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Dissertação

Produção e caracterização de anticorpos monoclonais contra a região idêntica das proteínas LigA e LigB de *Leptospira interrogans*

Leonardo Garcia Monte

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Produção e caracterização de anticorpos monoclonais contra a região idêntica das proteínas LigA e LigB de *Leptospira interrogans*

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Orientador: José Antonio Guimarães Aleixo

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RESUMO

MONTE, Leonardo Garcia. **Produção e caracterização de anticorpos monoclonais contra a região idêntica das proteínas LigA e LigB de *Leptospira interrogans***. 2009. 52f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A leptospirose é uma zoonose causada por bactérias patogênicas pertencentes ao gênero *Leptospira*. Diversos mamíferos podem albergar o agente, sendo o rato a principal fonte de infecção para humanos no ambiente. As manifestações clínicas da doença variam desde os sintomas leves, como febre e dores de cabeça, até os mais graves com insuficiência renal e hepática que podem levar o indivíduo à morte. Esse amplo espectro de sintomas faz com que a leptospirose seja freqüentemente confundida em sua fase aguda com outras doenças febris, como gripe e dengue. O teste de aglutinação microscópica (MAT) é considerado o "padrão ouro" para o diagnóstico laboratorial da leptospirose, entretanto, o teste apresenta limitações relacionadas com a sensibilidade na fase aguda da doença. Recentemente, as proteínas de superfície LigA e LigB foram identificadas e relacionadas com a virulência de leptospiras patogênicas. Estas proteínas foram caracterizadas como adesinas, possuindo estrutura protéica semelhante a intimina de *Escherichia coli* e invasina de *Yersinia pseudotuberculosis,* que são importantes fatores de virulência nestes microrganismos. O objetivo desse estudo foi produzir e caracterizar anticorpos monoclonais (MAbs) contra um fragmento truncado de aproximadamente 54 kDa, que codifica a região idêntica das proteínas LigA e LigB (domínios 2-7), denominado rLigBrep. Foram obtidos 5 MAbs dos isotipos IgG1 (2) e IgG2b (3), com afinidades por rLigBrep que variaram entre 7 x 10^7 M^{-1} e 4 x 10^8 M^{-1}. Foi comprovado através de imunofluorescência indireta, *immunoblotting*, ELISA *whole-cell* e microscopia imunoeletrônica que os MAbs foram capazes de detectar o antígeno nativo presente em *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130. Estes resultados permitem concluir que os MAbs produzidos são importantes ferramentas para serem utilizados em estudos que visam entender o papel das proteínas Lig na patogênese das leptospiras e em testes diagnósticos para leptospirose.

Palavras-chave: Leptospirose, Anticorpos Monoclonais, Diagnóstico.

ABSTRACT

MONTE, Leonardo Garcia. **Production and characterization of monoclonal antibodies against the identical region of LigA and LigB proteins from *Leptospira interrogans***. 2009. 52f. Dissertação (Mestrado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a zoonotic disease caused by pathogenic bacteria belonging to the *Leptospira* genus. Several mammals may carry the agent, and rats are the most important source of human infection in urban settings. The wide spectrum of clinical manifestations varies from mild cases, with fever and headaches, to severe presentations, with liver and kidney failure, which may lead to death. As a result of the various degrees of severity, leptospirosis is frequently mistaken, in its acute stage, with other tropical diseases such as influenza and dengue. The microscopic agglutination test (MAT) is considered the "gold standard" when diagnosing leptospirosis; however, the test presents limitations regarding sensitivity in the acute phase of the disease. Recently, surface proteins LigA and LigB have been identified to be related with leptospiral virulence. These proteins have been characterized as adhesins, with a proteic structure similar to *Escherichia coli* intimin and *Yersinia pseudotuberculosis* invasin, which are important virulence factors in these organisms. The goal of this study was to produce and characterize monoclonal antibodies (MAbs) against a truncated fragment of approximately 54 kDa, named rLigBrep, that comprise a identical portion 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 varied from 7×10^7 M^{-1} to 4×10^8 M^{-1}. The MAbs were able to react with the native antigen in *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 by indirect immunofluorescence, immunoblotting, whole-cell ELISA and immunoelectron microscopy. These results allow concluding that these MAbs are important tools for studies aiming understanding the role of Lig proteins in *Leptospira* pathogenesis and in the development of tests for diagnosis of leptospirosis.

Keywords: Leptospirosis, Monoclonal Antibodies, Diagnosis.

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LISTA DE ABREVIATURAS E SIGLAS

Big - *Bacterial Immunoglobulin-like*
Lig - *Leptospiral Immunoglobulin-like*
LPS -Lipopolissacarídeo
mL - mililitro(s)
µg - micrograma(s)
µL - microlitro(s)
ºC - grau(s) Célcius
Nm - nanômetros
DMEM - *Dulbecco's modified Eagle's Medium*
MI - Meio Incompleto
MC - Meio Completo
UV - Ultravioleta
mM - Milimolar
min - Minutos
h - Hora
MG - Miligramas
V - Volts
mA - Miliampere
BSA - Albumina Sérica Bovina
DNA - Ácido Desoxirribonucléico
cm^2 - Centímetros quadrados
g - Força da gravidade
NI - Não idêntico
SDS - Dodecil sulfato de sódio
kDa - kilodalton
FBS - Soro fetal bovino
LCM - Meio de cultura de *Leptospira*
FCS - Soro fetal de bezerro
NMS - Soro normal de camundongo
OMP - Proteína de membrana externa
LipL - Lipoproteína de *Leptospira*
OMF - Fator de membrana externa
MFP - Proteína de fusão de membrana
MAbs - Anticorpos monoclonais
PCR - Reação em cadeia da polimerase
PEG - Polietilenoglicol
PBS - Tampão fosfato salino
OPD - Ortofenildiamina
MAT - Teste de soroaglutinação microscópica
ELISA - *Enzime-linked Immunos-sorbent assay*
HAT - *Hypoxanthine Aminopterin Thymidine*

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 A leptospirose e a *Leptospira*

A leptospirose é uma zoonose de importância global causada por espécies patogênicas de bactérias pertencentes ao gênero *Leptospira*, o qual atualmente é composto por 19 espécies e mais de 300 sorovares (ADLER; MOCTEZUMA, 2009). A evolução da leptospirose no indivíduo infectado é caracterizada por dois períodos: o primeiro, denominado de fase aguda ou leptospirêmica, é o que possui espiroquetas presentes na circulação sanguínea e tecidos; o segundo, denominado de fase imune humoral, é aquele onde uma resposta imunológica é estabelecida, mediada pela produção de anticorpos circulantes específicos contra o antígeno (LEVETT, 2001; WHO, 2003).

