-test was used to compare the proteolytic activity of each *Leptospira* strain at different incubation periods in relation to T_0, and also to assess differences between the strains LPF and Patoc I in the resazurin reduction tests using LL-37. $P < 0.05$ was considered statistically significant. The analyses were performed with the *software* GraphPad Prism 6.

3. Results

3.1. Electrophoretic and proteolytic profiles of supernatant proteins secreted by *Leptospira* strains

In this work, 4 pathogenic and 2 saprophytic *Leptospira* strains were evaluated for their capacity to degrade human cathelicidin. The electrophoretic profiles of supernatant proteins of all 6 strains are shown in Fig. 1 A. Protein profiles of the two saprophytic strains are quite similar to each other but differ from those exhibited by the attenuated (Cop 10A and Cynopteri) or virulent (L1-130 and LPF) strains.

Proteolytic properties of the above-mentioned supernatants were evaluated by gel-zymography. Proteases of approximately 48 and 75–100 kDa with a pronounced gelatinolytic activity were detected in the Cynopteri and LPF supernatants. A faint proteolytic activity was observed in the L1-130 strain, but Cop 10A as well as the saprophytic strains Patoc I and Andamana supernatants did not present gelatinolytic activity (Fig. 1 B).

3.2. Extracellular proteases from pathogenic *Leptospira* degrade LL-37

Proteolytic activity of secreted *Leptospira* proteases against LL-37 was assessed. Proteases present in the supernatants of pathogenic leptospires (strains LPF, L1-130, Cop 10A, and Cynopteri) cleaved the peptide, which migrates as a 4 kDa band. Degradation was more pronounced as the incubation time increased, that is, cleavage was time-dependent, requiring 20 h of incubation for maximum degradation. Given the small size of the peptide, no intermediate cleavage fragments could be observed. Interestingly, the strain Cynopteri was exceptionally efficient in inactivating LL-37 (Fig. 2), and also displayed a pronounced gelatinolytic activity (Fig. 1 B). Conversely, proteases from supernatants of the 2 saprophytic strains (Patoc I and Andamana) did not degrade LL-37 in any of the incubation times tested (Fig. 2). Assays were performed in triplicate and a representative gel of three is shown on the left for each strain. Densitometry analysis considering the relative band intensities of LL-37 was performed based on the average of the three assays using the Alliance HD 6 v16.15 UVITEC software (bar graphs on the right). Under the same experimental conditions, no proteolytic activity was observed against α-defensins HNP-1 and -2, and β-defensins 1 and 2 (Suppl. Fig. 1).

3.3. *Leptospira* metalloproteinases are responsible for LL-37 degradation

To assess the class(es) of proteases responsible for LL-37 inactivation, proteolytic assays were performed in the presence of specific inhibitors with the four *Leptospira* strains shown to degrade LL-37. Degradation of LL-37 was fully inhibited only in the presence of 1,10-phenanthroline, strongly pointing to a role of metalloproteinases in the proteolytic inactivation of human cathelicidin (Fig. 3). When we used the LPF and L1-130 supernatants, a slight inhibition of LL-37 degradation was also observed in the presence of PMSF (Fig. 3 A, B).

In an attempt to identify potential metalloproteases with the ability to degrade LL-37, we performed additional proteolytic assays employing two extracellular enzymes specific to or conserved among pathogenic *Leptospira* species. One of them, the thermolysin encoded by *LIC13322* (a metalloprotease from the peptidase M4 family), was previously shown to cleave the complement system proteins C3 and C6 [6,7]. The other, pappalysin-1 domain protein (a metalloprotease from the M23 family), was identified in a proteomic study that aimed to uncover extracellular proteases that could be responsible for the degradation of host

