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Failure of LIC13435 Protein of *Leptospira interrogans* Serovar Copenhageni to Confer Protection in Immunized Hamsters

Vivian L Hashimoto[1,3], Zenaide M Moraes[2], Amane P Gonçales[2], Silvio A Vasconcellos[2] and Paulo L Ho[1,3]*

[1]*Centro de Biotecnologia, Instituto Butantan, São Paulo, SP, Brazil*
[2]*Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brazil*
[3]*Interunidades em Biotecnologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, SP, Brazil*

Abstract

Leptospirosis is a re-emergent zoonosis characterized by an acute febrile and systemic illness in humans caused by pathogenic spirochetes belonging to the genus *Lepstospira*. This disease has global distribution, and it is more frequent in tropical and subtropical areas. The complete genomic sequence of *Leptospira* species offered the possibility to identify potential vaccine candidates for leptospirosis, since environmental control measures are difficult to implement and there is not an ideal vaccine available for human use. Secreted and surface exposed molecules are potential targets for inducing protective immune response in the host. Although we selected six predicted sequences coding for putative outer membrane proteins with unknown function to be analyzed as vaccinal candidates against leptospirosis and for biological characterization, only the *lic13435* gene was expressed and purified. The *lic13435* gene is specific for pathogenic leptospires suggesting a possible virulence and/or pathogenicity associated function. The recombinant protein was purified and tested as vaccine candidate against leptospirosis. The immunization with the recombinant protein was able to produce a significant immune response in hamsters. Nevertheless, the animals were not protected against leptospirosis.

Keywords: Leptospira interrogans Copenhageni; Leptospirosis; Leptospira; Recombinant protein purification

Introduction

Leptospirosis, a widespread zoonosis caused by spirochetes belonging to the genus *Leptospira*, remains a major public and veterinary health problem in developing countries [1,2]. Mortality remains significant, related both to delays in diagnosis due to lack of infrastructure and adequate clinical diagnosis, and to other poorly understood reasons that may include inherent pathogenicity of some leptospiral strains or genetically determined host immunopathological responses [3]. Moreover, the overall disease burden is underestimated, since leptospirosis is a significant cause of undifferentiated fever frequently observed in other disease symptoms [4].

Since environmental control measurements are difficult to implement and there is not a universal available vaccine for human use, the complete genomic sequence of *Leptospira* species provides a window of opportunity to identify potential vaccine candidates for leptospirosis.

The immunogenic proteins, especially the outer membrane surface proteins of pathogenic *Leptospira*, may be effective as vaccinogens. The identification of proteins, which are conserved among pathogenic *Leptospira* that could generate cross-protection against various serovars, has become a major focus of leptospirosis research [5-17].

In the present study we have selected six genes (*lic10359, lic11211, lic13435, lic10291, lic20087, lic10544*) coding for predicted outer-membrane proteins, which function still have not been determined. All genes were cloned but only recombinant LIC13435 protein ($rLIC13435_{25\text{-}190}$) could be expressed and characterized. Orthologous proteins can also be observed in sequenced genome of pathogenic *Leptospira* but not in the saprophytic *L biflexa,* suggesting that this protein may play an important role during the infection. The *lic13435* gene was amplified from several pathogenic leptospiral strains and transcripts were also detected only in pathogenic retro-transcribed RNAs tested. The recombinant protein was expressed and purified in heterologous system. Peptide sequencing confirmed the correct purified protein and, the circular dichroism spectrum indicated that the protein was structured as predicted. Although the ELISA test indicates that the recombinant protein is very immunogenic and immunoblot detection indicates specificity of the antiserum generated, in an animal challenge model, it did not confer protection against leptospirosis.

Materials and Methods

In silico identification of surface proteins

Protein coding gene sequences were identified from the *L interrogans* sorovar Copenhageni genome by using GeneMark progam [18]. Six putative protein, presenting signal peptide according to Smart program [19], and/or predicted to be an outer membrane protein according to Psort program [20] were selected (LIC10359, LIC11211, LIC13435, LIC10291, LIC20087 and LIC10544). Blast analyses [21] were performed to identify orthologous genes in pathogenic *Leptospira* sequenced genomes.