Indivíduos com leptospirose possuem um amplo espectro de sintomas desde febre, vômitos e dores de cabeça, muitas vezes confundidos com os de outras doenças febris como gripe, malária e dengue, até os sintomas mais graves onde a infecção pode conduzir a hemorragia pulmonar, falha renal e hepática; porém, algumas vezes, conduz a falhas em múltiplos órgãos e até mesmo a morte (LEVETT, 2001; McBRIDE et al. 2005; WHO, 2003).

Os hospedeiros pertencentes ao gênero *Rattus* são as fontes mais importantes de infecção humana no ambiente urbano, sendo a espécie *Rattus norvegicus* considerada o principal reservatório de *Leptospira*, especialmente do sorogrupo Icterohaemorrhagiae, que é o responsável pelo maior número de casos de leptospirose humana no Brasil e na América Latina (KO et al. 1999; ATHANAZIO et al. 2008).

Os surtos de leptospirose humana estão associados a períodos de intensa precipitação pluviométrica e conseqüentes inundações, pois são nesses períodos que aumenta a exposição das mucosas e pele à água contaminada com as leptospiras patogênicas. Além disso, os riscos de infecção também aumentam devido à capacidade do organismo poder sobreviver em ambiente úmido por um

longo período (WHO, 2003; HAAKE et al. 2006). O homem pode se infectar através do contato com água, urina e tecidos de animais infectados, mas não é considerado parte do ciclo de vida do patógeno e sim um hospedeiro acidental (HAAKE et al. 2006).

Regiões de clima tropical e subtropical, onde as temperaturas são mais elevadas, proporcionam condições ideais para o desenvolvimento e manutenção da bactéria no meio-ambiente (VINETZ et al. 2001; BHARTI et al. 2003). No Brasil a população de baixa renda está continuamente exposta aos fatores de risco, uma vez que geralmente vive em locais de precárias condições de saneamento (KO et al. 1999). O Sul do Brasil é a segunda região mais afetada pela leptospirose, sendo o Rio Grande do Sul o terceiro estado no ranking nacional com o maior número de casos confirmados, estando o Rio de Janeiro em 1° e Pernambuco em 2°, em pesquisa realizada entre os anos de 1980 e 2005 (www.saude.gov). Dentre as cidades da região sul Pelotas possui um número de casos superior à média do Estado do Rio Grande do Sul, ou seja, acima de 10-50 casos por 100 mil habitantes (BARCELLOS et al. 2003).

As taxas de morbidade e mortalidade aumentam pela falta de medidas preventivas como a vacinação de animais e humanos, políticas de informação e orientação à população, tratamento médico adequado e principalmente técnicas eficazes para identificação e confirmação da enfermidade, especialmente na fase inicial da doença (LEVETT 2001; VINETZ et al. 2001).

A *Leptospira* é uma bactéria espiralada, aeróbica, helicoidal que não sobrevive em ambientes secos ou com forte incidência de raios ultravioletas, os ambientes úmidos e quentes proporcionam as condições ideais para o crescimento da bactéria (FAINE et al. 1999; OKUDA et al. 2004). O ciclo de vida na natureza é mantido devido ao potencial genético em codificar uma diversidade de proteínas que contribuem tanto para sobrevivência no meio ambiente como para a infecção da variedade de espécies mamíferos encontrados como hospedeiros (LO et al. 2006).

Atualmente o gênero *Leptospira* é constituído pelas seguintes espécies: *L. alexanderi, L. alstonii* (genomospecies 1), *L. borgpetersenii, L. inadai, L. interrogans, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae* (genomospecies3), *L. weilii, L. wolffii, L. biflexa, L. meyeri, L. yanagawae* (genomospecies 5), *L. kmetyi, L. vanthielii* (genomospecies 4), e *L. wolbachii* (ADLER; MOCTEZUMA, 2009).

1.2 O diagnóstico

Considerando as centenas de sorovares patogênicos pertencentes ao gênero *Leptospira* e a capacidade de associação desses com diversas espécies de mamíferos hospedeiros, melhorias no diagnóstico da leptospirose tornam-se necessárias. Para a confirmação da leptospirose os procedimentos devem ser baseados nos diagnósticos clínico, epidemiológico e laboratorial (WHO, 2003).

O teste de referência para o diagnóstico da leptospirose é a soroaglutinação microscópica (MAT), um ensaio complexo que utiliza cepas vivas de leptospiras para a triagem dos soros de humanos e animais suspeitos de leptospirose, entretanto possui baixa sensibilidade, especialmente na fase inicial da doença ((WHO, 2003; McBRIDE et al. 2005). A baixa sensibilidade do MAT atribui-se ao fato de que o sistema imune do indivíduo infectado leva alguns dias para produzir níveis detectáveis de anticorpos contra o agente infectante (WHO; 2003). Além das dificuldades enfrentadas para o diagnóstico durante a fase inicial da leptospirose, existe um alto grau de reações cruzadas entre diferentes sorovares, onde muitas vezes o resultado revela títulos elevados de anticorpos contra sorovares que não são os causadores da enfermidade ou surto (LEVETT, 2003).

A confirmação de um caso de leptospirose pode também ser realizada com o isolamento do agente através de cultivo em meios de cultura específicos, porém esta técnica não é realizada como rotina laboratorial e pode durar mais de dois meses para a confirmação, o que a torna inviável na maioria dos casos e faz com que o diagnóstico laboratorial da leptospirose seja baseado principalmente em testes sorológicos (FAINE et al. 1999; WHO, 2003).

Devido à complexidade e desvantagens do MAT a busca por novos testes diagnósticos é constante tanto do tipo ELISA (PALANIAPPAN et al. 2004; OOTEMAN et al. 2005; SUWIMONTEERABUTR et al. 2005) como PCR (LUCCHESI et al. 2004; OOTEMAN et al. 2005, PALANIAPPAN et al. 2005), o que os torna importantes para o desenvolvimento de futuras técnicas mais eficazes para o diagnóstico de certeza da leptospirose.

1.3 A importância das proteínas de leptospiras como ferramentas laboratoriais

A habilidade das leptospiras patogênicas de penetrar, disseminar e permanecer nos tecidos do hospedeiro mamífero parece residir na capacidade desse organismo em aderir às proteínas da matriz celular e fibrinogênio, descrita como propriedade importante das leptospiras virulentas, embora ainda pouco se saiba sobre a patogênese da enfermidade (TSUCHIMOTO et al. 1984; BALLARD et al. 1986; THOMAS; HIGBIE 1990, CHOY et al. 2007).

Um grande número de proteínas de membrana externa de leptospiras já foram caracterizadas como importantes ferramentas para o desenvolvimento de vacinas e de ensaios de imunodiagnóstico como a OmpL1 (HAAKE et al. 1993), LipL41 (SHANG et al. 1996), LipL36 (HAAKE et al. 1998), LipL32 (HAAKE et al. 2000), LipL21 (CULLEN et al. 2003), LipL46 (MATSUNAGA et al. 2006), OmpA-like Loa22 (KOIZUMI AND WATANABE, 2003) e a proteína LenA (VERMA et al. 2006).