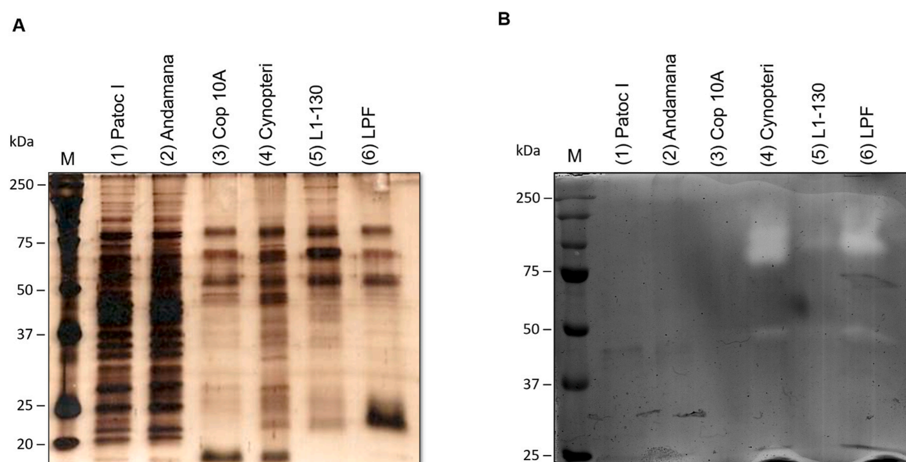
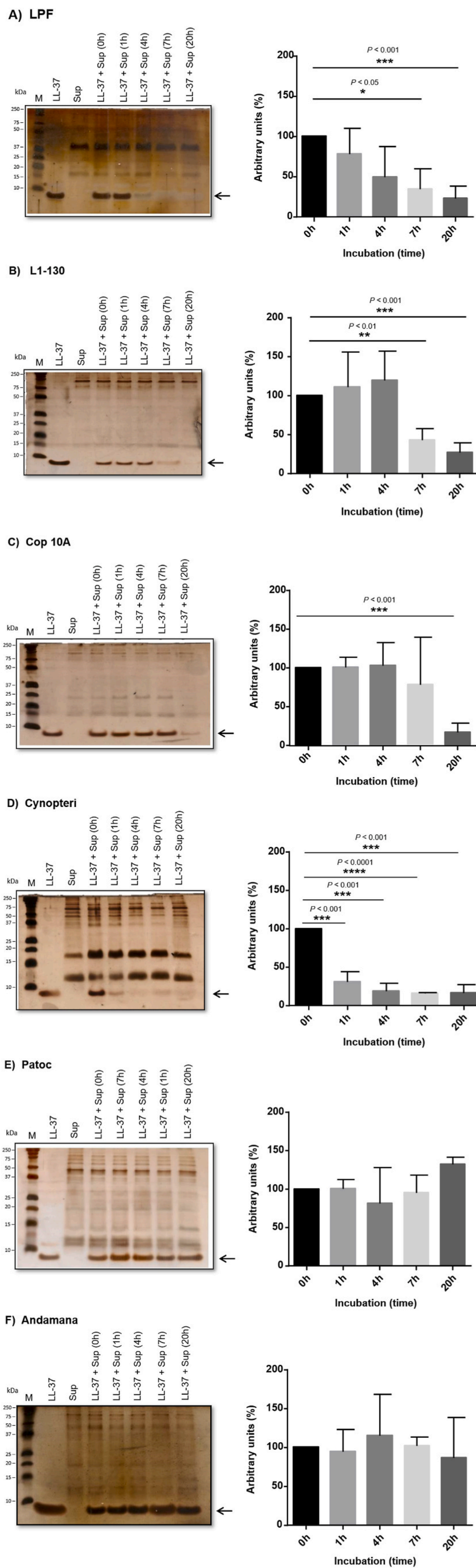


Fig. 1. Electrophoretic and proteolytic profiles of supernatant proteins secreted by *Leptospira* strains. A) Total proteins obtained from 1×10^9 bacteria were analyzed by 12% SDS-PAGE. The gel was silver stained. **B)** Proteins present in the supernatants (5 μg of total secreted proteins) were submitted to zymography on a 12% SDS-PAGE gel copolymerized with gelatin (1 mg/mL) and Coomassie Brilliant Blue stained. (M) Kaleidoscope molecular mass marker (Bio-Rad); (1) *L. biflexa* serovar Patoc strain Patoc I; (2) *L. biflexa* serovar Andamana strain CH11; (3) *L. interrogans* serovar Copenhageni strain 10A; (4) *L. kirschneri* serovar Cynopteri strain 3522C; (5) *L. interrogans* serovar Copenhageni strain Fiocruz L1-130; (6) *L. interrogans* serovar Kennewick strain Fromm (LPF).



