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Expression and characterization of HlyX hemolysin from *Leptospira interrogans* serovar Copenhageni: Potentiation of hemolytic activity by LipL32

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Abstract

The HlyX, a putative hemolysin identified from the *Leptospira* genomes, was cloned, expressed in *Escherichia coli*, purified, and its hemolytic activity was confirmed. Mouse polyclonal antiserum against the recombinant HlyX recognized HlyX-related antigens in a panel of *Leptospira* species extracts and it was also able to abolish the hemolytic activity of HlyX. A mixture of HlyX and LipL32, a known hemolysin from *Leptospira*, induced hemolysis in a synergistic way that was fully inhibited by antiserum against either protein. Moreover, sera from patients with leptospirosis also recognized the recombinant HlyX, showing that it is presented to the host immune system during *Leptospira* infection.

Keywords: HlyX; Hemolysin; LipL32; *Leptospira interrogans* serovar Copenhageni; Leptospirosis

Leptospirosis is a widespread antropozoonosis caused by pathogenic spirochetes belonging to the genus *Leptospira* which in recent years has emerged as an important global and veterinary health problem. The genus *Leptospira* presents more than 200 pathogenic serovars, which are classified by their agglutination after cross-adsorption with homologous antigen, related to the variations in the carbohydrate side chains of the lipopolysaccharide (LPS) [1,2]. The diseases caused by pathogenic *Leptospira* include subclinical infection, self-limited anicteric febrile illness (both with and without meningitis), and the severe and potentially lethal Weil's syndrome that is characterized by hemorrhage, renal failure, and jaundice. The primary lesions to small

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endothelial blood vessels caused by *Leptospira* lead to hemorrhage and localized ischemia in multiple organs [1,2]. The *Leptospira interrogans* serovar Lai and serovar Copenhageni genomes have recently been sequenced and compared [3,4]. Both *Leptospira* genomes consist of two circular chromosomes comprised of around 4.3 Mbp and 350 kbp for Chromosome I (CI) and Chromosome II (CII), respectively. Both the serovar Lai and the serovar Copenhageni genomes include orthologues of the hlyX hemolysin gene from *Leptospira borgpetersenii* serovar hardjo type hardjobovis (GenBank Accession No. AAF09252; unpublished results), which contains five tetratricopeptide repeats (TPR). LIC10325 from serovar Copenhageni was annotated as a hemolysin containing TPR domains related to *Leptospira borgpetersenii* HlyX whereas serovar Lai orthologous gene (LA0378) was annotated as a TPR-repeat-containing protein [3,4]. Only recently, it was reported that the lysate of *Escherichia coli* cells expressing HlyX (LA0378 from serovar Lai) displayed a detectable hemolytic activity on sheep blood agar plates [5]. Nevertheless, this result has to be confirmed and extended in order to a better understanding of what role HlyX plays in the host infection. Here, we present data showing that purified HlyX (LIC10325) is indeed a hemolysin, and provide experimental evidence showing that its hemolytic activity is potentiated by LipL32, the major lipoprotein presented by *Leptospira* in the outer membrane that also presents hemolytic activity. HlyX is widely distributed among pathogenic *Leptospira* serovars and is probably presented to the host immune system during the infection.

Materials and methods

Bacterial strains, plasmids, and culture conditions. Several leptospiral strains and sera from patients infected with leptospirosis were obtained from the Instituto Adolfo Lutz collection or from the Faculdade de Medicina Veterinária da Universidade de São Paulo, Brazil. *E. coli* DH5α was used as the cloning host strain and *E. coli* BL21(DE3) Star [pLysS] (Novagen) was used as the host strain for the expression of the recombinant HlyX using the T7 promoter based expression plasmid pAE [6]. *E. coli* cells were grown in 2YT medium supplemented with specific antibiotics (ampicillin and/or chloranphenicol).

