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Tese

Desenvolvimento de Testes Imunoquímicos e Moleculares para o Diagnóstico da Leptospirose

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RESUMO

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A Leptospirose é uma zoonose de ocorrência mundial causada por bactérias do gênero *Leptospira*. As manifestações clínicas da leptospirose são similares a outras doenças febris e este fato frequentemente atrasa o diagnóstico e o início do tratamento. Portanto, o diagnóstico precoce e acurado da doença é um prerequisito para o tratamento adequado. Sorovares patogêncios de *Leptospira* possuem uma grande variação antigência e esta diversidade é atribuída principalmente ao lipopolissacarídeo presente na membrana externa. Contrastando com esta característica, antígenos conservados de sorovares patogênicos são principalmente representados por proteínas de membrana externa. Recentemente foi comprovada a exposição da proteína LipL32 na superfície da membrana externa de leptospiras patogênicas. Neste estudo, LipL32 em sua forma recombinante (rLipL32) foi utilizada para imunizar camundongos BALB/c e produzir anticorpos monoclonais (mAbs). Três mAbs contra rLipL32 foram produzidos e caracterizados quanto ao seu potencial para uso em testes diagnósticos usando diferentes metodologias. Os mAbs foram conjugados à peroxidase e avaliados quanto a reação com proteina nativa em células de leptospiras íntegras e rompidas, conjugados com isotiocianato de fluoresceína (FITC) para uso em imunofluorescência para marcar células de leptospira intactas e tratadas com metanol, e usados para imunoprecipitar células de leptospira. Os anticorpos monoclonais anti-LipL32, utilizados em ELISA tanto conjugados com peroxidase ou como anticorpo primário, ligaram-se às células de leptospiras intactas ou rompidas pelo calor, provando que podem ser usados em testes imunoenzimáticos para detecção da proteína nativa. Na imunofluorescência, os mAbs foram capazes de marcar células da bactéria tanto intactas como fixadas com metanol. Dois mAbs foram capazes de imunoprecipitar proteina nativa de leptospiras vivas e móveis, e quando adsorvidos em partículas magnéticas foram capazes de capturar bactérias para amplificação por PCR. Na seqüência deste estudo, o mAb 1D9 foi utilizado em estudos de padronização da metodologia de

imunoseparação magnética associada a PCR (IMS/PCR) para diagnóstico de leptospirose. O anticorpo 1D9 foi adsorvido em partículas magnéticas e utilizado para capturar leptospiras em soro e urina humanas artificialmente contaminadas com leptospiras para posterior amplificação. Para assegurar a acurácia da PCR foi construído um controle interno de amplificação (IAC) específico para a metodologia desenvolvida utilizando como alvo sequências de primers já padronizados para exclusiva amplificação de leptospiras patogências e uma sequencia de DNA não relacionada. A metodologia de IMS/PCR – IAC permitiu usar somente um par de primers na reação de PCR e mostrou ser promissora para diagnóstico de leptospirose, pois foi capaz de detectar 10^2 células por mL em amostras de soro e urina artificialmente contaminadas, correspondendo a amplificação a partir de aproximadamente 25 cópias do genoma. Os resultados obtidos evidenciam uma nova perspectiva no diagnóstico da leptospirose através da utilização da proteína LipL32 em métodos imunoquímicos e moleculares ou pela associação destas metodologias. A metodologia de imunoseparação com o mAb anti-LipL32 pode ser utilizada previamente a amplificação de outros alvos do genoma bacteriano por PCR, já que ela possibilita a separação e concentração exclusiva de *Leptospira* patogênica.

Palavras chave: Leptospirose, diagnóstico laboratorial, anticorpos monoclonais, PCR, LipL32.

ABSTRACT

FERNANDES, Cláudia Pinho Hartleben. **Development of Immunochemical and Molecular Assays for the Diagnosis of Leptospirosis.** 2008. 84f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia Agrícola. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a zoonotic disease that occurs all over the world and is caused by pathogenic bacteria of the genus *Leptospira*. Clinical manifestations of leptospirosis are similar to other febrile illnesses and this fact frequently retards beginning of antibiotic therapy. Thus, early and accurate diagnosis is a prerequisite for proper treatment of leptospirosis. Pathogenic serovars of *Leptospira* have a wide antigenic diversity attributed mainly to the lipopolysacharide present in the outer membrane. In contrast, antigens conserved among pathogenic serovars are mainly represented by outer membrane proteins. Surface exposure of a major and highly conserved outer membrane lipoprotein (LipL32) was recently demonstrated on pathogenic *Leptospira*. LipL32 on its recombinant form (rLipL32) was used to immunize BALB/c mice to develop murine monoclonal antibodies (mAbs). Three mAbs against rLipL32 were produced, isotyped and evaluated for further use in diagnostic tests of leptospirosis using different approaches. The mAbs were conjugated to peroxidase and evaluated in a native protein ELISA with intact and heat-treated leptospiral cells, conjugated to fluorescein isothiocyanate (FITC) for direct immunofluorescence with intact and methanol fixed cells and were used for LipL32 immunoprecipitation from leptospiral cells. rLipL32 mAbs conjugated to peroxidase or used as primary antibody bounded to intact and heat-treated cells in ELISA, proving that they could be used in enzyme immunoassays for detection of the native protein. On immunofluorescence assay, mAbs labeled bacterial cells either intact or methanol fixed. Two mAbs were able to immunoprecipitate the native protein from live and motile leptospiral cells and, adsorbed onto magnetic beads, captured intact bacteria from artificially contaminated human sera for detection by PCR amplification. One mAb was utilized for the development of an immunoseparation assay coupled to PCR test (IMS/PCR) for diagnosis of leptospirosis. The antibody adsorved onto magnetic beads captured leptospires from urine and human sera artificially contaminated for further amplification of the *lipL*32 gene by PCR. To ensure PCR accuracy, an internal

amplification control (IAC) was constructed using as amplification targets sequences of standardized primers specific for pathogenic *Leptospira* and for a not-related DNA sequence. The IMS/PCR – IAC method developed was able to detect 10^2 cells per mL of sera or urine, corresponding to approximately 25 genomic copies per reaction. These results suggest that the association of LipL32-based immunochemical and molecular techniques could yield a novel method for the diagnosis of leptospirosis. Moreover, immunomagnetic separation with mAbs against LipL32 can be used previous to amplification of other targets in the *Leptospira* genome by PCR.