(caption on next column)

Fig. 2. Degradation of LL-37 peptide by *Leptospira* proteases. Bacterial supernatants were incubated with LL-37 (4 μg) for different times (1, 4, 7 and 20 h) at 37 °C. Peptide degradation was analyzed by SDS-PAGE 15%. The gels were silver stained (left) and the LL-37 peptide (4 kDa) is indicated by an arrow. Densitometry analyzes were performed from the average of three independent assays with the Alliance HD 6 v16.15 UVITEC software (right). (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$, (****) $P < 0.0001$ according to the t-Student test for independent samples. (M) Kaleidoscope molecular mass marker (Bio-Rad). A) *Leptospira interrogans* serovar Kennewick strain Fromm (LPF), B) *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (L1-130), C) *Leptospira interrogans* serovar Copenhageni strain 10A (Cop 10A), D) *Leptospira kirschneri* serovar Cynopteri strain 3522C (Cynopteri), E) *Leptospira biflexa* serovar Patoc strain Patoc I (Patoc I), and F) *Leptospira biflexa* serovar Andamana strain CH11 (Andamana).

extracellular matrix components and plasma proteins [8]. As shown in Suppl. Fig. 2, LL-37 was not degraded either by thermolysin or pappalysin-1 domain protein. Therefore, we assume that other not yet identified metallopeptidases are responsible for LL-37 inactivation.

3.4. Leptospira susceptibility to LL-37

Given that the supernatants of pathogenic *Leptospira* strains were capable of inactivating LL-37 whereas the supernatants of saprophytic strains did not present proteolytic activity against this particular peptide, we wondered if they would differ in susceptibility to the bactericidal activity of human cathelicidin. To assess leptospiral viability a resazurin reduction test was employed. At concentrations ≥25 μM of LL-37 both strains were susceptible to the action of LL-37 and, on the other hand, at concentrations ≤6 μM both survived. Differences in survival rate were noticed when 12 μM of LL-37 was used ($P < 0.0001$). At this LL-37 concentration Patoc I strain survival was reduced by half, whereas LPF strain did not present mortality (Fig. 4 A). The assay was performed six times, and a representative experiment is shown in Suppl. Fig. 3 A. To assess if LL-37 does become functionally inactive upon incubation with secreted proteases from pathogenic *Leptospira* strains we performed an additional assay in which we preincubated LL-37 (25 μM) with LPF or Patoc I supernatant for 20 h. Viability of both strains in the presence of the proteases-LL-37 mixtures was then evaluated. As shown in Fig. 4 B, LPF survival was not compromised in the presence of LL-37 preincubated with LPF supernatant. Conversely, Patoc I survival was quite affected in the presence of Patoc-LL-37 mixture, as expected. Both strains were susceptible to the action of LL-37 (25 μM) preincubated in PBS for 20 h. Based on these data we assume that LL-37 is functionally inactivated by proteases released by the virulent LPF strain. A representative experiment of three is shown in Suppl. Fig. 3 B.

4. Discussion

Antimicrobial peptides are part of the innate immune system of a variety of organisms including prokaryotes and eukaryotes [26]. Typically, they are short, amphiphilic, and positively charged molecules that induce cell damage mainly due to destabilization of the target cell membranes resulting from electrostatic interactions [27]. Certain pathogens of medical importance have mechanisms to counteract the action of antimicrobial peptides. Since leptospires are known to secrete proteases with a broad activity spectrum [6,8], in this work we evaluated the action of those proteases on human cathelicidin, and human α- and β-defensins, two important groups of mammalian antimicrobial peptides.