Cloning and expression of HlyX in E. coli. PCR was used to amplify LIC10325 (hlyX) using a genomic DNA purified from *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 isolated from a patient in Salvador, Brazil [4–7] as template, with the forward primer (5′-TACTCGAGGTGTATCAAACTACGATTCAAGAC-3′) containing a *Xho*I restriction endonuclease site (underlined) and the reverse primer (5′-AAGAATTCTCAATCCAATTTTTCGGTTTCC-3) containing an *Eco*RI (underlined) site. Plasmid pCR4 Blunt-TOPO (Invitrogen) was used to clone the amplified gene. The resulting 1.1 kb fragment was cloned into *Xho*I/*Eco*RI sites in the pAE expression plasmid [6]. This vector allows the expression of recombinant proteins with a minimal His_6 tag at the N-terminus. The final construct presents the translated N-terminal sequence MHHHHHHLE**VYQT** (where the start of predicted HlyX signal peptide is shown in bold). This construct was used to transform *E. coli* BL21(DE3) Star [pLysS] which was cultured in 1 liter of 2YT medium containing ampicillin–chloranphenicol and grown until the optical density reached 0.6 at 600 nm. The expression of the recombinant protein was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and cultivated for additional 3 h at 37 °C. The cells were collected by centrifugation and resuspended in 100 ml of phosphate-buffered saline, pH 7.4 (PBS), and lysed in a French press (Thermo Spectronic). The soluble and insoluble fractions were isolated by centrifugation at 5000 rpm for 10 min.

Purification of the recombinant HlyX protein. Briefly, 5 ml of Ni^{2+}-charged chelating Sepharose (GE Healthcare) column (1 cm diameter) was equilibrated with PBS. After adsorption of HlyX protein in the supernatant fraction, the resin was washed with 10 column volumes of PBS containing 5, 20, 40, and 60 mM imidazole, respectively. Proteins were eluted with 5 volumes of PBS containing 1 M imidazole and the eluted fractions were analyzed by 15% SDS–PAGE. Proteins were dialyzed in one step using 2 liters of a 10 mM Tris–HCl, pH 8.0, 20 mM NaCl, 0.1% (m/v) glycine solution. LPS was further removed from the final purified protein by Triton X-114 extractions [8].

Circular dichroism spectroscopic studies. Circular dichroism (CD) measurements were acquired using a Jasco J-810 Spectropolarimeter at 20 °C equipped with a Peltier unit for temperature control. 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. The protein concentration was 10 μM as estimated from absorbance at 280 nm, using an extinction molar coefficient of $\varepsilon_{280} = 46{,}510\ M^{-1}\,cm^{-1}$ based on the expected amino acid sequence of the recombinant protein. All measurements were made using 10 mM Na-phosphate buffer.

Production of HlyX antiserum. Five-to-eight-week-old female BALB/C mice were immunized intraperitoneally with 10 μg of purified HlyX in $Al(OH)_3$. The immunizations were performed over a period of 4 weeks, with booster doses at every week. Mice were bled by the retrorbital plexus and the blood was incubated for 30 min at 37 °C. After this, the clot was removed by centrifugation and the serum was collected from the supernatant.

Enzyme-linked immunosorbent assay (ELISA). A Microtiter plate (Maxisorp-NUNC) was incubated at 4 °C with 10 μg/ml of purified HlyX 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 anti-HlyX 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-mouse IgG (Sigma) 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 4 M H_2SO_4, and the absorbance was measured at 492 nm.

Immunoblot analysis. Leptospira extracts were fractionated on a 15% SDS–PAGE and electro-eluted to nitrocellulose membranes. The membranes were incubated with 10% (m/v) non-fat dried milk in PBS-T and, after washing three times for 10 min with PBS-T, they were further incubated with mouse anti-HlyX serum in 5% non-fat dried milk–PBS-T for 1 h. Following a repeat of the PBS-T wash as described above, the membranes were incubated with a proper dilution of goat anti-mouse IgG peroxidase conjugate (Sigma) in 5% non-fat dried milk–PBS-T, washed, and revealed with ECL reagent (GE Healthcare). Alternatively, the SDS–PAGE fractionated recombinant HlyX or LipL32 was fractionated on a SDS–PAGE, transferred to nitrocellulose membranes, and incubated with sera from patients diagnosed with leptospirosis. The blot was developed using goat anti-human IgG peroxidase conjugate (Sigma) as described above.