Key words: Leptospirosis, laboratory diagnosis, monoclonal antibodies, PCR, LipL32.

SUMÁRIO

1

1 INTRODUÇÃO GERAL

A leptospirose é uma doença infecciosa de distribuição mundial causada por bactérias do gênero *Leptospira* (FAINE, 1999). O gênero *Leptospira* possui vários sorovares que são reunidos em sorogrupos antigenicamente relacionados. Esta variedade antigênica deve-se a mudanças na composição do lipopolissacarídeo (LPS) presente na membrana externa da bactéria (ZUERNER et al., 2000). Atualmente estão descritos mais de 260 sorovares, distribuídos em 29 sorogrupos (LEVETT, 2001). O número de sorovares e sorogrupos descritos aumenta com os esforços despendidos no isolamento e caracterização da bactéria.

Antes de 1989 o gênero *Leptospira* era dividido, usando-se critérios antigênicos, em duas espécies: *L. interrogans*, da qual faziam parte todas as cepas patogênicas, e *L. biflexa*, contendo cepas saprófitas isoladas do ambiente (LEVETT, 2001). Porém, a análise molecular, que se baseia em características genéticas, tem sido aplicada na classificação de *L. interrogans lato sensu* (FAINE, 1999) e tem conduzido a várias espécies dentro deste gênero: *L. interrogans stricto sensu*, *L. santorosai*, *L. weiliii*, *L. inadai*, *L. wolbachii*, *L. borgpetersenii*, *L. kirschnerii*, *L. meyeri* e *L. noguchii* (YASUDA, 1987; RAMADASS et al., 1992). A essa classificação foram adicionadas 5 genomoespecies (BRENNER et al., 1999). Recentemente, uma nova espécie denominada *L. broomii,* isolada a partir de casos humanos de leptospirose, foi relatada (LEVETT et al., 2006).

A leptospirose é uma antropozoonose, sendo transmitida principalmente através da urina de animais infectados. Os roedores, dentre os animais sinantrópicos, são os principais reservatórios da doença disseminando leptospiras viáveis no ambiente através da urina que contamina o solo, a água e alimentos destinados ao consumo humano e animal (LEVETT, 2001).

A leptospirose humana tem sido associada ao contato direto ou indireto com a urina de animais infectados, sendo as profissões de médico veterinário, magarefes, aguadores de arroz e funcionários da limpeza urbana fatores de risco associados à doença (LEVETT, 2001; NATARAJASEENIVASAN et al., 2002). Surtos epidêmicos em populações urbanas estão associados à veiculação hídrica da bactéria, com ocorrência de casos relacionados ao nível de precipitação e ausência de saneamento básico (VIJAYACHARI et al., 2008). Na América do Sul e Central esses períodos têm sido intensificados pelo fenômeno climatológico “El Ninõ” (KO et al.,

1999; PLANK; DEAN, 2000; LEVETT, 2001; MCBRIDE et al., 2005) que é caracterizado por um significativo aumento nos níveis pluviométricos. A leptospirose é considerada uma doença re-emergente em países desenvolvidos e possui distribuição endêmica com surtos epidêmicos em países em desenvolvimento, ocorrendo em regiões de clima tropical, subtropical e temperado (BHARADWAJ, 2004; MCBRIDE et al., 2005; VIJAYACHARI et al., 2008).

No Brasil a leptospirose é uma zoonose importante, apresentando alta prevalência em vários estados. No período de 2004 a 2006 foram notificados 39.494 casos de leptospirose no país, sendo que destes, 10.341 foram confirmados e identificada uma taxa de letalidade de 11% (SINAN, 2007).

A penetração das leptospiras no hospedeiro pode ocorrer através da pele íntegra, sendo favorecida por portas de entrada como escoriações ou abrasões. As leptospiras quando atingem a corrente sanguínea se multiplicam por um período de até sete dias, com ocorrência de febre e dores musculares. Anticorpos circulantes são detectáveis no período chamado imune ocorrendo no período de colonização renal (LEVETT, 2001; BAL, 2005).

A sintomatologia da doença é ampla, podendo ocorrer na forma leve, benigna e dificilmente distinguível de outros quadros febris, até as formas graves caracterizando a síndrome de Weil e a síndrome pulmonar aguda. Os sintomas relatados associados à leptospirose incluem a cefaléia, febre, vômitos, dores no abdômen, artralgias e mialgias. A apresentação inicial benigna pode evoluir para quadros graves associados à falência renal e hepática com taxa de mortalidade descrita de 5 a 15% e hemorragia pulmonar com mortalidade de até 40% (SEHGAL; MURHEKAR; SUGUNAN, 1995; MURHEKAR et al., 1998; BHARTI et al., 2003).

O diagnóstico laboratorial da leptospirose está baseado principalmente na detecção de anticorpos circulantes no sangue aproximadamente de 5 a 7 dias após o início dos sintomas. A técnica padrão de diagnóstico, chamada soroaglutinação microscópica (MAT), pode ser realizada com antígenos vivos ou formolizados, e tem sido utilizada tanto em soros animais como de humanos. O diagnóstico através da MAT prevê o pareamento de amostras para identificação de soroconversão ou segundo critérios regionais de ocorrência da doença (FAINE, 1999; LEVETT, 2001; WHO, 2003). No Brasil, o pareamento de amostras de soro pode ser excluído quando um título de 800 é identificado na primeira amostra (MINISTÉRIO DA SAÚDE, 1995). A MAT, porém, apresenta baixa sensibilidade na fase inicial da

doença (FERNANDES, 2001; VIJAYACHARI; SUGUNAN; SEHGAL, 2001), além de ser uma técnica de alto custo e laboriosa, pois necessita de coleções de bactérias em constante cultivo em meio líquido, e requerer profissionais treinados para realização da leitura comparativa com sorovares controles. Além disto, em muitos pacientes não é identificada a soroconversão, inclusive nos quadros que resultam em óbito (RIBEIRO; BRANDAO; ROMERO, 1996; CUMBERLAND; EVERARD; LEVETT, 1999; SLACK et al., 2006; WUTHIEKANUN et al., 2007).

