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Monoclonal antibodies against the leptospiral immunoglobulin-like proteins A and B conserved regions

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ABSTRACT

Leptospirosis is an infectious disease caused by pathogenic spirochetes of the genus *Leptospira* that affects humans and a wide variety of animals. Recently the genomes of *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira biflexa* species were sequenced allowing the identification of new virulence factors involved in survival and pathogenesis of bacteria. LigA and LigB are surface-exposed bacterial adhesins whose expression is correlated with the virulence of *Leptospira* strains. In this study, we produced and characterized five monoclonal antibodies (MAbs) against a recombinant fragment of LigB (rLigBrep) with approximately 54 kDa that comprise the portions of LigA and LigB (domains 2–7). The 5 MAbs obtained were of the IgG1 (2) and IgG2b (3) isotypes and their affinity constants for rLigBrep ranged from 7×10^7 M^{-1} to 4×10^8 M^{-1}. The MAbs were able to react with the native antigen on the *L. interrogans*, *L. borgpetersenii* and *Leptospira noguchii* surfaces by indirect immunofluorescence, immunoblotting and immunoelectron microscopy. These results demonstrate that the MAbs anti-rLigBrep can be useful to complement genetic studies and to aid studies aiming understanding the role of Lig proteins in *Leptospira* pathogenesis and the development of Lig-based vaccines and improved diagnostic tests for leptospirosis.

1. Introduction

Leptospirosis is an infectious disease of global distribution that represents a challenge to public health, mainly in developing countries [1]. Outbreaks and epidemics are mostly associated with periods of intense rainfall and flooding [2]. Human and animal hosts can be infected by direct or indirect contact with the urine of infected carriers or through contaminated water and soil [3]. The disease usually produces symptoms such as fever, vomiting and headache, being many times confused with other febrile illnesses, i.e. influenza, dengue and malaria [4]. However, in some cases, it may evolve to serious complications such as renal and hepatic failures, uveitis, pulmonary damage with severe pulmonary hemorrhage and death [5].

The clinical presentation of leptospirosis is traditionally biphasic and includes an acute or septicemic phase,

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characterized by the presence of spirochetes in the bloodstream and tissues that usually last for 1 week, followed by a chronic or immune phase that culminates with the production of serovar-specific circulating antibodies and the dissemination of leptospires to the environment through the urine [2,5].

The microscopic agglutination test (MAT), that measures the antibodies levels in sera from suspected patients with a panel of live serovars of pathogenic leptospires, is considered to be the "gold standard" for laboratory diagnosis of leptospirosis. Although this technique has been performed in clinical laboratories worldwide for several years, it presents a high number of cross-reactions among serovars and low sensitivity in the acute phase of the disease [4,5].

Search for antigens suitable for use in vaccines and diagnostic tests of leptospirosis led to the identification of the Lig family of proteins that are characterized by the presence of repeated immunoglobulin-like domains, similar to *Yersinia pseudotuberculosis* invasin and *Escherichia coli* intimin that play important role in the colonization of host tissue [6]. Recent studies demonstrated the adherence of LigA and LigB to fibronectin, collagen, laminin, fibrinogen, elastin, human tropoelastin (HTE) [7] [8], and their capacity to induce immunoprotection using different approaches and animal models [9–14]. Both LigA and LigB were recognized by serum antibodies of infected human patients; LigA is the first to be expressed during *Leptospira* infection [15] and LigB is a novel bacterial Ca-binding protein that plays a pivotal role in the pathogenesis of leptospirosis [16,17], what suggests that they can be used as markers for serodiagnosis of leptospirosis [18]. In addition, a recombinant antigen encompassing the region of LigA and LigB was able to differentiate between vaccinated and naturally infected animals [19].

In this study we report on the production and characterization of monoclonal antibodies (MAbs) against a recombinant protein fragment of approximately 54 kDa encoded by the conserved regions of *ligA* and *ligB* genes (domains 2–7). The five MAbs obtained against this recombinant fragment were able to detect the native proteins in several *Leptospira* serovars and are expected to be useful in studies aiming understanding the role of Lig proteins in pathogenesis and the development of Lig-based vaccines and improved diagnostic tests for leptospirosis.