Hemolytic activity assay. Hemolytic activity was determined by spectrophotometry as described previously [9]. Briefly, a reaction mixture consisting of 0.1 ml of 10× PBS (pH 7.4), 0.80 ml of distilled water, and 0.1 ml of 10% washed human erythrocyte suspension was incubated with different concentrations of recombinant LPS free proteins at 37 °C for 5 min, followed by centrifugation (12,000 rpm, 0.5 min). LipL32- and HlyX-murine specific neutralizing antisera (SNA) (at titres of 1:64,000 and 1:512,000, respectively) were diluted 100-fold and preincubated for 10 min at 37 °C before addition of the erythrocytes. The hemolytic activity was estimated by measuring the absorbance of supernatant at 420 nm after a 10-fold dilution in 1× PBS (pH 7.4). Results were expressed as the percentage of hemolytic activity in relation to the 100% hemolysis, defined as complete lysis of the same concentration of erythrocytes in distilled water. Results were expressed as means ± standard error of mean (SEM) of data obtained from *N* separate experiments (see figure legends). Statistical significance was determined by two-tailed Student's paired *t* test. *P* values lower than 0.05 were considered statistically significant.

Results

LIC10325 is orthologous to HlyX

Sequencing of the *L. interrogans* serovar Copenhageni genome [4,7] revealed the presence of an ORF (LIC10325) related to the HlyX from *L. borgpetersenii* serovar hardjo type hardjobovis (GenBank Accession No. AAF09252). A gene encoding an orthologous protein was also observed in the genome of *L. interrogans* serovar Lai [3]. In this case, it was suggested that the N-terminus (MVEALSVSDLRRKS) does not start with the start codon ATG but with an alternative TTG start codon. However, we considered that it would be more plausible if the correct ORF would start with ATG start codon instead of the alternative TTG start codon (Fig. 1). The amino acid sequence alignment shows that the three putative *Leptospira* HlyX proteins share 93–99% of amino acid identity. Interestingly, there are no other similar proteins in the databank, indicating that these proteins are *Leptospira* specific. Although a signal peptide was observed, suggesting that these proteins are secreted, they also possess five tetratricopeptide repeats (TPR), which are involved in protein–protein interactions via a ''knobs and holes'' mechanism of proteins with an intracellular location [10].

Expression and purification of the recombinant LIC103225-HlyX related protein

Recombinant HlyX (42.3 kDa) was expressed in *E. coli* BL21(DE3) Star [pLysS], both in the soluble or in the insoluble fractions (data not shown). Although higher level of HlyX was found in the inclusion bodies of the insoluble fractions, we preferred to purify the HlyX protein from the soluble fraction, since this material is likely to be in the native and correct conformation. The soluble HlyX was purified by Ni^{2+}-charged chelating Sepharose in a single step chromatography (Fig. 2). The final purification yield using the soluble fraction was approximately 5 mg/L culture. As shown in Fig. 3, the minima at 209 and 222 nm, and the maximum at 196 nm in the circular dichroism spectra demonstrated the high α-helical secondary structure contend (approximately 81.5%) of the recombinant protein (Fig. 3), confirming the secondary structure contend predicted by computational analysis (JUFO, http://www.expasy.gov).

LIC10325-HlyX related protein presents hemolytic activity

To compare the hemolysis induced by LipL32 or HlyX, hemolytic activities were measured by spectrophotometric assay. Results showed that both LipL32 (15–20 μg/ml) and HlyX (25–30 μg/ml) exerted hemolytic activity on human erythrocytes (Fig. 4). LipL32 (also described as Hap-1, hemolysis-associated protein 1) was used as a positive control since it is known to possess hemolytic activity [13]. Moreover, this effect was completely abolished when LipL32- or HlyX-SNA was previously added to the reaction mixture. When subthreshold concentrations of proteins were added in combination, a synergistic hemolytic activity was observed that was also completely suppressed by SNAs (Table 1). These results suggest that both proteins alone trigger hemolysis and that their hemolytic activity is potentiated when combined.