Os testes diagnósticos para leptospirose estão descritos como gênero específicos ou sorogrupo específicos, dependendo do antígeno utilizado para reação com soros de pacientes com quadros clínicos compatíveis com a doença (SAMBSIAVA et al., 2003). Como alternativa diagnóstica durante a fase aguda, caracterizada pela bacteremia, as leptospiras podem ser visualizadas no sangue através de exame direto, cultivadas *in vitro* ou inoculadas em animais de laboratório (WHO, 2003). Contudo, o exame direto onde é necessário a visualização em microscópio de campo escuro ou a coloração das bactérias, somente permite a identificação de bacteremia por espiroquetas. Sendo assim, não é considerado um teste definitivo, devendo ser confirmado por outros testes (FAINE, 1999). A partir da segunda semana após o inicio dos sintomas, as leptospiras podem ser visualizadas e cultivadas a partir de amostras de urina. A técnica de isolamento, embora possível de ser realizada nesta fase, é normalmente utilizada como diagnóstico retrospectivo, pois o cultivo somente pode ser considerado positivo após semanas (MERIEN; BARANTON; PEROLAT, 1995; FAINE, 1999). O exame direto em líquor é raramente utilizado (LEVETT, 2001).

Nos últimos anos tem havido um grande esforço para desenvolver testes diagnósticos mais sensíveis, seja através da detecção de anticorpos ou identificação de antígenos (LEVETT, 2001; MCBRIDE et al., 2005). Para obter uma melhora no diagnóstico laboratorial, novos testes sorológicos nos formatos ELISA, Dipstick, Immunoblot e Dot-ELISA foram descritos, mas, embora sensíveis, continuam sorovar-específicos (PETCHCLAI; HIRANRAS; POTHA, 1991; RIBEIRO; SOUZA; ALMEIDA, 1995; DA SILVA et al., 1997; GUSSENHOVEN et al., 1997; YAN et al., 1999; LEVETT; BRANCH, 2002). Testes moleculares como o “dot-blotting” (TERPSTRA; SCHOONE; TER, 1986) ou hibridização *in situ* (TERPSTRA et al., 1987) foram descritos. Porém, a técnica molecular mais amplamente avaliada para diagnóstico da leptospirose é a da reação em cadeia da enzima polimerase (PCR).

A técnica de PCR vem sendo avaliada para o diagnóstico da leptospirose humana ou animal, seja na fase em que dificilmente são detectáveis anticorpos ou ainda detectando portadores da bactéria (VINETZ et al., 1996; CHU et al., 1998; HEINEMANN et al., 2000;). Para este propósito foram descritos como alvos da amplificação genes codificadores de rRNA 16S ou 23S (MERIEN et al., 1992; ZHANG; LI; DAI, 1993; WAGENAAR; SEGERS; VAN DER ZEIJST, 1994) ou elementos repetitivos no genoma (WOODWARD et al., 1991; ZUERNER; ALT; BOLIN, 1995). Contudo, a amplificação de cepas não patogências e baixa sensibilidade foram observadas nestes estudos.

Portanto, faz-se necessário o desenvolvimento de métodos diagnósticos rápidos, sensíveis e específicos, que estejam baseados na detecção de todas as leptospiras patogênicas ou na identificação de anticorpos gerados contra antígenos presentes e conservados entre todas estas espécies.

A proteomica é considerada como importante ferramenta para identificação de alvos para uso em diagnóstico e vacinas (THONGBOONKERD, 2008). Nesta perspectiva, as proteínas presentes exclusivamente em cepas patogênicas (HAAKE et al., 1993; SHANG; SUMMERS; HAAKE, 1996; HAAKE et al., 1998; HAAKE et al., 1999; HAAKE et al., 2000; HAAKE; MATSUNAGA, 2002; CULLEN et al., 2003) são importantes alvos para desenvolvimento de testes diagnósticos, seja utilizando métodos imunoquimicos ou moleculares. Entre estas proteínas, as localizadas na membrana externa (OMPs) de leptospiras patogênicas constituem um alvo promissor para melhorar o diagnóstico laboratorial da leptospirose. Uma família de proteínas chamadas de immunoglobulin-like (Ligs) foi descrita recentemente (PALANIAPPAN et al., 2002) e seu potencial diagnóstico deve-se as características de localização na membrana externa, presença exclusiva em sorovares de leptospiras patogênicas e de serem expressas somente durante a infecção do hospedeiro (MATSUNAGA et al., 2003). Estas características têm sido avaliadas para o desenvolvimento de métodos moleculares ou imunológicos de diagnóstico na fase inicial da doença (PALANIAPPAN et al., 2004; PALANIAPPAN et al., 2005; CRODA et al., 2007).

Porém, entre as OMPs de leptospira já descritas, a proteína de 32 kDa, LipL32, destaca-se por ser a proteína da membrana externa mais proeminente (CULLEN et al., 2005), sendo reconhecida por anticorpos em mais de 95% de pacientes com leptospirose durante a infecção (GUERREIRO et al., 2001), o que

justificou a sua avaliação em diagnóstico através da detecção de anticorpos em testes ELISA (FLANNERY et al., 2001; DEY et al., 2004; BOMFIM; KO; KOURY, 2005; TAHILIANI et al., 2005; DEY et al., 2007b;) ou de aglutinação, utilizando partículas de látex (DEY et al., 2007a) . Ainda, o gene *lipL32* quando usado como alvo para amplificação por PCR em soros e tecidos de animais (CHEEMAA et al., 2007) e em Nested-PCR (JOUGLARD et al., 2006; BOMFIM; BARBOSA-STANCIOLI; KOURY, 2007) em amostras de urina, resultou em ensaios sensíveis e específicos, com detecção de leptospiras patogênicas e não amplificação em cepas saprófitas.