Com o sequênciamento do genoma de *L. interrogans* cepa Fiocruz L1-130 no ano de 2003 foi possível identificar genes que codificam para outros tipos de proteínas de membrana externa (OMPs), como as OMPs TonB-dependentes que são envolvidas na aquisição de nutrientes e genes envolvidos em três sistemas de efluxo, que consistem de um fator de membrana externa (OMF), proteína de fusão de membrana (MFP) e um transportador da membrana interna (CzcA) que é importante na detoxificação de metais pesados (NASCIMENTO et al. 2004, GAMBERINI et al. 2005).

Além desses, foram identificados três novos genes que codificam para as já descritas "*Bacterial immunoglobulin-like proteins* (Big)", estes antígenos presentes em outros patógenos são caracterizados pela presença de domínios repetitivos de aminoácidos que conferem o nome a família protéica. Os genes de *Leptospira*, que contém tais domínios, foram denominados de "*Leptospiral immunoglobulin-like* (Lig)" e codificam para as proteínas LigA, LigB e LigC, encontradas somente em leptospiras patogênicas (MATSUNAGA et al. 2003). Essa familia de três proteínas LigA, LigB and LigC, pertencem a superfamilia *Bacterial immunoglobulin (Ig)-like (Big)* com domínio repetitivos semelhantes aos encontrados em determinantes de virulência como a intimina de *Escherichia coli* enteropatogênica, invasina de *Yersinia*

pseudotuberculosis (PALANIAPPAN et al. 2002; MATSUNAGA et al. 2003; KOIZUMI; WATANABE, 2004).

Essa superfamília medeia à interação do patógeno com as células do hospedeiro facilitando a invasão e o ancoramento durante a infecção. Em estudos recentes foi demonstrado que as Ligs recombinantes podem mediar *in vitro* a interação com as proteínas da matriz extracelular (fibronectina, colágeno, laminina) e fibrinogênio (CHOY et al. 2007; LIN; CHANG, 2007). Além disso, os genes *ligs* são regulados pela osmolaridade fisiológica codificando proteínas de superfície fortemente reconhecidas por anticorpos de pacientes com leptospirose humana (MATSUNAGA et al. 2003; MATSUNAGA et al. 2005; CRODA et al. 2007; SRIMANOTE et al. 2008). Outros ensaios também demonstraram que as proteínas Ligs são antígenos protetores quando usados em modelos animais com leptospirose (KOIZUMI; WATANABE, 2004; PALANIAPPAN et al. 2006; SILVA et al. 2007).

1.4 Anticorpos monoclonais

Em 1975, Kohler & Milstein (Nobel de Medicina em 1984) desenvolveram a técnica dos anticorpos monoclonais que são altamente específicos e reconhecem um único sítio de ligação do antígeno (epítopo). Essa técnica representa um excelente exemplo de biotecnologia, com vantagens de padronização, sensibilidade e confiabilidade em testes de imunodiagnóstico. A habilidade de produzir anticorpos monoclonais contra proteínas expressas por organismos patogênicos é reconhecida como uma das mais significantes realizações da biotecnologia (BERRY et al. 2005).

A uniformidade dos anticorpos monoclonais é uma característica decisiva no desenvolvimento de pesquisas e trabalhos que melhorem a sensibilidade e especificidade dos ensaios (BERRY et al. 2005). Esses anticorpos fornecem as bases para um grande número de imunoensaios altamente reprodutíveis e específicos para diagnóstico de doenças (PAYNE et al. 1998; ANDREOTTI et al. 2003). A produção de anticorpos monoclonais oferece vantagens para sua realização, entre elas o fácil manuseio com animais de pequeno porte, a possibilidade de estocagem dos hibridomas por um longo período, sem alteração das características estruturais da molécula e a utilização de pequenas doses de antígeno.

1.5 Hipótese e Objetivo

A hipótese deste estudo é a de que hibridomas obtidos a partir de esplenócitos de animais imunizados com um antígeno recombinante e células de mieloma produzirão anticorpos monoclonais capazes de reconhecer o antígeno nativo em *L. interrogans* sorovar Copenhageni cepa Fiocruz L1-130.

Assim, o objetivo do trabalho foi produzir anticorpos monoclonais contra uma proteína recombinante, que corresponde a um fragmento de aproximadamente 54 kDa codificado nos domínios 2-7 de LigB e LigA, e comprovar através de diversas técnicas imunológicas que os anticorpos obtidos reagem com as proteínas LigA e LigB expressas somente por leptospiras patogênicas.

2 ARTIGO

Production and Characterization of Monoclonal Antibodies Against the Leptospiral Immunoglobulin-like Proteins A and B Identical Region

(Artigo científico formatado de acordo com as normas do periódico Veterinary Microbiology)

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST THE LEPTOSPIRAL IMMUNOGLOBULIN-LIKE PROTEINS A AND B IDENTICAL REGION

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ABSTRACT

Leptospirosis is an infectious disease that affects humans and a wide variety of animals and is of difficult diagnosis in its acute phase. Search of novel antigens suitable for use as diagnostic reagents led to the identification of surface immunoglobulin-like proteins LigA and LigB. Lig proteins are expressed only by virulent low-passage strains and contribute for the attachment and invasion of host cell. In this study, we produced and characterized five monoclonal antibodies (MAbs) against a recombinant fragment of LigB (rLigBrep) with approximately 54 kDa, that comprise a identical portion 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 varied from 7×10^7 M^{-1} to 4×10^8 M^{-1}. The MAbs were able to react with the native antigen in *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 detected by indirect immunofluorescence, immunoblotting, whole-cell ELISA and immunoelectron microscopy. These results allow concluding that these MAbs are important tools for studies aiming understanding the role of Lig proteins in *Leptospira* pathogenesis and in the development of tests for diagnosis of leptospirosis.

Key Words: *Leptospira*, Leptospirosis, Diagnosis, Recombinant Antigen.

INTRODUCTION

Leptospirosis is an infectious disease of global distribution that represents a challenge to public health, mainly in developing countries (Bharti et al., 2003). Outbreaks and epidemics are mostly associated to periods of intense rainfall and flooding (Levett, 2001). Human and animal hosts can be infected by direct or indirect contact with the urine of chronically infected carriers or through contaminated water and soil (Faine et al., 1999). The disease routinely produces symptoms such as fever, vomiting and headache, being many times confused with other febrile illnesses, i.e. influenza, dengue and malaria (McBride et al., 2005). However, in some cases, it may involve to serious complications such as renal and hepatic failures, uveitis, pulmonary damage with severe pulmonary hemorrhage and death (WHO, 2003).