Here, we provide *in vitro* evidence that proteases secreted by four different *Leptospira* strains functionally inactivate the LL-37 peptide. Proteolytic degradation was only observed with culture supernatants from pathogenic species, strongly suggesting that the proteases involved in LL-37 degradation are not secreted by the two saprophytic strains Patoc I and Andamana. In all four strains, LL-37 inactivation was

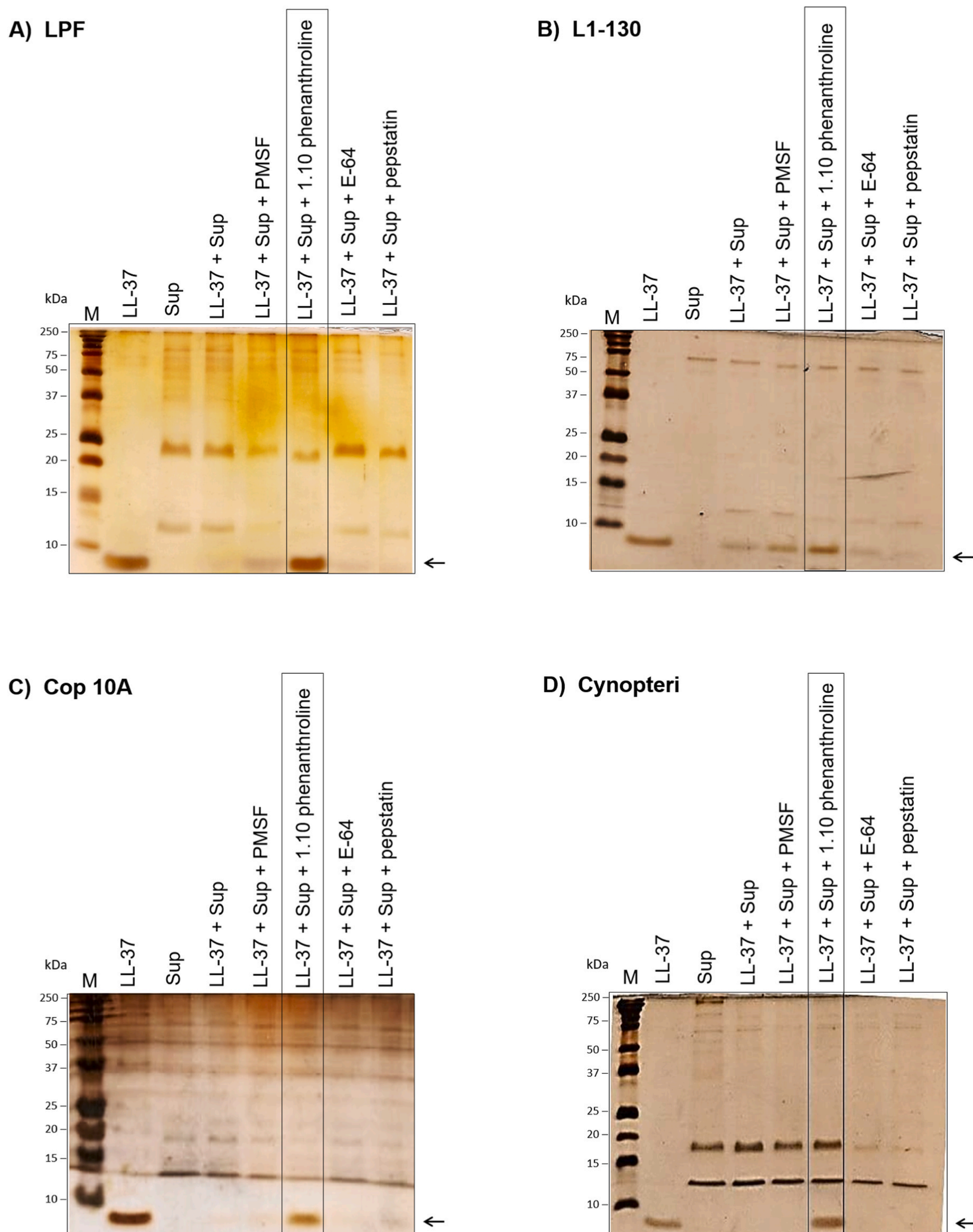


Fig. 3. Leptospiral metalloproteinases are involved in the degradation of the antimicrobial peptide LL-37. Bacterial supernatants were preincubated for 30 min at 37 °C with the following inhibitors: 5 mM PMSF, 5 mM 1.10-phenanthroline, 28 μM E−64 or 5 μM pepstatin. LL-37 (4 μg) was subsequently added to the reactions and the incubations continued for further 20 h at 37 °C. The reaction products were analyzed by SDS-PAGE 15%. The gels were silver stained and the LL-37 peptide (4 kDa) is indicated by an arrow. (M) Kaleidoscope molecular mass marker (Bio-Rad). **A)** *Leptospira interrogans* serovar Kennewick strain Fromm (LPF), **B)** *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 (L1-130), **C)** *Leptospira interrogans* serovar Copenhageni strain 10A (Cop 10A), **D)** *Leptospira kirschneri* serovar Cynopteri strain 3522C (Cynopteri).