A utilização desta proteína em métodos moleculares e imunológicos justifica-se por ser altamente conservada entre espécies de leptospiras patogênicas e estar ausente em leptospiras não patogênicas, por sua capacidade imunogênica e antigênica em mamíferos, proeminência e exposição na membrana externa, o que permite o reconhecimento por anticorpos do hospedeiro infectado e também por anticorpos monoclonais e policlonais obtidos com o intuito de servirem como insumos diagnósticos.

A hipótese deste trabalho foi que o antígeno LipL32, em sua forma recombinante, pode ser utilizado para gerar anticorpos capazes de reconhecer a proteína nativa exposta na superfície da membrana de leptospiras patogênicas. Sendo assim, os objetivos deste trabalho foram produzir anticorpos monoclonais contra rLipL32; caracterizar os anticorpos obtidos através de imunofluorescência indireta, Western blotting, ELISA e imunoprecipitação de superfície; avaliar a reatividade dos anticorpos quando conjugados a enzimas e fluoróforos ou partículas inertes; desenvolver uma metodologia sensível e específica para diagnóstico através da técnica de PCR utilizando estes anticorpos para separação e concentração do leptospiras em amostras clínicas.

A tese está apresentada na forma de artigos científicos, o que a nosso ver propicia uma divulgação objetiva e rápida dos resultados obtidos. O artigo 1 trata da produção e caracterização dos anticorpos monoclonais produzidos, quanto a sua capacidade de reconhecimento da proteína nativa e aplicabilidade em testes diagnósticos. Esse trabalho foi publicado no periódico **Hybridoma** no início do ano de 2007.

O seguimento do trabalho descreve a utilização de um dos anticorpos produzidos e caracterizados em uma metodologia ainda pouco explorada para

diagnóstico de leptospirose, e que consiste da separação imunomagnética de leptospiras a partir de fluídos biológicos e sua detecção através da amplificação do gene *lipL32* por PCR. Ainda neste segundo trabalho descreve-se a construção de um controle interno de amplificação que usa o mesmo par de primers para a seqüência alvo para assegurar a acurácia da PCR. Este trabalho será submetido para publicação no periódico **Microbiology and Imunology.**

Os artigos estão formatados conforme exigência dos periódicos científicos em que foram ou serão publicados.

2 ARTIGO 1

MONOCLONAL ANTIBODIES AGAINST LipL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *Leptospira*: PRODUCTION, CHARACTERIZATION AND TESTING IN DIAGNOSTIC APPLICATIONS

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MONOCLONAL ANTIBODIES AGAINST LipL32, THE MAJOR OUTER MEMBRANE PROTEIN OF PATHOGENIC *Leptospira*: PRODUCTION, CHARACTERIZATION AND TESTING IN DIAGNOSTIC APPLICATIONS

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ABSTRACT. Pathogenic serovars of *Leptospira* have a wide antigenic diversity attributed mainly to the lipopolysaccharide present in the outer membrane. In contrast, antigens conserved among pathogenic serovars are mainly represented by outer membrane proteins. Surface exposure of a major and highly conserved outer membrane lipoprotein (LipL32) was recently demonstrated on pathogenic *Leptospira*. LipL32 on its recombinant form (rLipL32) was used to immunize BALB/c mice to develop murine monoclonal antibodies (mAbs). Three mAbs against rLipL32 were produced, isotyped and evaluated for further use in diagnostic tests of leptospirosis using different approaches. mAbs were conjugated to peroxidase and evaluated in a native protein ELISA with intact and heat-treated leptospiral cells, were conjugated to FITC for indirect immunofluorescence with intact and methanol fixed cells and were used for LipL32 immunoprecipitation from leptospiral cells. rLipL32 mAbs conjugated to peroxidase or used as primary antibody bounded to intact and heat-treated cells in ELISA, proving that they could be used in enzyme immunoassays for detection of the native protein. On immunofluorescence assay mAbs labeled bacterial cells either intact or methanol fixed. Two mAbs were able to immunoprecipitate the native protein from live and motile leptospiral cells and, adsorbed onto magnetic beads, captured intact bacteria from artificially contaminated human sera for detection by PCR amplification. Results of this study suggest that the mAbs produced can be useful for the development of diagnostic tests based on detection of LipL32 leptospiral antigen in biological fluids.

Key words: LipL32, recombinant antigens; monoclonal antibody, ELISA; immunofluorescence; immunoprecipitation

INTRODUCTION

Leptospirosis, a zoonotic disease widespread in the world, is caused by pathogenic bacteria of the genus *Leptospira* (1). There are over 230 pathogenic leptospiral serovars identified and this antigenic diversity is mainly attributed to the lipopolysacharide (LPS) covering bacterial surface (2). Diagnostic approaches based on detection of antibodies generated during infection or on detection of antigens using antibodies generated against *Leptospira* strains have poor sensitivity since LPS is the dominant antigen and antibody reaction is serovar specific. Because of the LPS diversity, recent research focused on the use of outer membrane proteins (OMPs) conserved among pathogenic *Leptospira* species to increase sensitivity of diagnostic tests.

A small number of leptospiral outer membrane lipoproteins is exposed on the cell surface, among which are LipL32, LipL21 and LipL41 (3). Expression of the major OMP LipL32 has been demonstrated both in culture and in host infections (4), and its surface exposure on the bacterial membrane has recently been proved (3). Sera from patients with leptospirosis react strongly with the recombinant form of LipL32 (5) and an enzyme assay using this antigen was able to detect human (6) and animal cases of leptospirosis (7,8).