2. Materials and methods

2.1. Bacterial strains and culture

Six *Leptospira* isolates were used: *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130 and serovar Canicola strain Kito, *Leptospira borgpetersenii* serogroup Ballum strain Cau 42, *Leptospira noguchii* serovar Autumnalis strain Bonito and *Leptospira biflexa* serovar Patoc strain Patoc I. Strain L1-130 was kindly provided by Albert Ko, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, Brasil. All other strains were obtained from the Laboratory of Molecular Biology, Center of Technological Development (CDTec), Universidade Federal de Pelotas, Brazil. Bacteria were grown for up to 7 days at 30 °C in tubes containing 5 mL of Ellinghausen–McCullough–Johnson–Harris liquid medium (EMJH, Difco-USA) with the addition of 10% supplement Difco-USA, without antibiotics [20].

2.2. Preparation of recombinant LigBrep

A fragment of LigB corresponding to amino acids 131–649 [6] was cloned into the pAE expression vector that allows fusion of the protein with a N-terminal 6× His tag. This plasmid was used to transform *E. coli* BL21 (DE3) pLysS for recombinant protein (rLigBrep) production. Purification of the protein was accomplished by affinity chromatography with Ni-NTA resin using the ÄKTAPrime chromatography system (GE Healthcare, USA). Fractions of the purified rLigBrep were analyzed by SDS-PAGE (12%) in reducing conditions and quantified by the Bradford method [21].

2.3. MAb production and purification

The animals used in this study were treated according to the guidelines of animal experimentation recommended by COBEA (Colégio Brasileiro de Experimentação Animal). Two 6-week-old BALB/c mice were injected via intraperitoneal with 150 μg of rLigBrep on days 0, 14, 21 and 28. Freund's complete adjuvant was used in the first dose and incomplete in the subsequent ones. Four days before cellular fusion the mouse with the highest titer in indirect ELISA (1:64,000) was boosted with 20 μg of protein intravenously. Splenic lymphocytes were fused to murine Sp2/O-Ag14 myeloma cells in the presence of PEG 1450 (Sigma–Aldrich, USA). Fused cells were cultivated in Dulbecco's modified Eagle medium (DMEM Sigma–Aldrich, USA) containing 20% fetal calf serum (FCS, Cultilab, Campinas, Brazil) and supplemented with hypoxanthine, aminopterin and thymidine (HAT Sigma–Aldrich, USA). Hybridomas growing in HAT medium were screened for specific antibodies by indirect ELISA; those positive were cloned twice by the limiting dilution technique and expanded for storage in liquid nitrogen or injection in mice for ascites production. Supernatants from culture flasks were used for MAb isotype determination with an ELISA isotyping kit (Sigma–Aldrich, USA). For other characterization experiments MAbs were purified from ascitic fluids by affinity chromatography on a protein G-Sepharose column according to manufacturer instructions (GE Healthcare Company, USA), dialyzed against PBS and concentrated with PEG 20000. Concentrations of purified MAbs were determined at 280 nm, standardized to 5 mg mL^{-1} with PBS and the preparations were stored at −20 °C until use.

2.4. Affinity constants

MAbs functional affinity constants (K_a) for rLigBrep were determined by the ELISA method [22]. Briefly, different antigens concentrations from 10.8×10^{-3} M to 0.05×10^{-3} M were incubated in solution with constant amounts of each MAb until equilibrium was reached (16 h at 20 °C). After incubation, unbound antibodies in the liquid phase were determined by indirect ELISA using microtiter

plate wells coated with rLigBrep (1 μg mL^{-1}) and goat anti-mouse Ig-peroxidase conjugate (Sigma–Aldrich, USA). The optical densities (OD) were read at 450 nm and graphics were built according to ref. [23]. The linear relationships between values $(A_0 - A_i)/A_i$ and l_i (where A_0 is the OD of MAb without reacting with antigen, A_i is the OD of MAb after reacting with each antigen concentration and l_i is the antigen concentration) were determined and the values of functional K_a were defined by the slope of these linear relationships.