The clinical presentation of leptospirosis is traditionally biphasic and it includes an acute or septicemic phase characterized by the presence of spirochetes in the bloodstream and tissues, that usually lasts for one week, and this is followed by a chronic or immune phase, culminating with the production of serovar-specific circulating antibodies and the dissemination of leptospires to the environment through the urine (Levett, 2001; WHO, 2003).

The microscopic agglutination test (MAT), that confront 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. This technique has been performed in clinical laboratories worldwide for several years, however, presents a high number of cross-reactions among serovars and low sensitivity in the acute phase of the disease (WHO, 2003; McBride et al., 2005).

The *Leptospira* genus is composed of saprophytic and pathogenic spirochetes belonging to 19 species and more than 300 serovars (Adler et al. 2009). The diverse antigens encoded by the genome of *Leptospira* spp. are predicted to influence in survival, invasion and adaptation of leptospiras in host and ambient. For this reason, several surface proteins were already identified and characterized including the outer membrane proteins (OMP) as OmpL1, LipL41, LipL32, LipL21, Loa22, Len family and the Lig proteins (Haake et al., 1999; Haake et al., 2000; Matsunaga et al., 2003; Cullen et al., 2005, Stevenson et al., 2007).

Search for antigens suitable for diagnostic reagents against leptospirosis led to the identification of Lig superfamily proteins that are characterized by the presence of domains repeats, identified each of these 90 residue repeats, an structural organization similar to *Y. pseudotuberculosis* invasin and *E. coli* intimin that play to role important in the colonization of host tissue (Matsunaga et al., 2003). Several adhesins that bind host extracellular matrix proteins have been identified in pathogenic spirochetes, recent studies demonstrated the adherence of LigA and LigB to fibronectin, collagen, laminin and fibrinogen (Choy et al., 2007). In addition, these two proteins were able to induce immunoprotection in a number of studies involving different approaches and animal models (Koizumi; Watanabe, 2004; Palaniappan et al., 2006; Silva et al., 2007; Faisal et al., 2008; Yan et al., 2008; Faisal et al., 2009). Also the usefulness of the Lig proteins as markers for serodiagnosis was previously described where LigA and LigB were strongly recognized by serum antibodies of infected human patients (Croda et al., 2007).

Considering the large number of *Leptospira* serovars able of association with a wide range of host reservoirs for life cycle maintenance in nature improvements in diagnosis are necessary (Bharti et al., 2003). In this context, our study focused on

the production and characterization of monoclonal antibodies (MAbs) against a recombinant fragment of approximately 54 kDa encoded from identical region of LigA and LigB proteins (domains 2-7). The five MAbs obtained against rLigBrep were able to detect the native proteins in *L. interrogans* and are expected to be useful in clinical diagnosis of leptospirosis at the early stage of the disease.

MATERIALS AND METHODS

Bacterial strains and culture

L. interrogans serovar Copenhageni strain Fiocruz L1-130 low passage and *L. biflexa* serovar Patoc strain Patoc I were obtained from Laboratory of Molecular Biology, Center for Biotechnology, Universidade Federal de Pelotas, Brazil. Briefly, according to Silva et al. 2008, bacteria were grown for up to 7 days at 30^{o}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. After, leptospiral cultures were treated with NaCl 120mM overnight to increase expression of Lig proteins (Matsunaga et al., 2005).

Preparation of recombinant LigBrep

The protein LigBrep, corresponding to amino acid 131–649 of the LigB (Matsunaga, 2003) 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 (Amersham Biosciences, USA). Fractions of the

purified rLigBrep were analyzed by 12% SDS-PAGE and quantified by the Bradford method (Bradford, 1976).

Monoclonal antibody generation and characterization

MAbs production and purification

The animal used in this study was treated in agreement with the ethical beginnings in the 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 e 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 (1:64000) 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 in the indirect ELISA and those positive were cloned twice by limiting dilution, expanded and stored in liquid nitrogen. For ascites production the hybridomas cultivated on DMEM with 10% FCS, collected by centrifugation, washed five times in DMEM without FCS and injected into pristane primed BALB/c mice. Ascites were collected and MAbs isotypes were determined with an ELISA isotyping kit (Sigma-Aldrich, USA). MAbs were then purified from ascitic fluid by affinity chromatography on a protein G-Sepharose column (GE Healthcare Company, USA)

according to manufacturer instructions and final concentration was measured by spectrophotometry at 280 nm. Purified MAbs were stored at -20 °C.

Affinity constants

MAbs functional affinity constants (Ka) for rLigBrep were determined by the ELISA method Friguet et al. (1985). Briefly, concentrations of the antigen varying from 10,8 x 10^{-3} M to 0,05 x 10^{-3} M were incubated in solution with constant amounts of each MAb until equilibrium was reached (16 h at 20^{o}C). After incubation, unbound antibodies in the liquid phase were determined by indirect ELISA using microtiter plate wells coated with rLigBrep (1µg.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 Bobrovnik (2003). The linear relationships between values (A0 - Ai)/Ai and Ii (where A0 is the OD of MAb without reacting with antigen, Ai is the OD of MAb after reacting with each antigen concentration and Ii is the antigen concentration) were determined and the values of functional Ka were defined by the slope of these linear relationships.

Whole-cell ELISA

Initially, seven-day cultures of *L. interrogans* L1 130 and of *L. biflexa* Patoc I were harvested by centrifugation (15,000 × g, for 30 min) at 4°C and washed once in PBS. The cells were resuspended in PBS, counted in a Petroff-Hausser chamber and the concentration was adjusted to approximately 10^{9} cells per ml. For the ELISA, microtiter plates were first coated overnight at 4°C with 100 µl of a ten times diluted 0.1% poly L-lysine solution (Sigma-Aldrich, USA), washed three times with PBS and then reacted with the bacterial suspensions. After washing three times with PBS-T

the wells were treated with 100µL of Blotto (PBS containing 5% skim milk). Washing was repeated and two-fold dilutions of MAbs in PBS-T were reacted with leptospires retained in the wells. A MAb against LipL32 and normal mouse serum were added to the plates as positive and negative controls, respectively. Washing procedure was again repeated and goat anti-mouse Ig-peroxidase conjugate (Sigma-Aldrich, USA) was added to the wells. A final five times washing step was performed and the antigen-antibody complex was revealed as above. Unless indicated, assay steps were carried out in 50µL/well additions at 37ºC for 1h. Optical densities equal or higher than 2 times that obtained with the non-pathogenic serovar were considered positive.

Immunoblotting analysis

Aliquots from seven-day cultures (ca. 10^9 cells/mL) of *Leptospira* strains L1 130 and Patoc I were boiled in sample buffer and separated by SDS-PAGE using a 10% polyacrilamide gel and a discontinuous buffer system (Laemmli et al. 1970). After electrophoresis, proteins were transferred onto nitrocellulose membranes for testing specific LigA and LigB detection by MAbs. Membranes were blocked with Blotto, washed with PBS-T and and reacted with the MAbs for 2 hours. A rabbit polyclonal serum against rLigBrep and normal mouse serum were used as positive and negative control, respectively. Additionally, a MAb against LipL32 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 placed in a chromogen/substrate solution (0.6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl pH 8.0, and hydrogen peroxide 30 vol) for band visualization.