Bacterial strains and culture conditions

Leptospiral strains (*L. interrogans* serovars Canicola, Pyrogenes, Pomona, Autumnalis, Copenhageni, Hardjo, Bratislava, and Icterohaemorrhagiae; *L. kirchneri* serovar Grippotyphosa and *L. biflexa* serovar Patoc) were provided by the Laboratório de Zoonoses

***Corresponding author:** Paulo L. Ho, Centro de Biotecnologia, Instituto Butantan, 05503-900, São Paulo, SP, Brazil, Tel: (+ 55 11) 3726.7222; E-mail: hoplee@butantan.gov.br

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Bacterianas, FMVZ, Universidade de São Paulo. They were cultured as described [13]. *Escherichia coli* DH5 α was used as the cloning host strain, *E. coli* (DE3) Star pLysS (Novagen, Gibbstown, NJ, United States) and *E. coli* BL21 SI [22] was tested for expression strains of the recombinant proteins. *E. coli* cells were grown in 2YT or 2YT ON (2YT whithout NaCl) medium supplemented with ampicillin and/or chloramphenicol.

DNA isolation for PCR analysis

Leptospira cultures were harvested by centrifugation at 5000g for 30 min and gently washed twice in sterile phosphate-buffered saline (PBS). Genomic DNA was isolated from the pellets with a guanidine-detergent lysing solution (DNAzol Reagent, Invitrogen, Carlsbad, CA, United States), according to the manufacturer's recommendation.

RNA extraction and reverse transcriptase-PCR (RT-PCR) analysis

The total RNA extraction was done by the acid guanidinium thiocyanate phenol-chloroform method using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) was used for cDNA synthesis. The amplification was performed with the same oligonucleotides as in cloning. The amplified products were loaded onto 1.0% agarose gels for electrophoresis and visualized by ethidium bromide staining. *E. coli* cells were grown in 2YT or 2YT ON (2YT whithout NaCl) medium supplemented with ampicillin and/or chloramphenicol.

Cloning, expression and purification of recombinant proteins

The nucleotide sequence encoding the six selected genes was amplified by PCR using genomic DNA purified from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 isolated from a patient in Salvador, Brazil, as template [23]. The primer sequences with the respective restriction sites are shown in the Table 1. The coding DNA fragments exclude the sequence predicted to be the signal peptide. The amplification product was cloned in Plasmid pGEM-T Easy (Promega, Madison, WI, United States) and then subcloned in pAE expression plasmid, a vector with a promoter derived from T7 phage that adds an N-terminal hexa-histidine tag to the recombinant protein [24]. Correct insertions were confirmed by sequencing and the construct was transformed into *E. coli* BL21 SI or *E. coli* (DE3) Star pLysS. Bacteria were cultured in 1 liter of 2YT or 2YT ON medium containing the respective antibiotic, until the optical density reached 0.6 at 600 nm. The culture was then induced for 3 h at 30°C with the addition of 0.3 mM of NaCl in case of *E. coli* BL21 SI, or 10mM IPTG (isopropyl-beta-D-thiogalactopyranoside) in the case of *E. coli* (DE3) Star pLysS. The cells were collected by centrifugation and resuspended in 100 ml of PBS, pH 7.4, and lysed in a French Press Disrupter (Thermo Spectronic, Waltham, MA, United States). The soluble and insoluble fractions were isolated by centrifugation at 10,000 ×g, at 4°C for 20 min. The supernatant was loaded onto a 5ml of Ni^{2+} charged chelating Sepharose (GE Healthcare, Buckinghamshire, United Kingdon) column (1 cm diameter) equilibrated with PBS and the purification was performed according to described protocols [14]. After purification, the proteins were extensively dialyzed against PBS for imidazol removal before protein assays.

Protein sequencing

The N-terminal portion of the purified protein was sequenced by Edman degradation [25] using a Shimadzu PPSQ-21 (Kyoto, Japan) automated protein sequencer, following the manufacturer's standard instructions. The sequencing was performed by Laboratório de Bioquímica e Biofisica, Instituto Butantan, Brazil.

Circular dichroism spectroscopic studies

Secondary structure of the purified protein was assessed by circular dichroism (CD) spectroscopy as described [26]. Briefly, CD measurements were performed using a Jasco J-810 Spectropolarimeter at 20°C (Japan Spectroscopic, Tokyo, Japan) equipped with a Peltier unit for temperature control. Purified recombinant protein was diluted at a concentration of 20 mM and dialyzed into 10 mM sodium phosphate buffer before analysis. Far-UV CD spectra were measured using a 1 mm path length cell at 0.5 nm intervals over the wavelength range from 190 to 260 nm. Five scans were averaged for each sample from which an averaged blank spectrum was subtracted.