Ubiquitous distribution of LIC10325-HlyX related protein in L. interrogans serovars

The anti-HlyX antiserum was used to screen a large panel of *L. interrogans* extracts as shown in Fig. 5. A band corresponding to the expected size of HlyX was observed in all the pathogenic *L. interrogans* serovar extracts (serovars Canicola, Grippotyphosa, Pyrogenes, Pomona, Autumnalis, Hardjo, Bratislava, Copenhageni, and Icterohaemorrhagiae) but not in the non-pathogenic saprophytic *L. biflexa* serovar Patoc. This result shows that HlyX related protein is ubiquitously distributed only in pathogenic *L. interrogans* serovars. Due to the hemolytic activity described above, it is expected that HlyX would play an important role in the virulence of these spirochetes during the infection in the host.

LIC10325-HlyX related protein is presented to the host immune system

In the immunoblot analysis, the hemolysin *HlyX* was only recognized by serum of patients in the second week of leptospirosis infection with titer agglutination of 25,600, 6400, and 3200, indicating that the protein is presented to the host immune system during infection. However, no immunoreaction was observed using sera

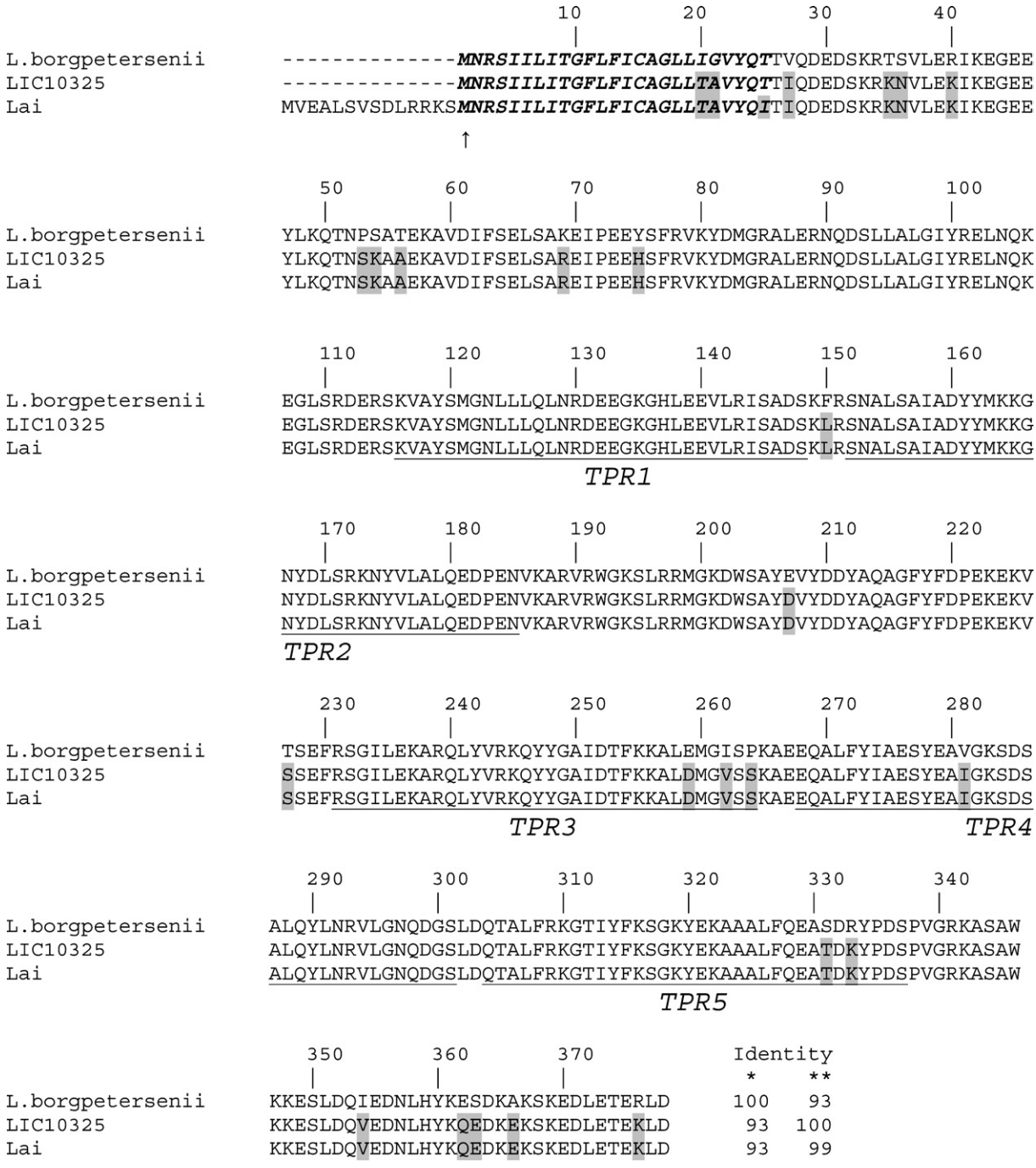

Fig. 1. Alignment of HlyX related sequences. Sequences from *L. borgpetersenii* serovar hardjo type hardjobovis (GenBank Accession No. AAF09252), *L. interrogans* serovar Copenhageni—LIC10325 (GenBank AAS68952) and *L. interrogans* serovar Lai (GenBank AAN47577). The alignment was performed