2.5. Immunoblotting analysis

Aliquots from 7-day cultures (10^9 cells mL^{-1}) of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and *L. biflexa* serovar Patoc strain Patoc I were separated by SDS-PAGE using a 10% polyacrylamide gel and a discontinuous buffer system [24]. After electrophoresis, proteins were transferred onto nitrocellulose membranes to test specific LigA and LigB detection by MAbs. Membranes were blocked with skimmed milk, washed with PBS-T and reacted with each MAb for 2 h. A rabbit polyclonal serum against rLigBrep and normal mouse serum was used as positive and negative control, respectively. Additionally, an MAb against LipL32 (1D9) was used as a control of protein electrotransference. After washing, the membranes were incubated for 1 h with goat anti-mouse or anti-rabbit Ig peroxidase conjugate (Sigma–Aldrich, USA). After that, membranes were developed with chromogen/substrate solution (6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris–HCl, pH 8.0, and hydrogen peroxide 30 vol%) for visualization of protein bands.

2.6. Indirect immunofluorescence

Slide chambers (ICN Biomedicals Inc., CA, USA) were coated with a 0.1% poly-L-lysine solution for 1 h at 30 °C. Seven-day cultures of all leptospira samples were washed once in PBS and resuspended to a density of approximately 10^8 cells mL^{-1}. Aliquots of 10 μL were pipetted into the slide chambers and incubated at 30 °C until dry. The slides were then blocked with 10% FBS in PBS, washed twice with EMJH and coated for 1 h at 30 °C with MAbs diluted in EMJH. The slides were washed again twice with EMJH and a 1:100 dilution of goat anti-mouse FITC conjugate (Invitrogen, USA) was added and incubated for 1 h in a dark humid chamber at 30 °C. After washing with EMJH a drop of mounting medium was added and labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm. Confirmation of bacteria in the microscopic field was achieved by DNA staining with Hoechst 33258.

2.7. Epitope mapping

Polystyrene ELISA microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY) were coated with 50 μL of five recombinant fragments from LigA and LigB proteins (10 μg mL^{-1}) encompassing different *ligA* and *ligB* gene domains, a gift from Dr. Marco Medeiros, FIOCRUZ, Rio de Janeiro (Table 1 and Fig. 1). The plates were then washed three times with phosphate-buffered saline with Tween 20 (PBS-T), and panel of antibodies added for reaction with each fragment. After another round of washing, goat polyvalent antibody against mouse IgG-peroxidase conjugate (Sigma–Aldrich, USA) was added for MAb detection. All assay steps to this point were carried out in 50 μL/well additions at 37 °C for 1 h. Wells were finally washed five times with PBS-Tween and the presence of the antigen–antibody complex was revealed by adding 50 μL of a substrate solution containing OPD (0.4 mg mL^{-1} in 0.1 M citrate buffer, pH 5.0) and 0.03% hydrogen peroxide. Color development was measured at 450 nm in an ELISA reader (Multiskan MCC/340, Titertek Instruments, Huntsville, AL, USA). The epitope corresponding to immunodominant region was analyzed in the NCBI database using the algorithm BLASTp to identify the most similar sequence among the leptospiral proteins deposited.

Table 1
Molecular characteristics of the recombinant protein fragments.

Fragments	Amino acids	Length (aa)	Domain[a]
LigAF1	625–1225	600	7–13
LigAF2	582–943	361	7–10
LigBF1	625–1257	632	7-Carboxyterminal
LigBF2	582–947	365	7–11
LigBF3	945–1257	312	11-Carboxyterminal
LigBrep	131–649	518	1–7

[a] Based on SMART and PFAM analysis.

2.8. Immunoelectron microscopy (IEM)

Cells from 7-day cultures of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 and *L. biflexa* serovar Patoc strain Patoc I were incubated under mild agitation during 12 h at 4 °C with purified MAbs R1 and R2 diluted in PBS (1:50). Bacteria were reacted with goat anti-mouse secondary antibody conjugated to 10 nm gold particles according to manufacturer instructions (Sigma–Aldrich, USA). Antigen/antibody complex was fixed in an aqueous solution containing glutaraldehyde 6%, paraformaldehyde 6% and sodium cacodylate 0.1 M at 4 °C for 1 h. The bacteria were washed three times for 15 min in an aqueous solution containing sodium cacodylate 0.1 M and sucrose 0.2 M, and a fixation step in osmium 2% and sodium cacodylate 0.4 M for 2 h was performed. After a new washing step with tetra distilled water for 15 min, the bacterial cells were embedded in resin (Epoxy) for 1 h under agitation at room temperature. Thin-sectioned samples from L1-130 and Patoc I embedded resins were placed onto grids and observed on a transmission electron microscope (ZEISS Germany EM 900). Between all steps the cells were harvested by centrifugation (5000 × *g* for 10 min) at 4 °C and washed three times with PBS, unless otherwise indicated.