Enzyme-Linked Immunosorbent Assay (ELISA)

A Microtiter plate (Maxisorp-NUNC, Kamstrup, Denmark)

Gene ID	Primer	Molecular mass (KDa)
LIC10359	Forward: GGATCCGATCCTGGAAGAGATAGACTAG	20,219
	Reverse: AAGCTTTTATTCCTTTTCGTTCAGAG	
LIC10544	Forward: GGATCCGATTCTCAAAAATCAGATTC	51,34
	Reverse: CAGCTGTTATATTTTAACCGAAATCG	
LIC11211	Forward: GGATCCCAAGAACAGAGAAAATCTACG	38,846
	Reverse: AAGCTTCTAATTCGTTTTAGAATCCTG	
LIC10291	Forward: GGATCCGATGAGTCCGAGAAAAAAACTTATTC	17,3
	Reverse: AAGCTTTTACTCTACGGTTGTAATTTC	
LIC10881	Forward: CTCGAGGGCACCTTATGGGCAAAGGCTTCTC	60.574
	Reverse: AAGCTTTCATTCTCTTGATTTATCGTAG	
LIC13435	Forward: GGATCCCAAACTTTGGATCAGAAC	20,189
	Reverse: AAGCTTTTAATTAGATGGATCTGTTTT	

Table 1: Sequence of primers employed for DNA amplification and the expected molecular mass of the recombinant protein. The restriction site (HindIII, BamHI and XhoI) of each sequence is underlined.

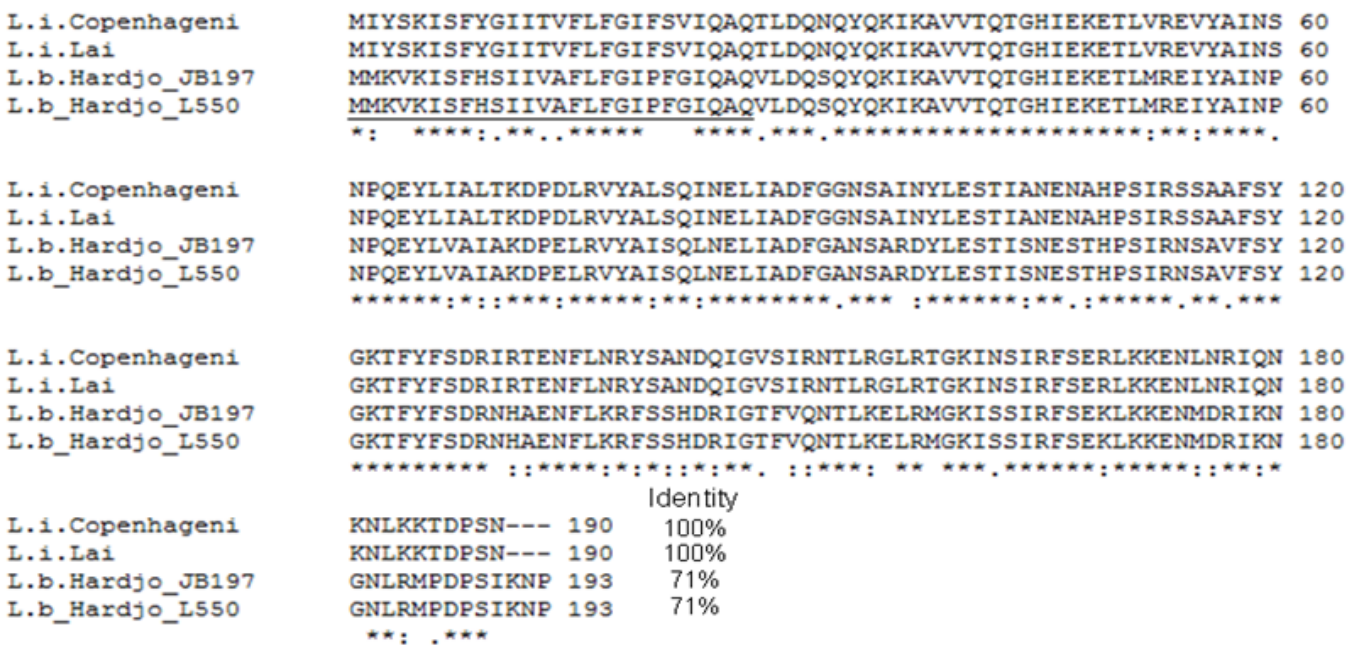

Figure 1: Alignment of LIC13435 related sequences. Sequences from *Leptospira interrogans* serovar Copenhageni (GenBank Accession AE016823.1), *L. interrogans* serovar Lai 56601 (GenBank Accession AE010300.2), *L. borgpetersenii* serovar hardjo-bovis JB197 (GenBank Accession CP000350.1) and *L. borgpetersenii* serovar hardjo-bovis L550 (GenBank Accession CP000348.1). Absolutely conserved (*), highly similar (:), and similar (.) residues are indicated below the aligment. The deduced signal peptide is underlined, according to SMART (http://smart.embl-heidelberg.de/). Alignments were performed using the ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Percentage of amino acid identity is related to LIC13435 from *L. interrogans* serovar Copenhageni