In this study we report on the production and characterization of three monoclonal antibodies (mAbs) against recombinant LipL32 (rLipL32) and their use on different approaches to demonstrate mAbs potential for diagnosis of leptospirosis. The mAbs recognized the native protein on the surface of pathogenic *Leptospira* cells and did not react with saprophytic strains or other microorganisms, suggesting they could be a useful diagnostic tool for leptospirosis.

MATERIAL AND METHODS

Leptospira strains and culture conditions

L. interrogans serovar Copenhageni strain FIOCRUZ L1 130 used in this study was provided by A.I.Ko (Centro de Pesquisa Gonçalo Moniz, FIOCRUZ, Salvador, BA, Brazil). Other *Leptospira* serovars used were obtained from the Center for Zoonosis Control (Universidade Federal de Pelotas, Brazil). Leptospires were grown at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton, Dickinson Co, MD, USA), supplemented with 8% of bovine serum albumin (1).

Antigen preparation

The *lipL32* gene, obtained by PCR using the DNA from *L. interrogans* L1 130 as template, was cloned into the pAE expression vector (9) that allows fusion of the protein with a 6×His tag. This plasmid was used to transform *E. coli* BL21 (DE3). Purification of the protein was accomplished by affinity chromatography with Ni-NTA resin using the QIA EXPRESSIONIST Kit (Qiagen Corporation, CA, USA) following the manufacturer instructions. The eluate was then dialyzed against phosphate-buffered saline (PBS) and glycine 0.1%, pH 8.0, for approximately 16 h at 4 °C. Protein in the final preparation was quantified by the Bradford method (10).

Generation and purification of monoclonal antibodies

Two 6-week-old BALB/c female mice were immunized intraperitoneally on day 1 with 100 µg of the recombinant protein LipL32 (rLipL32) mixed with Freund's complete adjuvant (Sigma Aldrich Co., St. Louis, MO). This was followed by three intraperitoneal injections of rLipL32 mixed with Freund's incomplete adjuvant (Sigma Aldrich) on days 14, 21 and 28. Three days before fusion the mouse with the highest

titer of serum antibodies against rLipL32 in an indirect ELISA was boosted with 20 µg of the protein intravenously. Hybridoma cells were obtained by established procedures (11). Splenic lymphocytes were fused to murine Sp2/O-Ag14 myeloma cells in the presence of PEG 1450 (Sigma Aldrich). Fused cells were cultivated in Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich) containing 20% fetal calf serum (FCS, Cultilab, Campinas, Brazil) and supplemented with hypoxanthine, aminopterin and thymidine (HAT, Sigma Aldrich). Hybridomas were primarily screened for specific antibodies by indirect ELISA with immunizing antigen. Supernatants showing at least 40% of the positive control (sera from rLipL32 immunized mouse) OD was considered positive and the hybridomas cloned twice by limiting dilution. A secondary screening was performed by immunoblotting and indirect ELISA using supernatant from cloned cells and heat-treated *Leptospira* from different serogroups as antigen. Specific hybrid cell lines identified in this second screening were expanded and stored in liquid nitrogen. For ascites production the hybridomas were removed from liquid nitrogen, cultivated on DMEM with 10% FCS, collected by centrifugation, washed five times in DMEM without FCS and injected into pristane primed BALB/c mice. mAbs were purified from ascitic fluid by affinity chromatography on a protein A-Sepharose CL-4B column (GE Healthcare Company, USA) according to manufacturer instructions. Purification efficacy was evaluated by SDS PAGE and final concentration was measured by spectrophotometry at 280 nm. Purified mAbs were stored at -20 °C. The mAbs were isotyped by ELISA with a mouse subisotyping kit following manufacturer instructions (Sigma Aldrich).

Gel electrophoresis and immunoblotting

Proteins immunoprecipitated from outer membrane or heat-treated leptospiral

cells were solubilized in final sample buffer 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 0.1% bromophenol blue, 2% SDS and 20% glycerol and separated in a 12% polyacrylamide gel using a discontinuous buffer system (12). After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid – 45% methanol) or transferred electrophoretically to a PVDF membrane (GE Healthcare, Piscataway, NJ, USA) at 25 volts for 60 min. The membranes were blocked with 0.1 M PBS containing 0.1% Tween 20 (PBS-T, pH 7.4) and 5% skim milk for 30 min, washed twice in PBS-T and incubated for 1 h with the mAbs diluted 1/1000 in PBS-T. After washing the membrane three times with PBS-T, a rabbit anti-mouse immunoglobulin (Ig) horseradish peroxidase conjugate (Sigma Aldrich) diluted 1:2,000 in PBS-T or a mAb anti-LipL32 peroxidase conjugate was added for 1 h and the membrane was again washed three times with PBS-T. The mAb-peroxidase conjugate was prepared according to established protocol (13). Bands were visualized after the addition of the substrate/chromogen solution (H_2O_2/4-chloro-1-naphtol).

ELISA with intact and heat-treated leptospiral cells

Seven-day cultures of *L. interrogans* serovar Copenhageni strain Fiocruz L1 130, and of saprophytic *L. biflexa* serovar patoc (strain Patoc I) were harvested by centrifugation (15,000 × *g*, for 30 min) at 4 °C and washed once in PBS (0.01 M, pH 7.2). 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 ELISA with intact leptospira cells, microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY, USA) were first coated overnight at 4 °C with 100 µL of a ten times diluted 0.1% poly L-Lysine solution and then with 100 µL of the bacterial suspension

in PBS for 2 hours at 30 °C. Wells were washed 3 times with leptospira culture medium (LCM) and 100 µl of twofold dilutions of each ascites mAb in LCM was added to the wells for 2 hours at 30 °C. Washing was repeated and 100 µL of rabbit anti-mouse Ig-POase conjugated was added for 2 h at 30 °C. After two washes with LCM and three washes with PBS, 100 µl of enzyme substrate/chromogen solution (H_2O_2/ ortophenylenediamine) in citrate-phosphate buffer, pH 5.0, was added and the reaction was allowed to take place in the dark for 10 min. Optical density was read at 450 nm in an ELISA reader (Multiskan MCC/340, Titertek Instruments, Huntsville, AL, USA). ELISA with heat-treated leptospiral cells was performed in the same way except that leptospiral cultures were first inactivated overnight at 56 °C and stored at -20 °C until use. As control of cell integrity, rat serum against cytoplasmatic protein GroEL was included in both tests. To investigate how would protein conjugation affect mAb performance in ELISA, a conjugate of mAb 1D9 and POase was also used in this experiment.