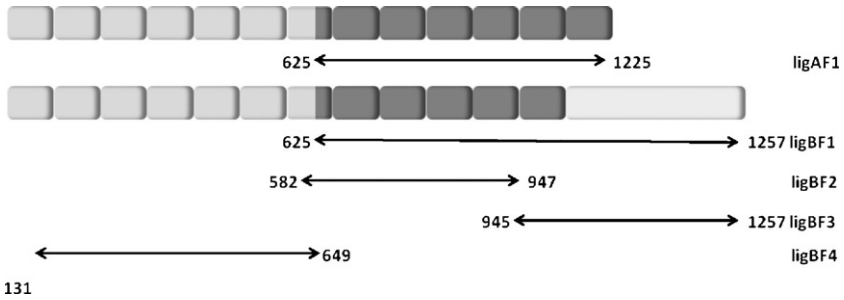

Fig. 1. Diagram of the leptospiral immunoglobulin-like (Lig) proteins expression as truncated fragments. (A) Representation of the conserved (gray) and non-conserved (dark gray) regions of LigA. (B) Representation of the conserved (gray), non-conserved (dark gray) and carboxyterminal (white) regions of LigB. Truncated peptides are represented as solid lanes and the first and last amino acids positions expressed are shown at the ends of each lane and in Table 1. Amino acids positions of the Lig domains are indicated into respective boxes.

3. Results

3.1. Preparation of recombinant LigBrep

Transformed *E. coli* BL21 (DE3) pLysS containing the expression plasmid pAE/*ligBrep* expressed an insoluble recombinant protein. Purification of rLigBrep from *E. coli* by affinity chromatography was efficient, resulting in approximately 12 mg L^{-1} of medium. A band of high intensity of the size expected (54 kDa) was observed when the fractions collected in the purification process were submitted to SDS-PAGE.

3.2. Generation of MAbs against rLigBrep

One cell fusion of mouse splenocytes with SP2/0 myeloma cells was performed and five hybridoma cell lines, named R1, R2, R3, R4 and R5, presenting stable production of antibodies that bound to rLigBrep, were cloned twice and expanded to obtain supernatants for MAb isotyping and cells for cryopreservation or injection in mice to obtain ascites for purification of Mabs for use in the other characterization experiments.

3.3. Isotypes and affinity constants

Results of MAbs isotyping and affinity constants (K_a) determination are shown in Table 2. Two of the MAbs selected were of the IgG1 isotype and the other three were

Table 2
Isotypes and affinity constants of MAbs against recombinant LigBrep.

Monoclonal antibody	Isotype	Affinity constant (M^{-1})
R1	IgG2b	4×10^8
R2	IgG1	1×10^8
R3	IgG2b	8×10^7
R4	IgG2b	8×10^7
R5	IgG1	7×10^7