was incubated at 4°C with 10 mg/mL of purified protein in 0.05 M carbonate-bicarbonate buffer, pH 9.6. The plate was washed three times with 0.05% Tween 20/phosphate-buffered saline, pH 7.4 (PBS-T). One hundred microliter blocking buffer (10% non-fat dried milk in PBS-T) was added and the plate was incubated at 37°C for 1 h. After removal of blocking buffer with three washes of PBS-T, dilutions of respective serum were added to the plate in 1% bovine serum albumin (BSA)–PBS-T and incubated at 37°C for 1 h. After washing, proper dilutions of a peroxidase-conjugated goat anti-hamster IgG (Sigma, St. Louis, MO, United States) were added to the plates and incubated for an additional hour at 37°C. The plates were developed by the addition of 100 µL of a solution containing 8 mg o-phenylenediamine (OPD) in 20 ml of a 0.2 M citrate-phosphate buffer, pH 5.0, in the presence of 10 µl H_2O_2. The reaction was stopped by adding 50 µL of 4M H_2SO_4, and the absorbance was measured at 492 nm.

Immunoblot analysis

Samples were fractionated on a 15% SDS–PAGE and electro-blotted onto a nitrocellulose membrane. The membrane was incubated with 10% non-fat dried milk in PBS-T overnight at 4°C. After the incubation, the membrane was washed three times for 10 min with PBS-T, and then incubated with the serum of interest in 10% non-fat dried milk-PBS-T for 2 h. Following a repeat of the PBS-T wash as described above, the membrane was incubated with an appropriate antibody-peroxidase conjugate in 5% non-fat dried milk-PBS-T for 1h. After this, the membrane was washed again with PBS-T, and detected with ECL reagent (GE Healthcare). The recombinant proteins LipL32 [13,27] and TlyC [28], a leptospiral extra-cellular matrix (ECM) binding proteins, were used as control.

Hamster challenge assays

Groups of 10 hamsters, 4-6 weeks old male Golden Syrian, were intradermally immunized with 50 µg of purified recombinant protein, in addition to 500 µg of aluminum ion (added in the form of $Al(OH)_3$), twice at 2 weeks intervals. Two weeks after the second immunization, the hamsters were challenged with approximately 1 LD_{50} ($6x10^3$) of *L. interrogans* serovar Copenhageni str. Fiocruz L1-130 (ATCC BAA-1198) injected intraperitoneally. The animals were monitored daily for 21 days after challenge, the surviving hamsters were sacrificed and their kidneys were removed and tested for the presence of *Leptospira* by culture in EMJH medium. The positive control group was immunized with the commercial vaccine (FarrowSure B, Pfizer, New York, United States) and the negative control group was immunized with saline (PBS). The experiment was three times reproduced.

All animal experiments were approved by Ethics Committee of Universidade de São Paulo and of Instituto Butantan, São Paulo, Brazil.

Results

LIC13435 is present in pathogenic Leptospira genome

Though we selected six predicted sequences coding for putative outer membrane proteins with unknown function, from *Leptospira interrogans* serovar Copenhageni genome, only the gene of LIC13435 could be expressed and purified. Orthologous genes of *Leptospira interrogans* serovar Copenhageni *lic13435* sequence (GenBank Accession AE016823.1) can be observed in sequenced genomes of pathogenic *L. interrogans* serovar Lai 56601 (GenBank Accession AE010300.2), *L. borgpetersenii* serovar hardjo-bovis JB197 (GenBank Accession CP000350.1) and *L. borgpetersenii* serovar hardjo-bovis L550 (GenBank Accession CP000348.1), but not in saprofitic *L. biflexa* serovar Patoc. Therefore, this protein seems to be specific of pathogenic *Leptospira* species. The amino acid sequence alignment (Figure 1) shows that the four putative *Leptospira* proteins share 71% - 100% of amino acid identity. It was clear using bioinformatics tools, the presence of a