Direct and indirect immunofluorescence

Slide chambers (ICN Biomedicals Inc, CA, USA) were coated with a 0.01% Poly L-Lysine solution (Sigma Aldrich) and dried for 1 h at room temperature. A 7-day culture of *L. interrogans* L1-130 was washed once in PBS, resuspended to a density of 10^8 cells per ml in PBS, and incubated in the slide chamber for 2 h at 30 °C. The slides were washed twice with leptospiral culture medium (LCM) and coated with mAbs ascites diluted 1:10 in LCM. After incubating for 1 h at 30 °C, the slides were washed again twice with LCM and a 1:100 dilution of rabbit anti-mouse FITC conjugate 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. In experiments with permeabilized membrane, slides were

incubated in 5 ml of methanol for 10 min at 4 °C followed by twice LCM washing. The following controls were used in this experiment: (i) mAb against S*almonella* OMP was used as primary antibody, (ii) rabbit anti-mouse FITC conjugate was applied to slides without primary antibody and (iii) normal mouse serum was used as primary antibody. For direct immunofluorescence a mAb 1D9 FITC conjugate was produced following established procedures (14) and used together a conjugate of rabbit antibodies against whole *Leptospira* and FITC (Ames Laboratories, NVSL, Iowa, USA) as positive control. Labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm.

Immunoprecipitation of native LipL32 and immunomagnetic separation of leptospiral cells

Surface immunoprecipitation of native LipL32 was performed according to Shang et al. (15) with modifications. A 2 ml volume of each heat-inactivated mAb ascites was mixed with 30 ml of a culture of *L. interrogans* L1-130 containing 3 x 10^{10} actively motile bacteria. After shaking the suspension gently for 1 h at 30 °C the cells were pelleted at 2,000 × *g* for 15 min at 4 °C, resuspended in PBS with 5 mM $MgCl_2$, centrifuged again and resuspended in 9 ml of 10 mM Tris-HCl (pH 8.8), 2 mM EDTA, 1 mM PMSF. A volume of 1 ml of 10% protein grade Triton X 100 (Sigma Aldrich) was added and the suspension was agitated for 30 min at 4 °C. The insoluble material was removed by centrifugation at 16,000 × *g* for 20 min and 1 ml of 2% deoxycholate, 50 µL of 20% SDS and 500 µL of Sepharose-SpA (Sigma Aldrich) were added to the supernatant. After agitating the mixture gently for 30 min at 4 °C, the complex of Sepharose SpA-mAb-antigen was washed by centrifugation two times with 0.01% Triton X 100 in 10 mM Tris-HCl (pH 8.8) and resuspended in final sample buffer. The complex was submitted to SDS-PAGE and immunoblotting using mAb

1D9-POase as probe. As negative controls two additional immunoprecipitation experiments were carried out in parallel: in the first one mAb against LipL32 was omitted and in the second one the culture of *L. interrogans* L1-130 was replaced for *L. biflexa* serovar patoc. For the immunomagnetic separation (IMS) procedure, 1D9 mAb was adsorbed onto protein A-coated microspheres (Bangs Laboratories Inc, Fishers, IN, USA). Briefly, 100 µl of particles with 1% solids was suspended with 900 µl of 50 mM pH 8.2 borate buffer (BB), washed twice with BB, resuspended in 1 ml of BB containing 1.2 mg of mAb and incubated with gentle agitation at 4 °C for 16 h. The mAb-coated particles were washed twice with BB and resuspended in stock buffer (100 mM borate pH 8.5, 0.1% BSA, 0.05 Tween 20, 10 mM EDTA e 0.1% NaN_3). An immunomagnetic separator (Invitrogen Corporation, CA, USA) was used in washing steps. Standard IMS was performed with 5 µL of mAb-coated particles per ml of different dilutions of a pool of sterile human sera artificially contaminated with pathogenic and saprophytic *Leptospira* species. The serum samples were gently agitated for 15 min at room temperature and then washed twice on a magnetic separator. The particles were then resuspended in 20 µL of lysis buffer (0.02 M Na_2HPO_4; 0.15 M NaCl; pH 7.2), boiled for ten min and stored at - 20 °C until use in PCR as described below.

PCR conditions

PCR primers *lipL32* F: 5' CGC TTG TGG TGC TTT CGG TGG T 3' and *lipL32* R: 5' CTC ACC GAT TTC GCC TGT TGG G 3' were used, resulting in a 264 bp amplicon of the *lipL32* coding region. Briefly, 2 µL of DNA-IMS template was added to a tube with 1 U *Taq* DNA polimerase (Invitrogen) 150 ng of primers, 2.5 µL of 10× reaction buffer containing $MgCl_2$ and 0.2 mM dNTP. For DNA template optimization,

volumes of 10 µL, 5 µL, 3 µL, 2 µL and 1 µL of DNA-IMS were tested in the same conditions. Amplification was carried out in a Perkin Elmer 2400 thermocycler (PE Biosystems, Foster City, CA, USA) with 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and an extension of 7 min at 72 °C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 1% agarose gel with ethidium bromide and visualized under UV transillumination.

RESULTS

Generation of monoclonal antibodies

From a total of 54 hybridomas tested in the primary ELISA, three, named 1D9, 36C4 and 412H4, have shown a specific reaction in the secondary ELISA screening and were selected for further characterization and testing in diagnostic applications. The immunoglobulin classes of mAbs 1D9, 36C4 and 412H4 were IgG2b, IgG2a and IgG3, respectively. All mAbs reacted with a band of an estimated molecular mass of 32 kDa and neither reacted with *E. coli* as seen by immunoblotting (Table 1).