completely abolished by 1,10-phenanthroline. Therefore, as reported for other hosts' molecules including ECM components, as well as complement and coagulation cascade molecules, metalloproteinases seem to be the main class of proteases responsible for degradation of LL-37. In previous studies, our research group demonstrated that the thermolysin encoded by *LIC13322* targets complement system proteins, and may contribute to leptospiral immune evasion [6,7]. Pappalysin-1 domain protein - another secreted metalloproteinase well-conserved among pathogenic *Leptospira* strains - was uncovered in a proteomic study aiming at identifying extracellular proteases that could be responsible

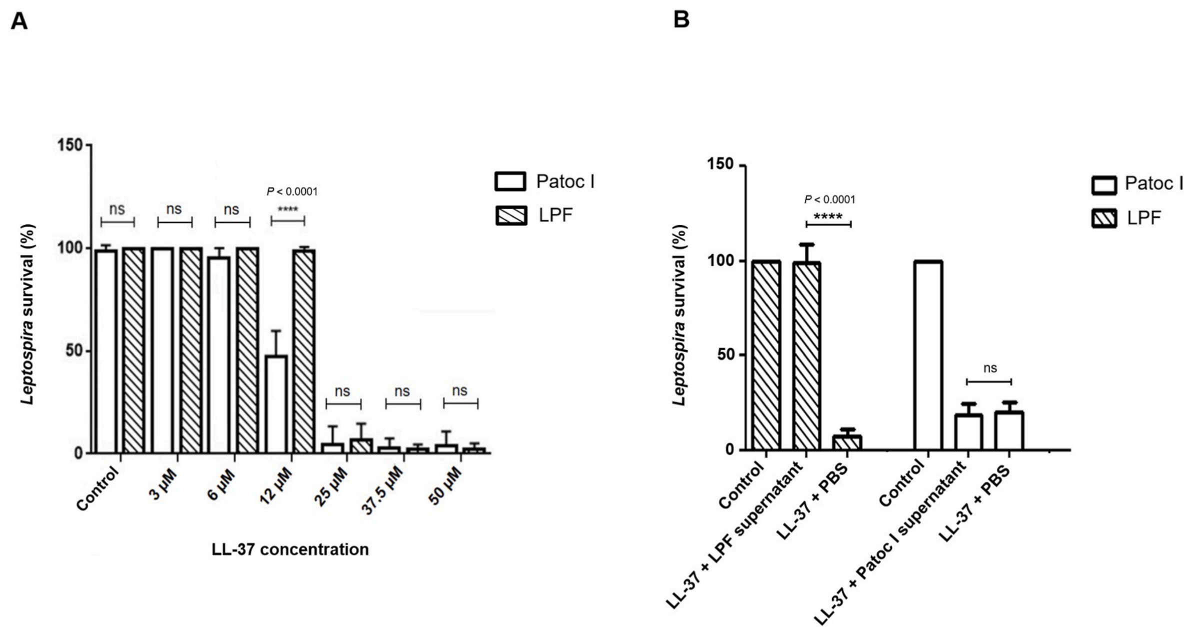


Fig. 4. Antileptospiral activity of LL-37. A) *Leptospira interrogans* serovar Kennewick strain Fromm (LPF) and *Leptospira biflexa* serovar Patoc strain Patoc I (Patoc I) (6,0 × 10^6 cells) were incubated with 3, 6, 12, 25, 37.5 or 50 μM of LL-37. The reactions were incubated for 2 h at 37 °C, and 20 μL of resazurin (Sigma-Aldrich™) at 0.02% w/v was added to each tube. Incubations proceeded for 2 additional hours in the dark. The reaction products were transferred to 96-well culture microplates, and the level of reduction was quantified using a spectrophotometer as described in Materials and Methods. The average of six assays is presented. B) *Leptospira* viability was also assessed in the presence of LL-37 (25 μM) preincubated with LPF or Patoc I supernatant (5 μg total protein) for 20 h, essentially as described above. LL-37 in PBS (also preincubated for 20 h) was used as a control. The average of three assays is presented. ****$P < 0.0001$; *ns*, not significant.