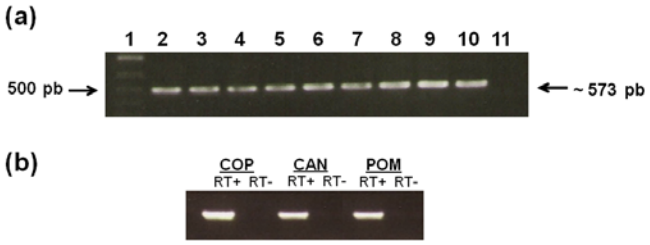

Figure 2: a Orthologous LIC13435 gene is detected in pathogenic leptospiras but not in saprophytic *L.biflexa*. Genomic DNAs of several strains of leptospires were subjected to PCR analysis with specific primers designed according to LIC13435 sequence from *L. interrogans* serovar Copenhageni genome. Lanes (serovar and strain are, respectively, shown after leptospire species) 1 – *L. interrogans* Copenhageni; 2 - *L. interrogans* Icterohaemorrhagie; 3 - *L. interrogans* Pomona; 4 - *L. kirschneri* Grippo-typhosa; 5 - *L. interrogans* Hardjo; 6 - *L. interrogans* Canicola; 7 – *L. interrogans* Bratislava; 8 - *L. interrogans* Autumnalis; 9 - *L. interrogans* Pyrogenes; 10 - *L. biflexa* Patoc; The size of the amplified DNAs was approximately 573pb. **b** Detection of LIC13435 transcripts by RT-PCR. The transcripts of LIC13435 from *L. interrogans* serovar Copenhageni (COP), *L. interrogans* serovar Canicola (CAN) and *L. interrogans* serovar Pomona (POM) are shown (RT+). A negative control (RT-) for samples not treated with reverse transcriptase, was used in each reaction. Reactions were performed with the same primer pairs mentioned above. RT+: reverse transcriptase present; RT -: reverse transcriptase omitted.

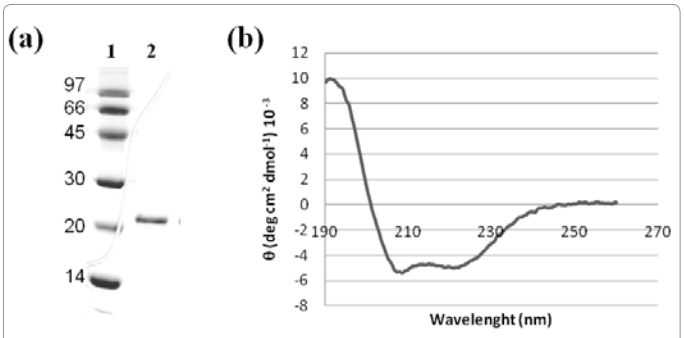

Figure 3: a Purification of rLIC13435$_{25\text{-}190}$ by Ni^{2+} affinity chromatography. SDS-PAGE (15%) showing lane 1- Molecular mass marker (KDa), lane 2 – Purified rLIC13435$_{25\text{-}190}$ with approximately 20.2 KDa. **b** Circular Dichroism spectrum of rLIC13435$_{25\text{-}190}$. Far UV spectra indicates that rLIC13435$_{25\text{-}190}$ has regular secondary sctructure, composed mainly of α-helix.

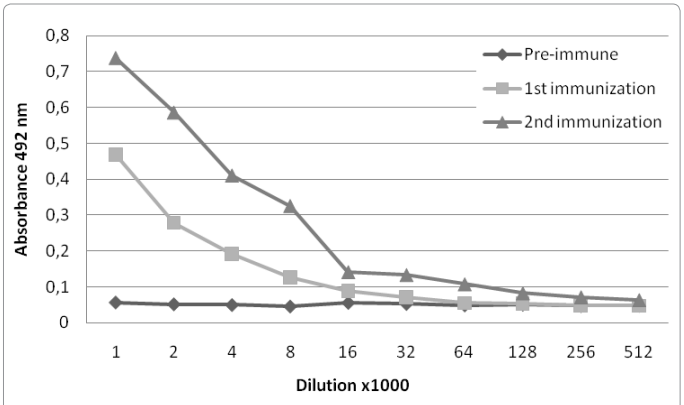

Figure 4: Antibody titers after each immunization were determined by Enzyme-linked immunosorbent assay. The titer after second immunization was approximately 64.000. The antisera were generated in hamsters.