Indirect immunofluorescence

Slide chambers (ICN Biomedicals Inc, CA, USA) were coated with a 0.01% poly L-lysine solution for 1 h at 30oC. Seven-day cultures of *Leptospira* strains L1 130 and Patoc I were washed once in PBS and resuspended to a density of approximately 10^8 cells/mL. Aliquots of 10 µL were pippeted onto the slide chambers and incubated at 30°C until dry. The slides were then blocked with 10% FBS in PBS, washed twice with leptospiral culture medium (LCM) and coated for 1 h at 30^{o}C with MAbs diluted in LCM. The slides were washed again twice with LCM 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 LCM a drop of mounting medium was added and a cover slip was sealed with acrylic. 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.

Mapping epitope region

Polystyrene ELISA microtiter plates (Microtiter plate Cral) were sensitized with 50µL of five recombinant fragments from LigA and LigB proteins (10µg.mL^{-1}) encompassing different *ligA* and *ligB* gene domains, a gentle gift from Dr. Marco Medeiros, FIOCRUZ, Rio de Janeiro (Table 1 and Figure 1). The plates were then washed three times with phosphate-buffered saline plus Tween 20 (PBS-T) and the panel of MAbs 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 50µL/well additions at 37ºC for 1h. Wells were finally washed five times with

PBS-Tween and the presence of the antigen-antibody complex was revealed by adding 50µL a substrate solution containing OPD (0.4 mg/mL 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).

Table 1

Molecular characterization of the recombinant proteins.

Fragments	Aminoacids	Lenght (aa)	Domain‡
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

‡ Based on SMART and PFAM analysis

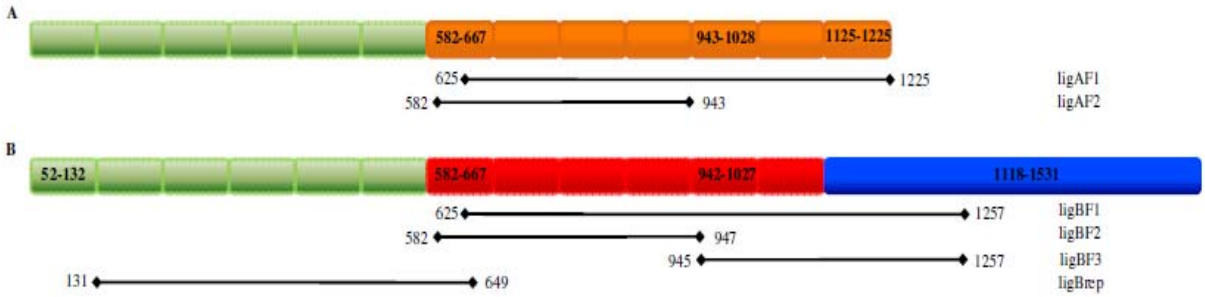

Fig. 1. Diagram of the immunoglobulin-like (Lig) proteins expression as truncated fragments. (A) Representation of the identical (green) and non-identical (orange) regions of LigA. (B) Representation of the identical (green), non-identical (red) and carboxyterminal regions of LigB. Truncated peptides are represented as solid lines and the first and last aminoacids positions expressed are shown at the ends of each

line and in table 1. Aminoacids positions of the Big2 domains are indicated into respective boxes.

Immunoelectron microscopy (IEM)

Cells from seven-day cultures of *Leptospira* strains L1 130 and Patoc I (ca. 10^8 cells/mL) were incubated under mild agitation during 12h at 4°C with ascites from Mabs R2 and R5 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% sodium cacodylate 0,1M at 4°C for 1h. The bacteria were washed three times for 15min in an aqueous solution containing sodium cacodylate 0,1 M and sucrose 0,2M, a new fixation step in osmium 2% and sodium cacodylate 0,4 M for 2h was performed. After a new washing step with tetradistilled H_2O for 15 minutes, the bacterial cells were embedded in resin (Epoxy) for 1h 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.

RESULTS

Expression and purification of rLigBrep

E. coli BL21 (DE3) pLysS transformed with 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 per liter of medium. A band of high intensity of the size expected (50 kDa) was observed when the fractions collected in the purification process were submitted to SDS- PAGE (Figure 2).

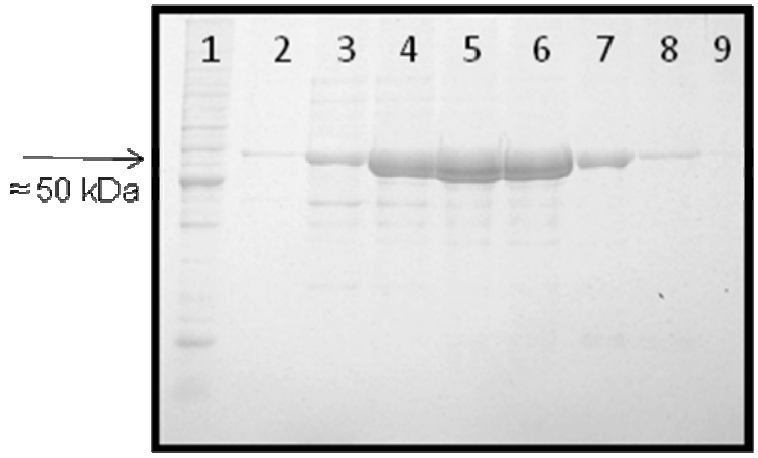

Fig. 2. SDS-PAGE of rLiBrep fractions collected in the purification process. Line 1, Protein Ladder (Invitrogen, USA); lines 2-9, rLigBrep fractions.

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 for cryopreservation and ascites production. Several hybridomas initially identified as producing antibodies against rLigBrep either died or stopped antibody secretion during the cloning steps.

MAbs characterization

Isotypes and affinity constants

Results of MAbs isotyping and affinity constants (*Ka*) determination are shown in Table 2. Two of the MAbs selected were of the IgG1 isotype and the other three were of the IgG2b isotype. After purification, the concentration of MAbs in the final preparations ranged from 0,5 mg/mL to 0,7 mg/mL. The MAbs showed intermediate *Ka*, varying from 7, 0 x 10^7 M^{-1} for R5 to 4, 0 x 10^8 M^{-1} for R1.

Table 2

Isotypes and affinity constants of MAbs against recombinant LigBrep.

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

Whole-cell ELISA

The five MAbs were tested in a whole-cell ELISA to evaluate reactivity with the identical region of the native forms of adhesins LigA and LigB. A pathogenic and a non-pathogenic serovar and different dilutions of MAbs were used in this experiment. Positive reactions were observed with the pathogenic serovar only (Figures 3 and 4).