ELISA with intact and heat-treated *Leptospira*

To evaluate mAbs reactivity against native LipL32 protein ELISA protocols with intact and heat-treated leptospiral cells were used. A decrease in the absorbance of ELISA with intact cells occurred as the concentration of antibodies decreased, demonstrating the specificity of the reaction (Figure 1, Panel A). In addition, antisera against the cytoplasmic protein GroEL did not reacted in this ELISA. A conjugate of mAb 1D9-POase reacted with native LipL32 in both intact and heat-treated cell ELISA, but higher absorbance values were observed with heat-treated bacteria (Figure 1, Panel B). In these ELISA experiments mAbs did not reacted with

saprophytic bacteria and sera against GroEL reacted with heat-treated cells (data not shown).

Direct and indirect immunofluorescence

Reaction of mAbs with native LipL32 on the outer membrane of pathogenic *Leptospira* was investigated by indirect immunofluorescence and by an in house prepared conjugate of mAb 1D9 and FITC. Bacterial cells were applied to slides with and without methanol treatment to evaluate the effect of the fixing method on mAb reactivity. The three mAbs labeled leptospiral cells in both fixing methods as could be seen by the intense fluorescence (Figure 2). Labeling of leptospiral cells with mAb anti-*Salmonella* or normal mouse serum was not observed (data not shown).

Immunoprecipitation of native LipL32 and use of a mAb on IMS-PCR

The mAbs were used in immunoprecipitation assays to investigate their ability in binding the surface of live and motile leptospiral cells in suspension. Two mAbs were able to precipitate LipL32 from the suspension of live pathogenic bacteria (Figure 3). In addition, mAb 1D9 was adsorbed onto protein A magnetic beads to explore its potential for use in diagnostic assays which associates IMS to PCR amplification. With the use of IMS for capturing bacterial cells prior to DNA extraction and PCR amplification, it was possible to detect as low as 10 leptospires per ml of artificially contaminated human sera (Figure 4). Sera contaminated with saprophytic *Leptospira* submitted to IMS and PCR did not show any amplification product (data not shown).

DISCUSSION

Leptospiral outer membrane protein LipL32 is prominent on pathogenic strains and is the major detergent-phase protein extracted by Triton X 114 (4,16). Human sera from cases of leptospirosis reacted with native LipL32 and its recombinant form expressed in *E. coli* (5). After these findings this antigen has been used for developing diagnostic tests that detects antibodies in human and animal leptospirosis (6,7,17,18). However, diagnostic tests based on antibody detection suffer from lack of sensitivity in the first 10 days of infection. Antigen detection tests should be preferred when early diagnosis is important for initiating treatment, as is the case of leptospirosis (1,19). Monoclonal antibodies are important tools for antigen detection tests due to their high specificity and permanent availability. The three mAbs reported in this study reacted with a 32 kDa polypeptidic band from different leptospiral serovars on immunoblotting, a molecular mass similar to that of LipL32. Moreover, anti-LipL32 mAbs 1D9, 36C4 and 412H4 did not react with the other microorganisms tested. The mAbs were also able to detect native LipL32 antigen when used as primary antibody in indirect ELISA with intact and heat-treated leptospiral cells. Furthermore, mAb 1D9 maintained antibody activity after conjugation with peroxidase suggesting it is suitable for two-site immunoassays such as sandwich ELISA.

Indirect immunofluorescence was used to investigate mAbs reaction after fixing leptospiral cells with or without methanol treatment. When anti-LipL32 mAb 1D9 was used conjugated to FITC, it was possible to visualize leptospiral cells by direct fluorescence. Monoclonal antibodies have been successfully used in immunofluorescence based diagnostic tests for lime and syphilis, two diseases also caused by spirochetes (20,21). The antibodies reported here were able to attach to

the bacterial outer membrane after the two fixing treatments, providing evidence of their usefulness for immunohistochemical tests.

Surface immunoprecipitation experiments were carried out to explore mAbs potential for use in immunoseparation techniques or chromatographic assays. IMS has been used to increase PCR sensitivity and to improve bacterial isolation from clinical samples containing inhibitory substances or contaminating organisms (22,24). Two of the mAbs obtained in this study were able to form complexes with live leptospiral cells suspended in a liquid phase suggesting that they coud be useful for IMS. One of these mAbs was adsorbed to magnetic particles and used to develop a method for *Leptospira* detection in association with PCR amplification of the *lipL32* gene. Using this novel method we were capable to detect approximately 10 leptospiral cells per milliliter of human sera experimentally contaminated. An assay that uses mAb-based magnetic separation coupled to PCR amplification for detection of *L. borgpetersenii* serovar hardjo in urine from experimentally infected cattle has been reported (25). Despite its good sensitivity, the assay consistently detected 10 organisms in urine samples spiked with leptospires, it may have an important limitation in specificity since the mAbs used for IMS are directed to LPS or a non-characterized outer envelope protein.

In conclusion, the results reported in this study demonstrated that mAbs generated against the recombinant form of LipL32 were able to recognize the native protein from pathogenic *Leptospira.* Since LipL32 is a surface protein conserved among pathogenic serovars and is not found on saprophytic strains, the mAbs against this target will be useful in the development of different test formats for diagnosis of human and animal leptospirosis.

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Table 1. Reactions of anti-LipL32 mAbs with different strains of *Leptospira* in immunoblotting.