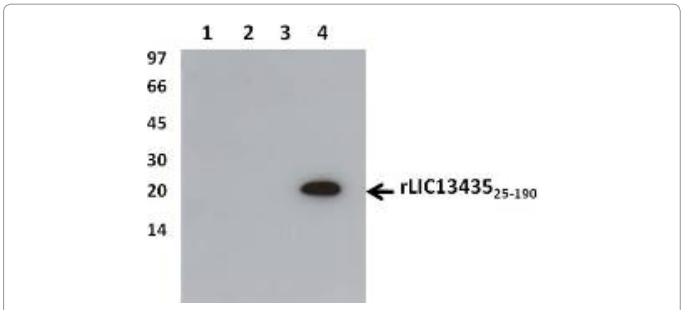

Figure 5: Anti-rLIC13435$_{25\text{-}190}$ recognizes rLIC13435$_{25\text{-}190}$ antigen in western blot. 1- Molecular Weight Marker; 2 – LipL32; 3 – TlyC; 4 - rLIC13435$_{25\text{-}190}$.

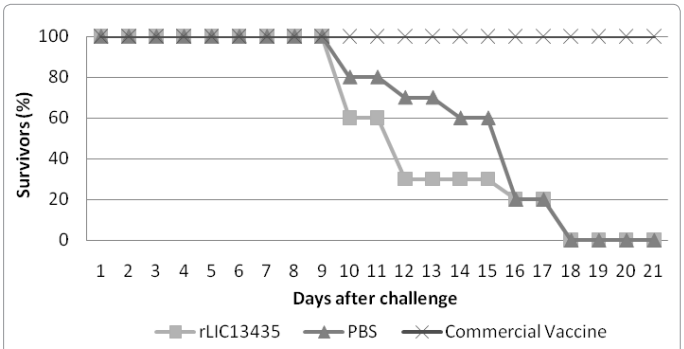

Figure 6: Survival of rLIC13435$_{25\text{-}190}$ immunized hamsters after challenge with virulent leptospires. Ten hamsters were immunized with rLIC13435$_{25\text{-}19}$, saline (PBS), or commercial vaccine and then challenged with *L. interrogans* serovar Copenhageni str. Fiocruz L1-130. None of the hamsters immunized with rLIC13435$_{25\text{-}190}$ or saline survived while commercial vaccine had effective protection.

hydrophobic core within a typical signal peptide as indicated in Figure 1. There are no other similar proteins in the databank.

Indeed, using the primer pair specific for *lic13435* gene, it was possible to detect orthologous genes by PCR among pathogenic leptospiral strains tested (*L. interrogans* serovars Canicola, Pyrogenes, Pomona, Autumnalis, Hardjo, Bratislava, Copenhageni, Icterohaemorrhagiae; and *L. kirchneri* serovar Grippotyphosa). This gene was not detected in saprofitic *L. biflexa* serovar Patoc (Figure 2a).

Moreover, the PCR performed on four reverse transcribed RNA detected transcripts only in pathogenic strains (*L. interrogans* serovars Copenhageni, Canicola and Pomona) (Figure 2b) but not in saprofitic *biflexa* serovar Patoc (data not shown).

Expression, purification and secondary structure of rLIC13435$_{25\text{-}190}$

The *lic13435* gene was amplified and the 513bp DNA insert cloned and expressed in *E. coli* BL21 (SI) in its soluble fraction. The purification was performed by metal chelating chromatography and the final yield was approximately 15 - 20 mg of purified protein per liter of induced culture (Figure 3a). Protein N–terminal characterization by Edman sequencing confirmed the correct purified protein. The circular dichroism spectra indicated a predominant α-helix secondary structure, consistent with the computational analysis prediction (NetSurf, http://www.cbs.dtu.dk/services/NetSurfP/) [29] (Figure 3b).

rLIC13435$_{25\text{-}190}$ generates high titers and specific antisera

The anti-rLIC13435$_{25\text{-}190}$ serum was produced by intraperitoneal

immunization of hamster. ELISA test indicates that the recombinant protein is immunogenic. The titer after second immunization was approximately 64.000 (Figure 4). The anti-rLIC13435$_{25\text{-}190}$ serum seems to be specific, it does not recognize recombinant LipL32 and TlyC, two well characterized leptospiral surface proteins but reacts strongly with rLIC13435$_{25\text{-}190}$ (Figure 5).

rLIC13435 is not protective against leptospirosis

Taking advantage of the titers presented by immunized hamsters (see above), they were challenged intraperitoneally with 6x10^3 virulent *L. interrogans* serovar Copenhageni. Although the serum titre after second immunization was approximately 64.000 (Figure 5), none of the hamsters immunized with rLIC13435$_{25\text{-}190}$ or with saline survived, while a commercial veterinary vaccine had effective protection as expected (Figure 6).