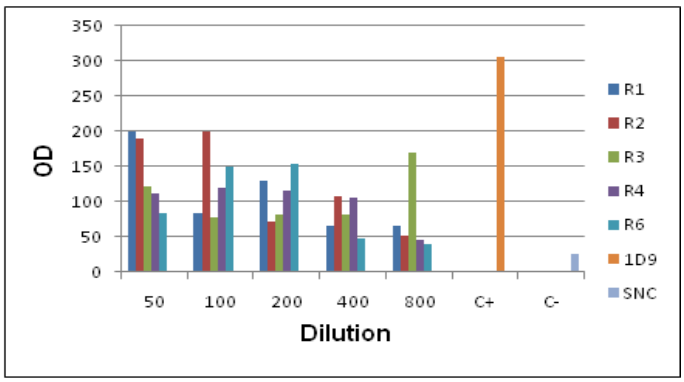

Fig. 3. Reactivies of MAbs anti-rLigBrep in a whole-cell ELISA using *L .interrogans* strain L1-130. A MAb anti-LipL32 and normal mouse serum were used as positive and negative controls, respectively.

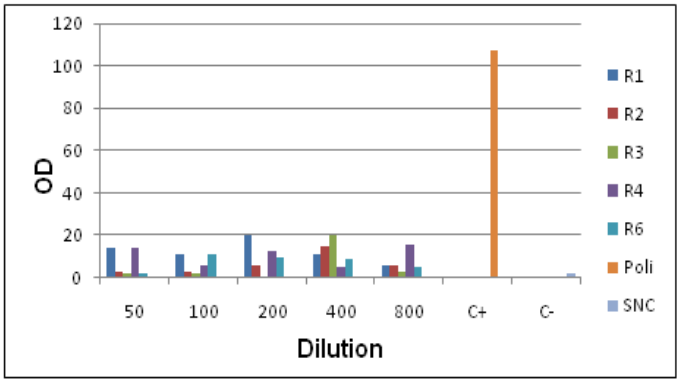

Fig. 4. Reactivies of MAbs anti-rLigBrep in a whole-cell ELISA using *L. biflexa* strain Patoc I. A policlonal mouse serum against *L. interrogans* and normal mouse serum were used as positive and negative controls, respectively.

Immunoblotting

In the figure 5 it is demonstrated the reactions of MAbs anti-LigBrep with LigBrep protein in immunoblotting assay. The accomplished analysis with leptospiras species revealed that the same MAbs identified two bands with sizes compatible with LigA (128 kDa) and LigB (201 kDa) in SDS denatured proteins from the pathogenic *Leptospira* only (Figures 6 and 7). This result also indicated that the MAbs recognized linear epitopes on both proteins.

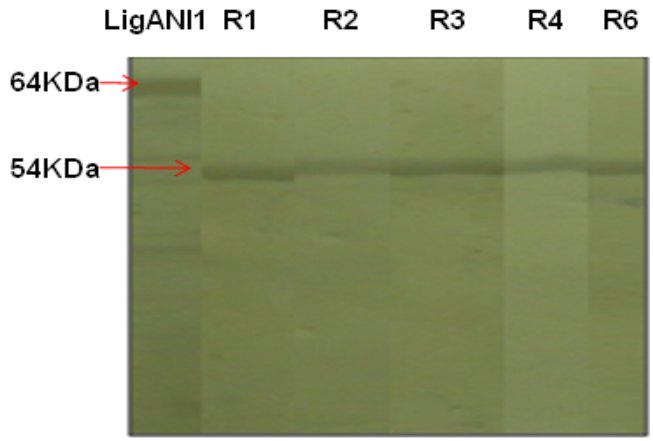

Fig 5. Immunoblotting analysis of the MAbs R1, R2, R3, R4 and R5 with rLigBrep. The reaction of MAb LigAni1 against the protein recombinante LigAni 64kDa was used as marker of molecular weight.

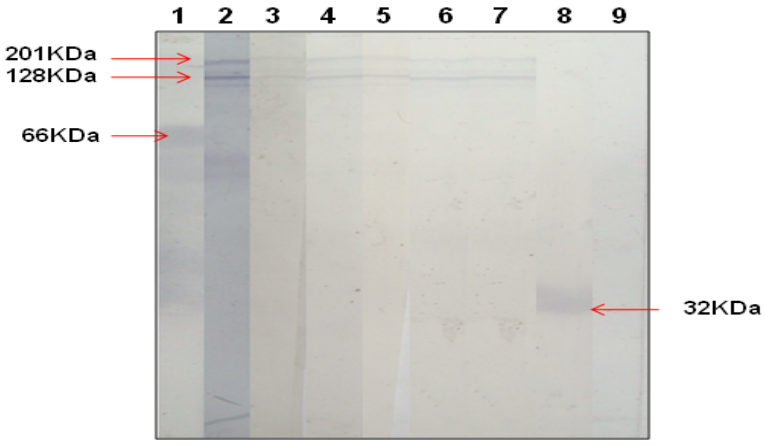

Fig. 6. Immunoblotting analysis of the MAbs anti-rLigBrep binding to denatured proteins from *L. interrogans* L1-130. Lines 3-7: MAbs R1, R2, R3, R4 and R5, respectively. Lines 2, 8: rabbit policlonal antibody anti-LigBrep and MAb 1D9 (anti-LipL32) (positive controls). Line 9: NMS (negative control). Arrows indicate bands corresponding to LigB (201kDa), LigA (128 kDa), LipL32 (32 kDa) and LigBNI (66kDa).

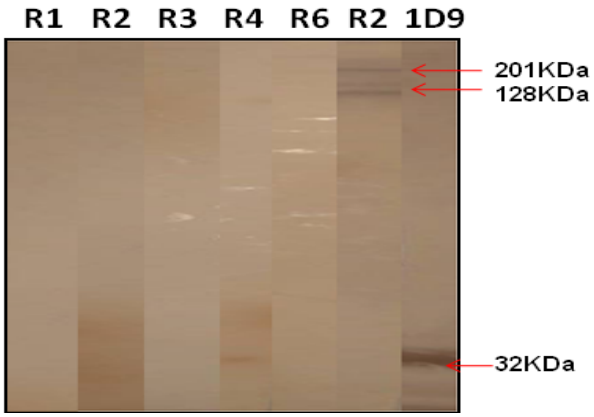

Fig. 7. Immunoblotting analysis of the MAbs R1, R2, R3, R4 and R5 reaction with *L.biflexa* strain Patoc I. Transference controls: MAb R2 and 1D9 (α-LipL32) reaction with *L interrogans* strain Fiocruz L1-130.

Mapping epitope region

Besides testing with rLigBrep, we tested the MAbs by indirect ELISA for binding to two recombinant fragments from LigA and three from LigB. Excepting fragment 3 from LigB, the other fragments have overlap regions with rLigBrep (Figure 8). 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 (Figura 3). Also, weak reactions with fragment LigBF3 were observed.