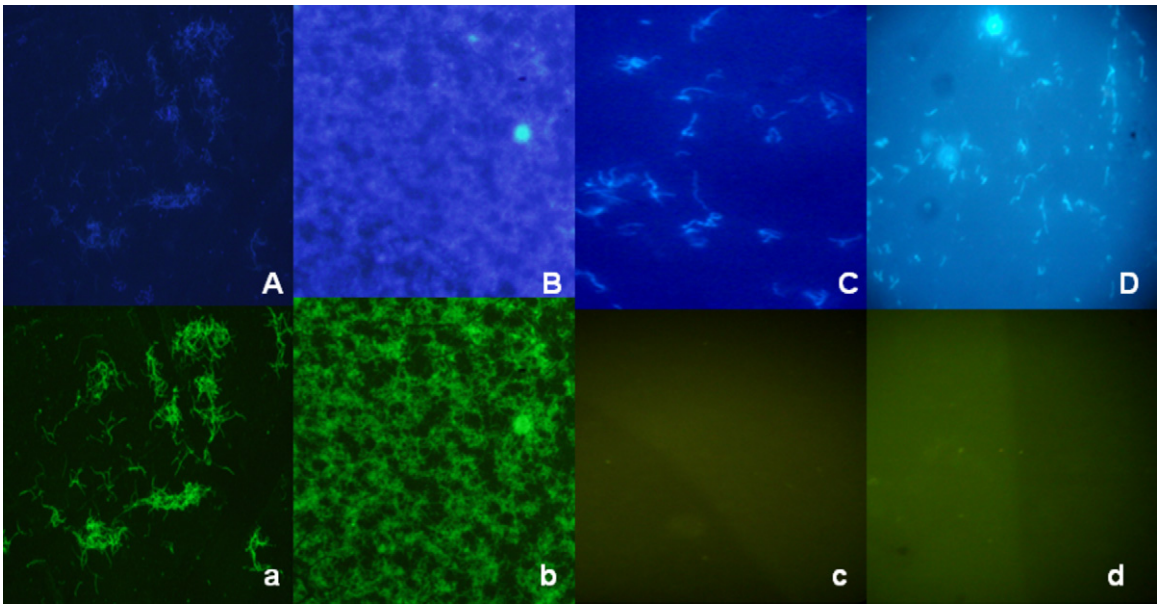

Fig. 2. Specific surface staining of *L. interrogans* L1-130 with monoclonal antibody R2. Panels (a) *L. interrogans* and MAb R2; (b) *L. interrogans* and MAb anti-LipL32 (positive control); (c) *L. interrogans* and normal mouse serum (negative control); (d). *L. biflexa* Patoc I and MAb R2. Panels A, B, C, and D: leptospiral DNA stained with Hoechst 33258. Visualization was performed with a 100× objective on an Olympus BX 51 fluorescence microscope.

of the IgG2b isotype. The MAbs showed intermediate K_a, varying from $7.0 \times 10^7\ M^{-1}$ for R5 to $4.0 \times 10^8\ M^{-1}$ for R1.

3.4. Immunoblotting analysis

The MAbs anti-rLigBrep were able to react with LigBrep protein in an immunoblotting assay. The analysis with two species of leptospires revealed that the MAbs identified two bands with sizes compatible with LigA (128 kDa) and LigB (201 kDa) in SDS denatured proteins from pathogenic *Leptospira* only. These results also indicate that the MAbs recognize linear epitopes on both proteins (data not shown).

3.5. Indirect immunofluorescence

Viable intact leptospires were attached to slide chambers coated with poly-L-lysine solution and subsequently exposed to MAbs anti-rLigBrep. The five MAbs stained cells from pathogenic *Leptospira* only. The assay performed with MAb R2 and *L. interrogans* L1-130 illustrates the reaction with native LigA and LigB expressed by pathogenic strains (Fig. 2).

3.6. Epitope mapping

Besides testing with rLigBrep, we tested the binding of MAbs to one recombinant fragment from LigA and three from LigB by indirect ELISA. Except for fragment 3 from LigB, the other fragments have overlap regions with rLigBrep (Fig. 1). ELISA results showed that with the exception of MAb R2, which gave a strong reaction with rLigBrep only, the MAbs reacted strongly with at least one of the other fragments tested (Fig. 3). Also, weak reactions with fragment LigBF3 were observed. Most of the MAbs bound to the region spanning amino acids 625–649, except for MAb R2, which reacted with an epitope located between residues 131 and 582. Data obtained from BLASTp analysis revealed 100% sequence similarity of the LigB lipoprotein immunodominant epitope (residues 625–649) with homologous regions from *L. interrogans* serovars Lai, Pomona, Copenhageni, Kennewicki, Canicola and Manilae; *L. noguchii* and *Leptospira kirschneri* serovar Grippotyphosa.

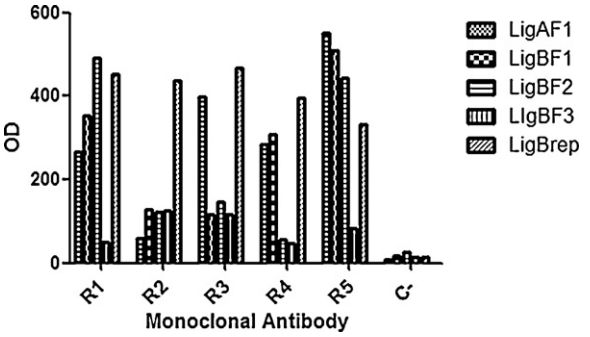

Fig. 3. Reactivities of monoclonal antibodies anti-rLigBrep with synthetic fragments from LigA and LigB in an indirect ELISA.