Discussion

Identification of genes that are regulated during interaction with host tissues and the mechanisms that regulate these infection-associated genes represent an important step toward the understanding of leptospiral pathogenesis. Efforts have been focused on discovering cross-species-conserved or cross-serovar-conserved protective antigens that may elicit longer-term protection against a broad range of *Leptospira.* Currently avaiable leptospiral vaccines have low efficacy, are serovar specific, and generally produce only short-term immunity in domestic livestock [17]. Surface associated molecules are potential targets for inducing protective immune responses in the host. Reverse vaccinomology approach was first described for *N. meningitides*[30]. This pioneering work was designed for high throughput cloning and expression of putative cell exposed antigens selelcted from *in silico* analysis. This kind of project needs a whole human and physical support not found in most of the laboratories aiming to use similar approaches. Small scale assaying of *in silico* selected antigens is a feasible alternative to some groups, like ours. Therefore, we have identified six potential targets by *in silico* analysis of the genome sequence of *L. interrogans* serovar Copenhageni. However, we were only successful to produce the recombinant protein of LIC13435 (rLIC13435$_{25\text{-}190}$). Sequence analysis of the deduced protein revealed the presence of a typical signal peptide with a hydrophobic core (Figure 1). Orthologous of this gene are present in sequenced pathogenic genomes of *Leptospira*, which prompted us to examine the gene/protein conservation among other important *Leptospira* strains. Genomic and retro-transcribed PCR indicate that this gene is conserved and transcribed among pathogenic *Leptospira* tested. The recombinant protein was expressed and purified from soluble fraction by Ni^{2+} affinity chromatography. Peptide sequencing confirmed the correct purified protein. Circular dichroism characterized the secondary structure as being mainly composed of α-helix.

The immunization of hamsters generated specific antisera (Figure 4 and Figure 5) against rLIC13435$_{25\text{-}190}$. Instead of using this serum to characterize the antigen localization, we took advantage of the presence of immunized animals to directly challenge them with pathogenic *Leptospira* in a more straightforward approach. Unfortunately the recombinant protein was not protective in the challenge assays. In conclusion, our findings suggest that, although the presence of the *lic13435 gene* and its transcripts in pathogenic *Leptospira*, the immunogenicity of the recombinant protein, LIC13435 does not present potential as vaccine antigen against leptospirosis.