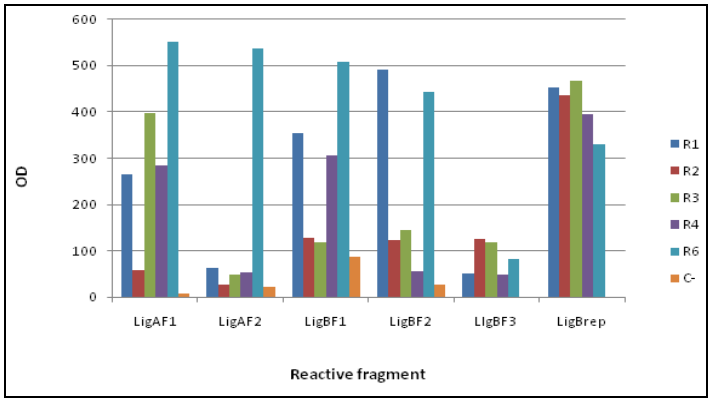

Fig. 8. Reactivities of MAbs anti-LigBrep with synthetic fragments from LigA and LigB in an indirect ELISA.

The fragments reacting and probable reaction region are shown in Table 3. Most of the MAbs reacted in the region spanning amino acids residues 625 to 649, except for MAb R2, which reacted with an epitope located between residues 131 and 582.

Table 3

Epitope region mapping of MAbs anti-rLigBrep as determined by indirect ELISA with recombinant fragments from LigA and LigB.

MAb	Reactive fragments	Region recognized*
R1	LigAF1, LigBF1, LigBF2, LigBrep	625 to 649
R2	LigBrep	131 to 582
R3	LigAF1, LigBrep	625 to 649
R4	LigAF1, LigBF2, LigBrep	625 to 649
R5	LigAF1, LigAF2, LigBF1, LigBF2, LigBrep	625 to 649

*Numbers refer to region position in the amino acid sequence of LigA and LigB

Indirect immunofluorescence

Reaction of MAbs with native LigA and LigB exposed on the surface of leptospiral cells was also investigated by indirect immunofluorescence. The five MAbs stained cells from pathogenic *Leptospira* only (Annex). The assay performed with MAb R2 illustrate these results (Figure 9).

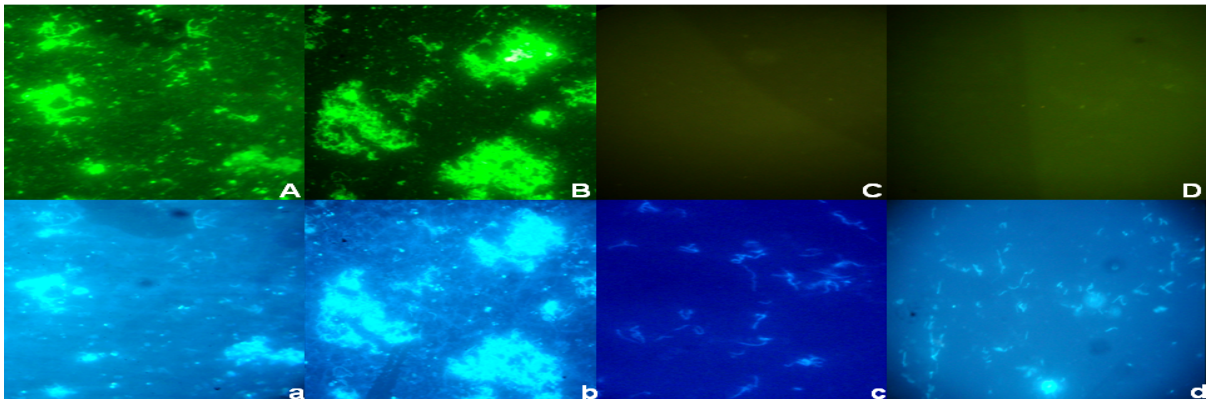

Fig. 9. Surface staining of pathogenic *Leptospira* with Mab R2 anti-rLigBrep. Panel A: *L interrogans* L1 130 and anti-LipL32 (positive control). Panels B: *L interrogans* and MAb R2. Panel C: *L interrogans* and NMS (negative control). Panel D: *L biflexa* Patoc I and MAb R2. Panels a, b, c, and d: DNA stained with Hoechst 33258 to document the presence of leptospires. Visualization was performed with a 100x objective on an Olympus BX 51 fluorescence microscope.

Immunoelectron microscopy (IEM)

IEM experiment using MAbs R2 and R5 anti-rLigBrep demonstrated their capacity to react with the native Ligs on the external surface of the outer membrane of leptospires. Results obtained with MAb R2 are shown to Illustrate specific surface labeling of pathogenic leptospires (Figure 10).

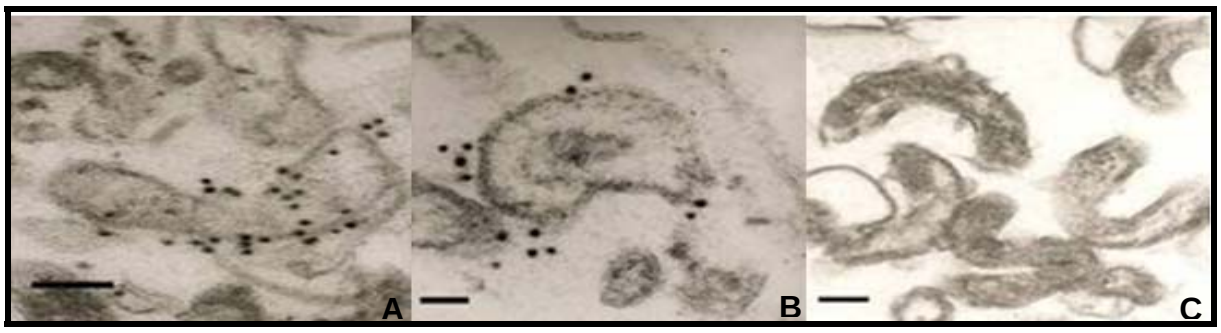

Fig. 10. Immunoelectron microscopy of thin-section samples of *L. interrogans* strain L1 130 (panels A and B) and *L. biflexa* strain Patoc I (panel C). Samples were incubated with MAb R2 anti-rLigBrep followed by anti-mouse secondary antibody

conjugated to 10 nm gold particles. Visualization was performed with 85000x (panels A and C) and 140000x (panel B) magnification on a ZEISS EM 900 transmission electron microscope. Bars represent 0.09um

DISCUSSION

The adhesion mechanism is an essential first step in leptospiral pathogenesis since during that process the interaction between pathogen and host cells is established to facilitate tissue invasion (Choy et al., 2007). Thus, it would be of great help for the development of diagnostic tests the identification of surface-exposed proteins from pathogenic *L. interrogans* that provide cross-protection against different serovars, as well as antibodies against them.