for the degradation of host extracellular matrix components and plasma proteins [8]. Functional and biochemical characterization of these metalloproteinases is currently under way, and will allow us to have a clearer picture concerning their substrate specificities. In the current study, these two potential candidates were evaluated for their capacity to degrade LL-37. However, none of them could inactivate the antimicrobial peptide. Thus the identity of the protease (s) responsible for the inactivation of LL-37 is yet to be determined. In previous studies, proteases secreted by the virulent LPF strain were shown to degrade complement components, plasma fibronectin, fibrinogen, small leucine-rich proteoglycans, besides gelatin [6,8]. Given the diversity of host molecules targeted by pathogenic *Leptospira*, these spirochetes are very likely to secrete an array of proteases with a certain degree of specificity against the various classes of molecules found in the host. Of note, the strains Cynopteri and LPF, which showed a marked gelatinolytic activity, were also quite effective in inactivating LL-37. It remains to be investigated whether *Leptospira* collagenases would also have proteolytic activity against LL-37.

Since LL-37 inactivation occurred only in the presence of proteases secreted by pathogenic *Leptospira* strains, we then assessed susceptibility of the LPF (pathogenic) and Patoc I (saprophytic) strains to LL-37. At high peptide concentrations both strains succumbed whereas at low LL-37 concentrations both strains survived. A difference in the survival rate was observed at 12 μM of LL-37, with total viability of the LPF strain but a 50% reduction in the viability of the Patoc I strain. Susceptibility of spirochetes to cathelicidins derived from different mammals including sheep, cattle, pigs, mice and humans has been evaluated by Sambri et al. [28]. According to their study cathelicidins have a fast action on these bacteria, but their efficacy varies depending on the specific peptide and on the strain evaluated. For leptospires, of the five peptides derived from cathelicidins, SMAP-29 from sheep and BMAP-28 from cattle were the most active against *L. interrogans* serovars, with Minimum Inhibitory Concentration (MIC) values varying between 3 and 51 mg/L (0.75–12.75 μM), depending on the strain studied. The MICs of the other cathelicidins varied between 4.3 and 224 mg/L (1.08–56 μM).

We also evaluated the action of proteases secreted by the pathogenic *Leptospira* strain Cynopteri, previously shown to efficiently degrade LL-37, on four other antimicrobial peptides, β – defensins 1 and 2 and α – defensins HNP-1 and 2. Unlike LL-37, all defensins tested under the same conditions were resistant to the action of proteases present in the supernatant of the bacterial culture regardless of the incubation period. Although still preliminary, these findings suggest that *Leptospira* proteases may not act on this class of antimicrobial peptides. However, more studies are required to further address this question.

In a previous work by Lindow and colleagues [29], the authors evaluated multiple parameters of patients hospitalized with acute leptospirosis aiming to detect factors associated with lethal cases of the disease. Interestingly, lower serum levels of LL-37 were associated with a higher bacterial load, and fatal cases. A more pronounced inflammatory response with major tissue damage was observed in this group of patients compared to those that survived. Their findings are important to propose cathelicidin as a therapeutic approach. In this regard, it may be relevant to study mechanisms originating from the pathogen during the infectious process that may interfere with the bactericidal activity of the host.

In conclusion, our study provides evidence that proteases of pathogenic leptospires have proteolytic effect on LL-37, a potential contributor to the outcome of leptospirosis in humans. Future studies are still necessary to clarify the *in vivo* relevance of our findings, and to elucidate whether inactivation of this host defense peptide by leptospires may influence the course of the disease.

Author statement

PNO and DSC: Performed the assays.
RMCC and LM: Purified the recombinant proteases.
GOS: Provided *Leptospira* strains.
MRF: Provided resources and contributed do data analysis.
EAWJ: Provided resources and contributed do data analysis.
MBH: Provided resources and contributed do data analysis.
ASB: Conceptualization and writing.

Declaration of competing interest

The authors declare they have no competing interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104704.

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