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 *Leptospira*. 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 vaccine 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 inadequate 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 measures 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) 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 leptospirosis 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* serovar Copenhageni genome by using GeneMark program [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.
Bacterianas, FMVZ, Universidade de São Paulo. They were cultured as described [13]. *Escherichia coli* DH5α 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 without NaCl) medium supplemented with ampicillin and/or chloramphenicol.

DNA isolation for PCR analysis

*Leptospira* cultures were harvested by centrifugation at 5000 g for 30 min and gently washed twice in sterile phosphate-buffered saline (PBS). Genomic DNA was DNA 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 without 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 lyzed in a French Press Disrupter (Thermo Spectronic, Waltham, MA, United States). The soluble and insoluble fractions were isolated by centrifugation at 10,000 x g, at 4°C for 20 min. The supernatant was loaded onto a 5ml of Ni2+ charged chelating Sepharose (GE Healthcare, Buckinghamshire, United Kingdom) 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-terminus 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)
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$_2$O$_2$. The reaction was stopped by adding 50 µL of 4M H$_2$SO$_4$, and the absorbance was measured at 492 nm.

Immunoblot analysis

Samples were fractionated on a 15% SDS–PAGE and electroblotted 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 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$_2$O$_2$. The reaction was stopped by adding 50 µL of 4M H$_2$SO$_4$, and the absorbance was measured at 492 nm.

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) could 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 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 alignment. 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.
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. kirschneri serovar Grippotyphosa). This gene was not detected in saprophytic 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 saprophytic biflexa serovar Patoc (data not shown).

The anti-rLIC13435 25-190 serum was produced by intraperitoneal injection of immunized hamsters with recombinant protein. The 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.

The rLIC13435 25-190 generates high titers and specific antisera. The anti-rLIC13435 25-190 serum was produced by intraperitoneal injection of immunized hamsters with recombinant protein. The 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.
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\textsubscript{25-190} serum seems to be specific, it does not recognize recombinant LipL32 and ThyC, two well characterized leptospiral surface proteins but reacts strongly with rLIC13435\textsubscript{25-190} (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\textsuperscript{7} virulent \textit{L. interrogans} serovar Copenhageni. Although the serum titre after second immunization was approximately 64,000 (Figure 5), none of the hamsters immunized with rLIC13435\textsubscript{25-190} 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 leptospirosis 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 \textit{Leptospira}. Currently available 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 vaccinology approach was first described for \textit{N. meningitidis}[30]. This pioneering work was designed for high throughput cloning and expression of putative cell exposed antigens selected from \textit{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 assay of \textit{in silico} selected antigens is a feasible alternative to some groups, like ours. Therefore, we have identified six potential targets by \textit{in silico} analysis of the genome sequence of \textit{L. interrogans} serovar Copenhageni. However, we were only successful to produce the recombinant protein of LIC13435 (rLIC13435\textsubscript{25-190}). 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 \textit{Leptospira}, which prompted us to examine the gene/protein conservation among other important \textit{Leptospira} strains. Genomic and retro-transcribed PCR indicate that this gene is conserved and transcribed among pathogenic \textit{Leptospira} tested. The recombinant protein was expressed and purified from soluble fraction by Ni\textsuperscript{2+} affinity chromatography. Peptide sequencing confirmed the correct purified protein. Circular dichroism characterized the secondary structure as being mainly composed of \alpha-helix.

The immunization of hamsters generated specific antisera (Figure 4 and Figure 5) against rLIC13435\textsubscript{25-190}. 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 \textit{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 \textit{Leptospira}, the immunogenicity of the recombinant protein, LIC13435 does not present potential as vaccine antigen against leptospirosis.

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