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References

1. Levett PN (2007) Sequence-based typing of leptospira: epidemiology in the genomic era. PLoS Negl Trop Dis 1: e120.
2. Levett PN, Edwards CN (2009) Leptospirosis. In: Brachman PS, Abrutyn E, editors. Bacterial Infections of Humans. Springer 439-460.
3. Bharti AR, Nally JE, Ricaldi JN, Matthias MA, Diaz MM, et al. (2003) Leptospirosis: a zoonotic disease of global importance. Lancet Infect Dis 3: 757-771.
4. McBride AJ, Athanazio DA, Reis MG, Ko AI (2005) Leptospirosis. Curr Opin Infect Dis 18: 376-386.
5. Nally JE, Artiushin S, Timoney JF (2001) Molecular characterization of thermoinduced immunogenic proteins Q1p42 and Hsp15 of Leptospira interrogans. Infect Immun 69: 7616-7624.
6. Haake DA, Walker EM, Blanco DR, Bolin CA, Miller MN (1991) Changes in the surface of Leptospira interrogans serovar grippotyphosa during in vitro cultivation. Infect Immun 59: 1131-1140.
7. Haake DA, Champion CI, Martinich C, Shang ES, Blanco DR, et al. (1993) Molecular cloning and sequence analysis of the gene encoding OmpL1, a transmembrane outer membrane protein of pathogenic Leptospira spp. J Bacteriol 175: 4225-4234.
8. Shang ES, Summers TA, Haake DA (1996) Molecular cloning and sequence analysis of the gene encoding LipL41, a surface-exposed lipoprotein of pathogenic Leptospira species. Infect Immun 64: 2322-2330.
9. Haake DA, Martinich C, Summers TA, Shang ES, Pruetz JD, et al. (1998) Characterization of leptospiral outer membrane lipoprotein LipL36: downregulation associated with late-log-phase growth and mammalian infection. Infect Immun 66: 1579-1587.
10. Park SH, Ahn BY, Kim MJ (1999) Expression and immunologic characterization of recombinant heat shock protein 58 of Leptospira species: a major target antigen of the humoral immune response. DNA Cell Biol 18: 903-910.
11. Haake DA, Mazel MK, McCoy AM, Milward F, Chao G, et al. (1999) Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infect Immun 67: 6572-6582.
12. Haake DA, Chao G, Zuerner RL, Barnett JK, Barnett D, et al. (2000) The leptospiral major outer membrane protein LipL32 is a lipoprotein expressed during mammalian infection. Infect Immun 68: 2276-2285.
13. Hauk P, Macedo F, Romero EC, Vasconcellos SA, de Morais ZM, et al. (2008) In LipL32, the major leptospiral lipoprotein, the C terminus is the primary immunogenic domain and mediates interaction with collagen IV and plasma fibronectin. Infect Immun 76: 2642-2650.
14. Hauk P, Negrotto S, Romero EC, Vasconcellos SA, Genovez ME, et al. (2005) Expression and characterization of HlyX hemolysin from Leptospira interrogans serovar Copenhageni: potentiation of hemolytic activity by LipL32. Biochem Biophys Res Commun 333: 1341-1347.
15. Guerreiro H, Croda J, Flannery B, Mazel M, Matsunaga J, et al. (2001) Leptospiral proteins recognized during the humoral immune response to leptospirosis in humans. Infect Immun 69: 4958-4968.
16. Branger C, Sonrier C, Chatrenet B, Klonjkowski B, Ruvoen-Clouet N, et al. (2001) Identification of the hemolysis-associated protein 1 as a cross-protective immunogen of Leptospira interrogans by adenovirus-mediated vaccination. Infect Immun 69: 6831-6838.
17. Palaniappan RU, Chang YF, Jusuf SS, Artiushin S, Timoney JF, et al. (2002) Cloning and molecular characterization of an immunogenic LigA protein of Leptospira interrogans. Infect Immun 70: 5924-5930.
18. Delcher AL, Harmon D, Kasif S, White O, Salzberg SL (1999) Improved microbial gene identification with GLIMMER. Nucleic Acids Res 27: 4636-4641.
19. Schultz J, Milpetz F, Bork P, Ponting CP (1998) SMART, a simple modular architecture research tool: identification of signaling domains. Proc Natl Acad Sci U S A 95: 5857-5864.
20. Nakai K, Kanehisa M (1991) Expert system for predicting protein localization sites in gram-negative bacteria. Proteins 11: 95-110.

21. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215: 403-410.

22. Bhandari P, Gowrishankar J (1997) An Escherichia coli host strain useful for efficient overproduction of cloned gene products with NaCl as the inducer. J Bacteriol 179: 4403-4406.

23. Ko AI, Galvao Reis M, Ribeiro Dourado CM, Johnson WD Jr, Riley LW (1999) Urban epidemic of severe leptospirosis in Brazil. Salvador Leptospirosis Study Group. Lancet 354: 820-825.

24. Ramos CR, Abreu PA, Nascimento AL, Ho PL (2004) A high-copy T7 Escherichia coli expression vector for the production of recombinant proteins with a minimal N-terminal His-tagged fusion peptide. Braz J Med Biol Res 37: 1103-1109.

25. Edman P, Begg G (1967) A protein sequenator. Eur J Biochem 1: 80-91.

26. Carvalho E, Barbosa AS, Gomez RM, Oliveira ML, Romero EC, et al. (2010) Evaluation of the expression and protective potential of Leptospiral sphingomyelinases. Curr Microbiol 60: 134-142.

27. Hauk P, Guzzo CR, Roman Ramos H, Ho PL, Farah CS (2009) Structure and calcium-binding activity of LipL32, the major surface antigen of pathogenic Leptospira sp. J Mol Biol 390: 722-736.

28. Carvalho E, Barbosa AS, Gomez RM, Cianciarullo AM, Hauk P, et al. (2009) Leptospiral TlyC is an extracellular matrix-binding protein and does not present hemolysin activity. FEBS Lett 583:1381-1385.

29. Petersen B, Petersen TN, Andersen P, Nielsen P, Lundegaard C (2009) A generic method for assignment of reliability scores applied to solvent accessibility predictions. BMC Struct Biol 9: 51.

30. Rappuoli R (2001) Reverse vaccinology, a genome-based approach to vaccine development. Vaccine 19: 2688-2691.