using the Multalin program (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_multalin.html) [11]. The deduced signal peptide is in bold and italic. The five tetratricopeptide repeats are underlined, according to SMART (http://smart.embl-heidelberg.de/) [12]. (* and **) Percentage of amino acid identity related to HlyX from *L. borgpetersenii* or to LIC10325, respectively. (↑) The methionine coded by the probable ATG start codon in the HlyX orthologous gene from *L. interrogans* serovar Lai.

from patients with lower titers or from the first week of infection. In contrast, LipL32, the leptospiral major outer membrane lipoprotein expressed during mammalian infection [14], was recognized by acute-phase sera from leptospirosis patients both in the first and second weeks of infection (Fig. 6).

Discussion

Recently, the genome of two serovars (Lai and Copenhageni) of *L. interrogans* has been described [3,4,7]. This represents an important contribution to the field and also an opportunity for functional genomic

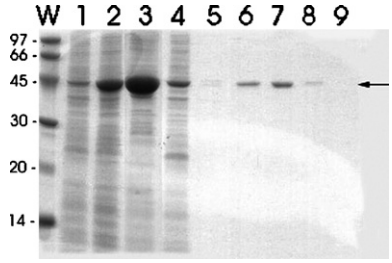

Fig. 2. Expression and purification of recombinant HlyX. *E. coli* BL21(DE3) Star [pLysS] transformed with the pAE-hlyX plasmid was grown in 2YT-ampicillin-chloranphenicol medium. IPTG (1 mM) was added when the OD_{600} reached 0.6. Lanes 1 and 2, cellular extracts before and after induction with IPTG, respectively; lane 3, the inclusion bodies fraction; lane 4, soluble cellular fraction. Lanes 6–8, purified protein. Lanes 5 and 9 are empty lines.

