Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Epitope mapping of pathogenic *Leptospira* LipL32

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Keywords
epitope, *Leptospira*, LipL32, mapping, peptides, serodiagnosis.

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2009/0744: received 26 April 2009, revised 25 July 2009 and accepted 14 August 2009

doi:10.1111/j.1472-765X.2009.02723.x

Abstract

Aims: To identify LipL32 epitopes and to evaluate their capability to recognize specific antibodies using ELISA.
Methods and Results: Epitope mapping by means of a library of overlapping peptide fragments prepared by simultaneous and parallel solid phase peptide synthesis on derivatized cellulose membranes (SPOT synthesis) was carried out. Eighty-seven overlapping decapentapeptides corresponding to the complete sequence of LipL32 were synthesized. According to spot-image intensities, the most reactive sequences were localized in regions 151–177 (sequence AAKAKPVQKLDDDDDGDDTYKEERHNK) and 181–204 (sequence LTRIKIPNPPKSFDDLKNIDTKKL). Two peptides (P1 and P2) corresponding to these sequences were synthesized, and their reactivity evaluated using ELISA test.
Conclusions: Epitope identification and analysis suggested the existence of two antigenic regions within LipL32. These LipL32 reactive regions were highly conserved among antigenically variants of *Leptospira* spp. isolates. Peptides containing these regions (P1 and P2) showed a good capability for anti-leptospiral antibody recognition.
Significance and Impact of the Study: This finding could have potential relevance not only for serodiagnosis but also as a starting point for the characterization of targets for vaccine design.

Introduction

Leptospirosis is a zoonosis of ubiquitous distribution, caused by infection with pathogenic *Leptospira* species. The spectrum of human disease it causes is extremely wide, ranging from subclinical infection to a severe syndrome of multi-organ infection with high mortality (Levett 2001; Vanasco *et al.* 2008).

Serological tests are considered to be the most useful tool for the detection of leptospiral antibodies. The international standard method – the microscopic agglutination test (MAT) – is not only technically complex but also time consuming (Faine 1982). The MAT and the enzyme immunoassays (ELISA) are the most frequently applied tests in serodiagnosis. Rapid diagnosis of leptospirosis is very important, because antibiotic treatment is most effective in altering the course of this life-threatening disease when it is initiated early on in the infection (Costa Ooteman *et al.* 2006).

LipL32 is one of the most antigenic outer membrane proteins from pathogenic leptospires and the most abundant antigen found in the leptospiral total protein profile (Zuerner *et al.* 1991).

This lipoprotein is highly conserved among pathogenic *Leptospira* species but has no orthologs in the saprophyte *Leptospira* species (Hauk *et al.* 2008). Moreover, the surface lipoprotein LipL32 was identified as a target by leptospires during natural infection (Wang *et al.* 2007).

These natural infection antibodies provide passive genus-specific immunity in some animal models against a number of different strains and species of *Leptospira* (Faine *et al.* 1999; Faisal *et al.* 2008). The usefulness of this lipoprotein in the serodiagnosis of leptospirosis has already been reported (Cullen *et al.* 2002; Tahiliani *et al.* 2005; Levett 2007; Hoke *et al.* 2008). Most of these studies used whole recombinant molecules or big LipL32 C-terminus fragments as an antigen for diagnosis or vaccine development (Cullen *et al.* 2002; Tahiliani *et al.* 2005; Levett 2007; Hoke *et al.* 2008). Some authors have, however, recently predicted epitopes of other proteins (Lig A, OmpL1, LipL 21) by using other methods such as bioinformatics (Wiwanitkit 2007; Lin *et al.* 2008a,b) or random peptide libraries (Tungtrakanpoung *et al.* 2006). Epitope mapping of LipL32 by the use of peptide arrays has yet to be reported.

Epitope mapping by the synthesis of an overlapped peptide library covering the full length of any protein and its immunoreactivity evaluation with specific antibodies allows study of the full linear epitope map of that protein. It enables us to obtain small molecules that could be used in peptide-based ELISA assays. This method is an easy and very flexible technique for simultaneous, parallel, chemical synthesis on membrane supports and gives a rapid and low-cost access to a large number of peptides for systematic epitope analysis (Frank and Overwin 1996).

The objectives of this study were to identify LipL32 epitopes and to evaluate their capability to recognize specific antibodies by means of an ELISA test.

Material and methods

Epitope identification and analysis

Epitope mapping was carried out by means of a library of overlapping peptide fragments corresponding to the complete sequence of *Leptospira interrogans* serovar Copenhageni LipL32 (http://aeg.lbi.ic.unicamp.br/world/lic/). Peptides were simultaneously synthesized by the SPOT method (Frank 1992) on derivatized cellulose membranes with an Ala-Ala linker, for the preparation of immobilized peptides. Assembly of the peptides was carried out utilizing Fmoc-chemistry essentially, as previously described (Frank and Overwin 1996). The prepared membrane consists of overlapping pentadecapeptides spanning the complete sequence of LipL32 (residues 1–272), with an offset of three amino acid residues. The reactivity of the SPOT membrane was evaluated according to the protocol described by Soutullo *et al.* (2007). Bound antibodies were detected with alkaline phosphatase (AP)-conjugated secondary antibody (anti-rabbit IgG; Sigma) followed by a colour reaction with 5-bromo-4-choro-indolylphosphate (BCIP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (Frank 1992).