Antibody reagents have provided the basis for a large number of highly specific and reproducible immunoassays for the rapid diagnosis and treatment of infectious diseases (Berry, 2005). Coutinho et al 2007, produced monoclonal antibodies against LipL32, the most abundant protein in the outer membrane of pathogenic leptospiras. The MAbs anti-LipL32, all of the isotype IgG2b, were capable to recognize the native protein only in pathogenic serovars when tested through ELISA and immunoblotting. As well as LigA and LigB proteins, LipL32 interacts with collagen and also with fibronectin, these interactions contribute for attachment to host cells (Hauk et al. 2008, Choy et al. 2007). Recently, a MAb to LipL32, protected hamsters against heterologous challenge (Maneewatch et al., 2008). Furthermore, this MAb was produced as a humanized single-chain antibody and also protected hamsters from a heterologous *Leptospira* lethal challenge (Maneewatch et al., 2009).

In this study we produced five stable hybridomas secreting MAbs against the leptospiral immunoglobulin-like proteins identical region that reacted to pathogenic *L.*

interrogans serovar Copenhageni strain Fiocruz L1-130 but not to non-pathogenic *L. biflexa* serovar Patoc strain Patoc I. The MAbs isotypes IgG2b and IgG1 demonstrated that a mixed response of cells TH_1 and TH_2 was respectively produced. The profile of cells TH_1 and TH_2 elicited by leptospires is of interest for the success of vaccine development (Srikram et al., 2008). The whole-cell ELISA, immunofluorescence and immunobloting assays demonstrated the reaction of MAbs either with the epitopes exposed in the intact protein on the bacterial cell surface or with the denatured protein. The mapping of epitope regions using cloned fragments from LigA and LigB identified the domain 7 from both proteins as an immunodominant region recognized by MAbs anti-rLigBrep. In addition, the LigBF3 fragment that does not contain domain 7 presented weak reactions with all MAbs confirming their specificity for this region.

After confirming by indirect immunofluorescence the ability of MAbs against rLigBrep to recognize LigA and LigB adhesins in the surface of pathogenic strain, an immunoelectron microscopy (IEM) study was accomplished to investigate the distribution of Lig proteins in the outer membrane of *Leptospira*. The IEM assay was executed with MAbs R2 and R5 revealed an expressive amount of Ligs on the surface of pathogenic *Leptospira* strain only.

Since the recent identification of immunoglobulin-like proteins A and B in pathogenic *Leptospira* species, this was the first report on the production and characterization of monoclonal antibodies against their identical region. The results obtained here suggest that the MAbs anti-LigBrep represent important tools of diagnostic for confirm the phase initial of the leptospirosis.

ACKNOWLEDGEMENTS

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ANNEX

Indirect Immunofluorescence of the MAbs anti-LigBrep and *L. interrogans* serovar Copenhageni Strain L 1-130.

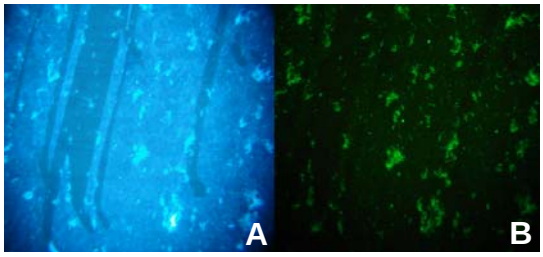

Figura 1 Surface staining of pathogenic *Leptospira* with Mab R1 anti-rLigBrep. Panel A: DNA stained with Hoechst 33258 to document the presence of leptospires. Panels B: *L interrogans* and MAb R2. Visualization was performed with a 100x objective on an Olympus BX 51 fluorescence microscope.

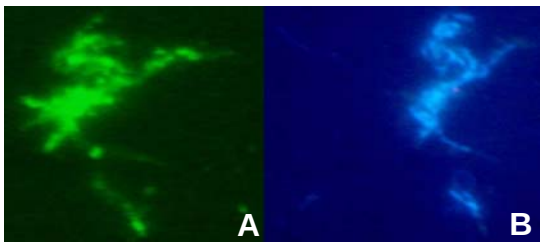

Figura 2 Surface staining of pathogenic *Leptospira* with Mab R3 anti-rLigBrep. Panel A: DNA stained with Hoechst 33258 to document the presence of leptospires. Panels B: *L interrogans* and MAb R2. Visualization was performed with a 100x objective on an Olympus BX 51 fluorescence microscope.

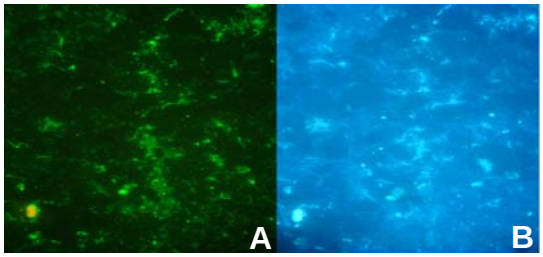

Figura 3 Surface staining of pathogenic *Leptospira* with Mab R4 anti-rLigBrep. Panel A: DNA stained with Hoechst 33258 to document the presence of leptospires. Panels B: *L interrogans* and MAb R2. Visualization was performed with a 100x objective on an Olympus BX 51 fluorescence microscope.

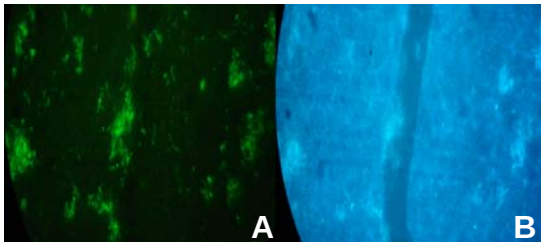

Figura 4 Surface staining of pathogenic *Leptospira* with Mab R5 anti-rLigBrep. Panel A: DNA stained with Hoechst 33258 to document the presence of leptospires. Panels B: *L interrogans* and MAb R2. Visualization was performed with a 100x objective on an Olympus BX 51 fluorescence microscope.

3. CONCLUSÕES

- Os cinco hibridomas obtidos são estáveis e secretam anticorpos monoclonais contra a região idêntica das proteínas LigA e LigB da cepa Fiocruz L1-130, a qual pertence à espécie considerada como a maior causadora de leptospirose urbana no Brasil e na América Latina;

- As reações dos MAbs com os fragmentos de LigA e LigB possibilitam a identificação de um epítopo formado no intervalo compreendido entre os aminoácidos 625 e 649 localizados no domínio 7;

- Os MAbs anti-LigBrep são capazes de reconhecer a proteína nativa presente na cepa Fiocruz L1-130, quando testados através de ELISA indireto, immunoblotting, imunofluorescência e microscopia imunoeletrônica;

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