3.7. Immunoelectron microscopy (IEM)

IEM experiment using MAbs R1 and R2 demonstrated their capacity to react with the native Ligs on the external surface of leptospires. Results obtained with MAb R2 are shown to illustrate specific surface labeling of pathogenic leptospires (Fig. 4). Gold particles were present on leptospires incubated with MAbs against the LigBrep (23 ± 4 particles per bacterium).

4. Discussion

Antibody reagents have provided the basis for a large number of highly specific and reproducible immunoassays for use in vaccine development and diagnosis of infectious diseases [25]. In this study, we have produced five stable hybridomas secreting MAbs against the conserved regions of LigA and LigB proteins that reacted with pathogenic *L. interrogans* serovars Copenhageni strain Fiocruz L1-130 and Canicola strain Kito, *L. borgpetersenii* serogroup Ballum strain Cau 42 and *L. noguchii* serovar Autumnalis strain Bonito, but not to non-pathogenic *L. biflexa* serovar Patoc strain Patoc I. The immunofluorescence and immunoblotting assays demonstrated the reaction of MAbs

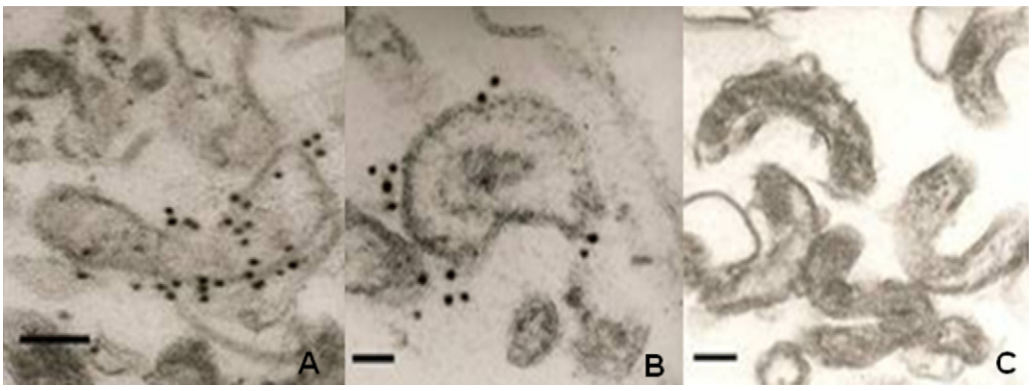

Fig. 4. Immunoelectron microscopy of thin-section samples of *L. interrogans* strain L1-130 (panels A and B) and *L. biflexa* strain Patoc I (panel C). Samples were incubated with MAb R2 followed by anti-mouse immunoglobulin secondary antibody conjugated to 10 nm gold particles. Visualization was performed with 85,000× (panels A and C) and 140,000× (panel B) magnification on a ZEISS EM 900 transmission electron microscope. Bars represent 0.09 μm.

with epitopes exposed either in the native protein on the bacterial cell or in the denatured protein.

The mapping of epitope regions targeted by the anti-rLigBrep MAbs with peptide fragments cloned from LigA and LigB revealed the domain 7 from both proteins as the immunodominant region. A BLAST protein analysis to compare the amino-acid sequence of the immunodominant region with the LigB lipoprotein from several serovars of *L. interrogans*, *L. noguchii* and *L. kirschneri* revealed 100% similarity, suggesting that the MAbs may recognize additional *Leptospira* species. Although there were some differences in the same region in *L. borgpetersenii* serovar Hardjo-bovis, our MAbs recognized, by indirect immunofluorescence, *L. borgpetersenii* serogroup Ballum.

After confirming by indirect immunofluorescence the ability of MAbs to recognize LigA and LigB adhesins expressed by pathogenic leptospires, an immunoelectron microscopy (IEM) study was performed to investigate the distribution of Lig proteins in *Leptospira* outer membrane. Two MAbs (R1 and R2) with the highest affinity constants were used in the IEM assay and both revealed a substantial amount of Ligs on pathogenic *Leptospira* surface. These results are similar to reactions of anti rLigB polyclonal antibodies in immunoelectron and immunofluorescence microscopy assays using *L. kirschenri* and *L. interrogans* [6]. These MAbs may be useful in studies aiming understanding the role of Lig proteins in *Leptospira* pathogenesis and in studies aiming the development of Lig-based vaccines and diagnostic assays for leptospirosis.

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