The epitope analysis was carried out with rabbit serum samples, a true negative sample and a pool of three hyperimmune samples against *Leptospira* Icterohaemorrhagiae serogroup, serovar Copenhageni (strains M20 and RGA) and serovar Icterohaemorrhagiae (strain Ictero no. 1).

After epitope identification, two peptides were chemically synthesized by 9-florenyl-methoxy-carbonyl chemistry and purified by HPLC as described previously (Tonarelli *et al.* 2000). These two peptides (P1 and P2) were subsequently used as antigens in an ELISA test on human serum samples.

ELISA tests

Three serological tests were carried out in parallel using a blind design, two peptide-based (P1 and P2) and a classical ELISA test.

The protocol used in the peptide-based ELISA was previously described by Lottersberger *et al.* (2004). Briefly, the peptides were adsorbed onto polystyrene plates (Costar EIA Microplates; Corning Costor, Oneonta, NY) using 100 μl of a solution containing 20 μg ml^{-1} of peptide in carbonate/bicarbonate buffer (pH 9·6). Microplates were incubated for 60 min at 37°C with 100 μl per well of a 1 : 100 dilution of human sera, followed by incubation with peroxidase-conjugated anti-human IgG (Dako, Glostrup, Denmark) for 30 min at 37°C. The plates were developed by adding 100 μl of a solution containing 3,3′,5,5′-tetramethylbenzidine and H_2O_2 as substrate. After 15 min, the reaction was stopped by adding 100 μl of 2 N H_2SO_4 and read at 450/630 nm.

The classical ELISA test was genus specific (whole *Leptospira* antigen) (Vanasco *et al.* 2007). Each sample was tested in duplicate, and an average optical density (AOD) for each was obtained. Results were expressed as checked optical density (COD), which was obtained by dividing the AOD by the optical density of the negative control pool of samples. Samples were considered positive when observance readings as a COD result were higher than the cut-off point. The optimal cut-off point was determined by means of a receiver operating characteristic curve using Medcalc® (Schoonjans *et al.* 1995).

Serum samples and case definition

One hundred and ten human serum samples of 50 confirmed and 60 unconfirmed cases of leptospirosis (in different stages of illness) were randomly selected from all samples referred to the National Leptospirosis Laboratory of the Instituto Nacional de Enfermedades

Respiratorias (INER). Case definition was based on MAT results, clinical presentation, unspecific findings and epidemiological data (Vanasco *et al.* 2007).

Results

Epitope identification and analysis

Eighty-seven overlapping decapentapeptides were synthesized, and their sequences are presented in Table 1. Each peptide sequence was offset by three amino acids from the previous peptide.

According to the SPOT image intensities, the most reactive regions were localized by spots 51–55 and spots 61–64. These results strongly suggest the existence of two antigenic regions within LipL32, which can be recognized by their anti-leptospiral antibodies. These LipL32 reactive regions are highly conserved among antigenic variants of *Leptospira* spp. isolates and do not show homology with sequences from other micro-organisms.

According to these findings, two synthetic peptides were prepared. Peptide P1: (region 151–177) AAKAKPVQKLDDDDDGDDTYKEERHNKYNS, Peptide P2: (region 181–204) YNSLTRIKIPNPPKSFDDLKNIDTKKLVRG.

Table 2 ELISAs results in serum samples from patients with confirmed and unconfirmed leptospirosis

	ELISA test results			
Case definition (*n*)	ELISA*	P1-ELISA	P2-ELISA	P1 + P2-ELISA
Confirmed (50)	47	44	42	46
Unconfirmed (60)	1	3	3	4

*Sonicated whole *Leptospira* molecules-based ELISA.

ELISA test

Cut-off points for the peptide-based ELISAs were COD >1·79 and >1·99 (P1 and P2 respectively). Results are provided in Table 2. Obviously, as MAT was part of the case definition, its results was not included in the table. The three confirmed cases negative to classical ELISA were positive to all peptide-based ELISAs. The six

Table 1 Eighty-seven overlapping decapentapeptides synthesized corresponding to the complete sequence of LipL32 *Leptospira interrogans*