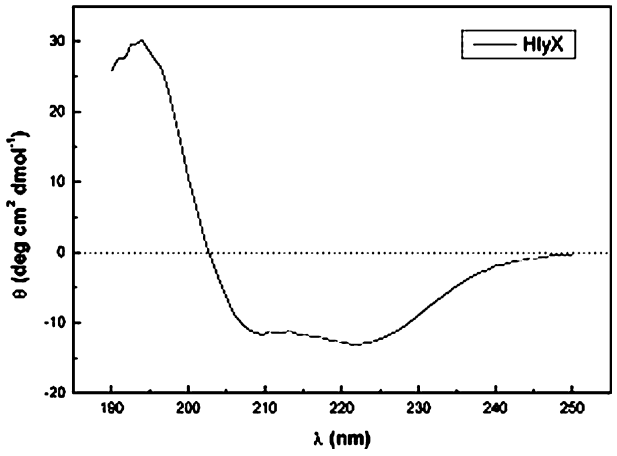

Fig. 3. Circular dichroism spectrum of the recombinant LIC10325. Far-UV CD spectrum of 10 μM of His_6-tagged recombinant LIC10325 in 10 mM Na-phosphate buffer performed at 20 °C.

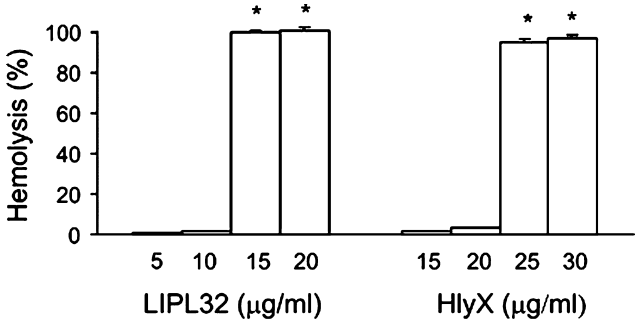

Fig. 4. Hemolytic activities of LipL32 and HlyX on human erythrocytes. Human erythrocytes were added to reaction mixture containing LipL32 or HlyX at the indicated concentrations and then incubated for 15 min. Results are expressed as the percentage of hemolytic activity in relation to the complete lysis of erythrocytes in distilled water. Control samples (PBS) failed to exert hemolytic activity (data not shown). Results are means ± SEM of five independent experiments. $*p < 0.05$ vs PBS.

studies. Reverse vaccinology [15] has been applied to the discovery of potential vaccine candidates using the genomic information of *Neisseria meningitides* [16] and

Table 1
Synergistic hemolytic activities of LipL32 and HlyX on human erythrocytes: inhibition by specific neutralizing antisera (SNA)

Treatments	Hemolysis (%)		
	15 min	30 min	90 min
PBS	0	0	0
LipL32 (20)	100	100	100
SNA + LipL32 (20)	0	0	1
HlyX (25)	100	100	99
SNA + HlyX (25)	1	5	4
LipL32 (10)	2	1	1
HlyX (20)	0	2	0
LipL32 (10) + HlyX (20)	52	75	100
SNAs + LipL32 (10) + HlyX (20)	4	4	4

Human erythrocytes were added to the reaction mixture containing LipL32 and/or HlyX at the concentrations indicated in parenthesis (μg/ml). SNA were preincubated with proteins for 10 min at 37 °C before erythrocyte addition. Results were expressed as the percentage of hemolytic activity relative to the complete lysis of erythrocytes in distilled water. Antiserum alone failed to exert hemolytic activity (data not shown). Results are means ± SEM of three independent experiments.

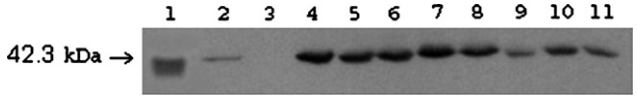

Fig. 5. Western blot of cellular extracts of different *L. interrogans* serovars probed with anti-HlyX antiserum. 1, recombinant HlyX; 2, Canicola; 3, *L. biflexa* serovar Patoc; 4, Grippotyphosa; 5, Pyrogenes; 6, Pomona; 7, Autumnalis; 8, Hardjo; 9, Bratislava; 10, Copenhageni; 11, Icterohaemorrhagiae.

Streptococcus pneumoniae [17]. This approach is also being explored in the case of *L. interrogans* [18]. Using genomic information, we have noticed that the annotated genome of the *L. interrogans* sorovar Lai also included a hlyX gene as a putative coding sequence for a hemolysin based on a description in the GenBank (AAF09252) of an orthologous gene from *L. borgpetersenii* serovar hardjo type hardjobovis. Recently, it was reported the hemolytic activity of HlyX from *L. interrogans* sorovar Lai, by the lysate of *E. coli* cells expressing the protein on sheep blood agar plates [5]. However, the activity of purified HlyX and its synergism with another hemolysin, LipL32, has not been reported. The characterization of the HlyX presented here represents the first characterization of a purified protein of this family of hemolysins that seems to be specific for pathogenic *Leptospira* (Fig. 5). Our results suggest an important role of HlyX in the pathogenesis of *Leptospira* which can be attributed to the experimentally confirmed hemolytic activity against erythrocytes in vitro (Fig. 4 and Table 1). During infection by pathogenic *Leptospira*, HlyX is presented to the host immune system (Fig. 6) and the hemolytic activity observed during infection would be an expected consequence of several hemolytic factors acting together synergistically, as

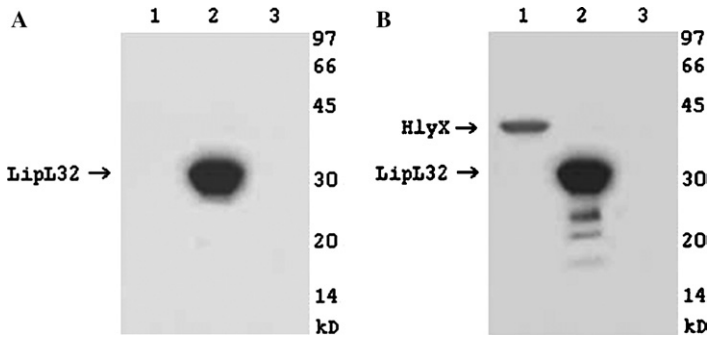

Fig. 6. Immunoblot panel of proteins probed with patient sera. 1, HlyX; 2, recombinant LipL32 (used as positive control); 3, Sm14 (a recombinant fatty-acid binding protein from *Schistosoma mansoni* used as negative control) using leptospirosis patient sera from the first (A) and second (B) week of infection. In (A) is shown the recognition of LipL32 by the first week serum, in (B) the immunoreaction of the second week serum with LipL32 and HlyX. Microagglutination titer of the patient serum was not detectable in the first week and for the second week, the titer was 25,600.

observed for HlyX and LipL32. Indeed, antibodies raised against these proteins were able to abolish the hemolytic activity (Table 1), raising the possibility to treat patients in severe cases of *Leptospira* infection by serumtherapy. In general, hemolysins have been considered potential vaccine antigens [18,19], and in this regard, further studies involving HlyX have to be performed to evaluate its use as a potential subunit vaccine against leptospirosis. LipL32 is the major antigen presented to the host during the infection [14], it is highly immunogenic and for these reasons, it has been evaluated as an antigen for immunodiagnosis of leptospirosis [20,21]. LipL32 plays an important role during *Leptospira* infection, during tubulointerstitial nephritis in proximal tubule cells [22], and we have confirmed here its hemolytic activity (Fig. 4 and Table 1). LipL32 was also described to be a promising vaccine antigen when presented by adenovirus [23]. It is possible that LipL32 in combination with other possible leptospiral antigens could represent the much needed vaccine for human use. It is interesting to note that the immune response against the currently available bacterin vaccine approved for animal use elicits primarily a humoral immune response against the carbohydrate portions of the *Leptospira* LPS that confers serovar specificity [24]. As a consequence, no memory immune response is developed and the use of multiple *Leptospira* serovars is therefore used in this vaccine formulation. In contrast, the use of antigenic proteins expressed in most, if not all, pathogenic *Leptospira* serovars (Fig. 5) suggested an alternative strategy to obtain not only a long lasting memory immune response, but also a more universal antigen against all the pathogenic *Leptospira* serovars. In this respect, the combination of HlyX with LipL32 is an interesting one which will be evaluated further as leptospirosis vaccine candidates.

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