Spot no.	Sequence	Spot no.	Sequence	Spot no.	Sequence
1	MKKLSILAISVALFA	30	AVIAEMGVRMISPTG	59	HNKYNSLTRIKIPNP
2	LSILAISVALFASIT	31	AEMGVRMISPTGEIG	60	YNSLTRIKIPNPPKS
3	LAISVALFASITACG	32	GVRMISPTGEIGEPG	61	LTRIKIPNPPKSFDD
4	SVALFASITACGAFG	33	MISPTGEIGEPGDGD	62	IKIPNPPKSFDDLKN
5	LFASITACGAFGGLP	34	PTGEIGEPGDGDLVS	63	PNPPKSFDDLKNIDT
6	SITACGAFGGLPSLK	35	EIGEPGDGDLVSDAF	64	PKSFDDLKNIDTKKL
7	ACGAFGGLPSLKSSF	36	EPGDGDLVSDAFKAA	65	FDDLKNIDTKKLLVR
8	AFGGLPSLKSSFVLS	37	DGDLVSDAFKAATPE	66	LKNIDTKKLLVRGLY
9	GLPSLKSSFVLSEDT	38	LVSDAFKAATPEEKS	67	IDTKKLLVRGLYRIS
10	SLKSSFVLSEDTIPG	39	DAFKAATPEEKSMPH	68	KKLLVRGLYRISFTT
11	SSFVLSEDTIPGTNE	40	KAATPEEKSMPHWFD	69	LVRGLYRISFTTYKP
12	VLSEDTIPGTNETVK	41	TPEEKSMPHWFDTWI	70	GLYRISFTTYKPGEV
13	EDTIPGTNETVKTLL	42	EKSMPHWFDTWIRVE	71	RISFTTYKPGEVKGS
14	IPGTNETVKTLLPYG	43	MPHWFDTWIRVERMS	72	FTTYKPGEVKGSFVA
15	TNETVKTLLPYGSVI	44	WFDTWIRVERMSAIM	73	YKPGEVKGSFVASVG
16	TVKTLLPYGSVINYY	45	TWIRVERMSAIMPDQ	74	GEVKGSFVASVGLLF
17	TLLPYGSVINYYGYV	46	RVERMSAIMPDQIAK	75	KGSFVASVGLLFPPG
18	PYGSVINYYGYVKPG	47	RMSAIMPDQIAKAAK	76	FVASVGLLFPPGIPG
19	SVINYYGYVKPGQAP	48	AIMPDQIAKAAKAKP	77	SVGLLFPPGIPGVSP
20	NYYGYVKPGQAPDGL	49	PDQIAKAAKAKPVQK	78	LLFPPGIPGVSPLIH
21	GYVKPGQAPDGLVDG	50	IAKAAKAKPVQKLDD	79	PPGIPGVSPLIHSNP
22	KPGQAPDGLVDGNKK	51	AAKAKPVQKLDDDDD	80	IPGVSPLIHSNPEEL
23	QAPDGLVDGNKKAYY	52	AKPVQKLDDDDDGDD	81	VSPLIHSNPEELQKQ
24	DGLVDGNKKAYYLYV	53	VQKLDDDDDGDDTYK	82	LIHSNPEELQKQAIA
25	VDGNKKAYYLYVWIP	54	LDDDDDGDDTYKEER	83	SNPEELQKQAIAAEE
26	NKKAYYLYVWIPAVI	55	DDDGDDTYKEERHNK	84	EELQKQAIAAEESLK
27	AYYLYVWIPAVIAEM	56	GDDTYKEERHNKYNS	85	QKQAIAAEESLKKAA
28	LYVWIPAVIAEMGVR	57	TYKEERHNKYNSLTR	86	AIAAEESLKKAASDA
29	WIPAVIAEMGVRMIS	58	EERHNKYNSLTRIKI	87	AAEESLKKAASDATK

confirmed cases negative to P1 ELISA were not included within the eight cases negative to P2 ELISA.

Discussion

Two epitopes from *L. interrogans* LipL32 were identified. Peptides P1 and P2, containing the identified epitopes, were able to recognize specific antibodies when used in the ELISA test. These preliminary findings suggest that these peptides can be useful not only for serodiagnosis but also as a starting point in the characterization of targets for vaccine design.

The three confirmed cases negative to classical ELISA were positive to all peptide-based ELISAs. Meanwhile, the six confirmed cases negative to P1 ELISA were not included within the eight cases negative to P2 ELISA. This fact could indicate different test habilities to detect the disease on different patients and stages of disease. Therefore, a combination of all test results interpreted in parallel will increase its sensitivity. Meanwhile, the four unconfirmed cases positive to P1 and/or P2 ELISAs may be the result of either an increased sensitivity of these tests or serum cross-reactivity with diseases other than leptospirosis.

Previous papers describing epitopes from other proteins of pathogenic leptospires (Lig A, OmpL1 and LipL21) either used bioinformatic techniques to predict possible epitopes (Wiwanitkit 2007; Lin *et al.* 2008a,b) or randomly selected peptides from libraries (Tungtrakanpoung *et al.* 2006) and subsequent evaluation on patient serum samples. However, none of these studies performed a complete LipL32 mapping, nor did they synthesize small protein fragments to evaluate their reactivity against specific leptospiral antibodies, as we did.

These encouraging results will allow further research in validating this ELISA test. To evaluate the sensitivity and specificity of these peptides in serological diagnosis, further studies will be needed using a larger sample size and a wider panel including serum samples from other relevant diseases, such as dengue, hantavirus and haemorrhagic fever.

Acknowledgements

This work was supported by the UNL-CAI+D 2006 program and PICTO-2004 15-22427 ANPCyT/UNL.

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