Serogroup	Serovar	Genomoespecies	Monoclonal antibodies		
			1D9	**412H**	**36C**
Australis	Australis	*L. interrogans*	+	+	+
Australis	Bratislava	*L. interrogans*	+	+	+
Autumnalis	Autumnalis	*L. interrogans*	+	+	+
Autumnalis	Butembo	*L. kirshneri*	+	+	+
Autumnalis	Rachmat	*L. interrogans*	+	+	+
Bataviae	Bataviae	*L. interrogans*	+	+	+
Canícola	Canícola	*L. interrogans*	+	+	+
Hebdomadis	Hebdomadis	*L. interrogans*	+	+	+
Icterohaemorrhagiae	Copenhageni	*L. interrogans*	+	+	+
Icterohaemorrhagiae	Icterohaemorrhagiae	*L. interrogans*	+	+	+
Pomona	Pomona	*L. interrogans*	+	+	+
Pyrogenes	Pyrogenes	*L. interrogans*	+	+	+
Sejroe	Wolffi	*L. interrogans*	+	+	+
Sejroe	Hardjo	*L. interrogans*	+	+	+
Djasiman	Sentot	*L. interrogans*	+	+	+
Djasiman	Djasiman	*L. interrogans*	+	+	+
Ballum	Castellonis	*L. borgpetersenii*	+	+	+
Ballum	Ballum	*L. borgpetersenii*	+	+	+
Celledoni	Withcombi	*L. borgpetersenii*	+	+	+
Javanica	Javanica	*L. borgpetersenii*	+	+	+
Tarassovi	Tarassovi	*L. borgpetersenii*	+	+	+
Cynopteri	Cynopteri	*L. kirshneri*	+	+	+
Grippotyphosa	Grippotyphosa	*L. kirshneri*	+	+	+
Louisiana	Louisiana	*L. noguchii*	+	+	+
Panamá	Panama	*L. noguchii*	+	+	+
Shermani	Shermani	*L. santarosai*	+	+	+
Celledoni	Celledoni	*L. weilii*	+	+	+
Andamana	Andamana	*L. biflexa*	-	-	-
Semaranga	Patoc	*L. biflexa*	-	-	-
Semaranga	Semaranga	*L. meyeri*	-	-	-

FIGURE 1 - ELISA reactions of mAbs anti-LipL32 using intact and heat-treated *L. interrogans* L1-130 cells. Panel A: ELISA using 10^9 intact leptospiral cells/ml (100 µl/well) and mAbs 1D9 (♦), 412H4 (■), 36C4 (▲) and GroEL antiserum (x) diluted 1:4 (1), 1:8 (2), 1:16 (3), 1:32 (4) and 1:64 (5). Panel B: ELISA reactions with intact (◘) and heat-treated cells (■) at 10^9 cell per ml (1), $4x10^8$ cell per ml (2) and $2x10^8$ cell per ml (3) and 1D9-POase conjugate diluted 1:1000.

FIGURE 2 - Staining of LipL32 from *L. interrogans* L1-130 by indirect immunofluorescence. *L. interrogans* L1-130 was fixed to microscope slides with and without methanol and probed with mAbs. Test control slides were made by reacting fixed bacteria with normal mouse sera and rabbit anti-mouse FITC.

FIGURE 3 - Immunoprecipitation of native LipL32 on the surface of *L.interrogans* L1-130. Bacterial cells were reacted with mAbs before Triton X-100 membrane fractionation and precipitated with protein A-Sepharose. Precipitated material was separated by SDS-PAGE and blots were probed with mAb 1D9-POase. 1- rLipL32; 2- *L biflexa* Patoc and mAb 1D9; 3- *L.interrogans* L1-130 and mAb 1D9; 4- *L biflexa* Patoc and 412H; 5- *L.interrogans* L1-130 and 412H; 6- *L biflexa* Patoc and 36C; 7- *L.interrogans* L1-130 and 36C and 8- *L.interrogans* L1-130 treated with protein A-Sepharose only.

FIGURE 4 - PCR amplification of the *lipL32* coding region from *L. interrogans* L1-130 after immunomagnetic separation from experimentally contaminated human sera. **1**- DNA ladder; **2**- *L biflexa* Patoc 10^7 cells/mL ; **3**- 10^7; **4**- 10^6; **5**-10^5; **6**- 10^4; **7**- 10^3; **8**- 10^2; **9-** 10^1 cells/mL.

Figure 1

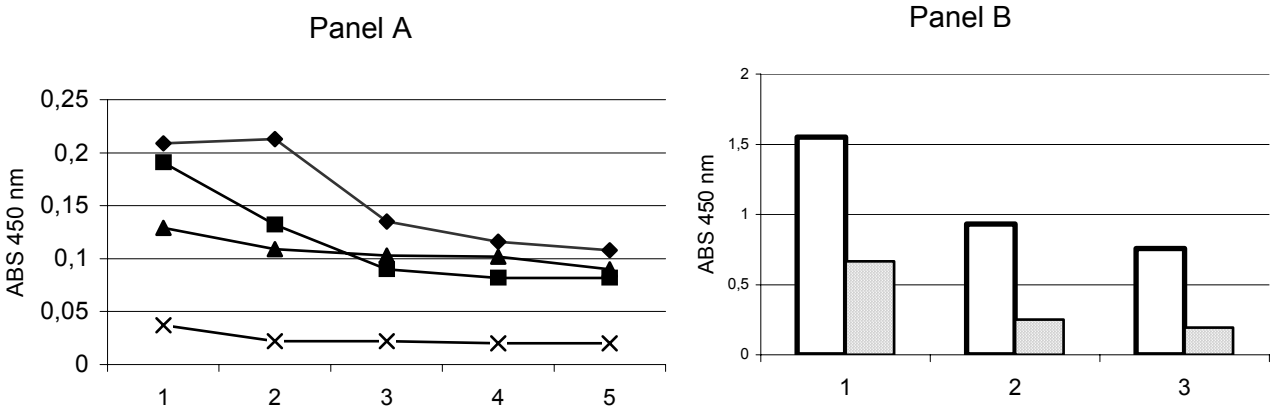

Panel A
ABS 450 nm
0,25
0,2
0,15
0,1
0,05
0
1
2
3
4
5
Panel B
ABS 450 nm
2
1,5
1
0,5
0
1
2
3

Figure 2

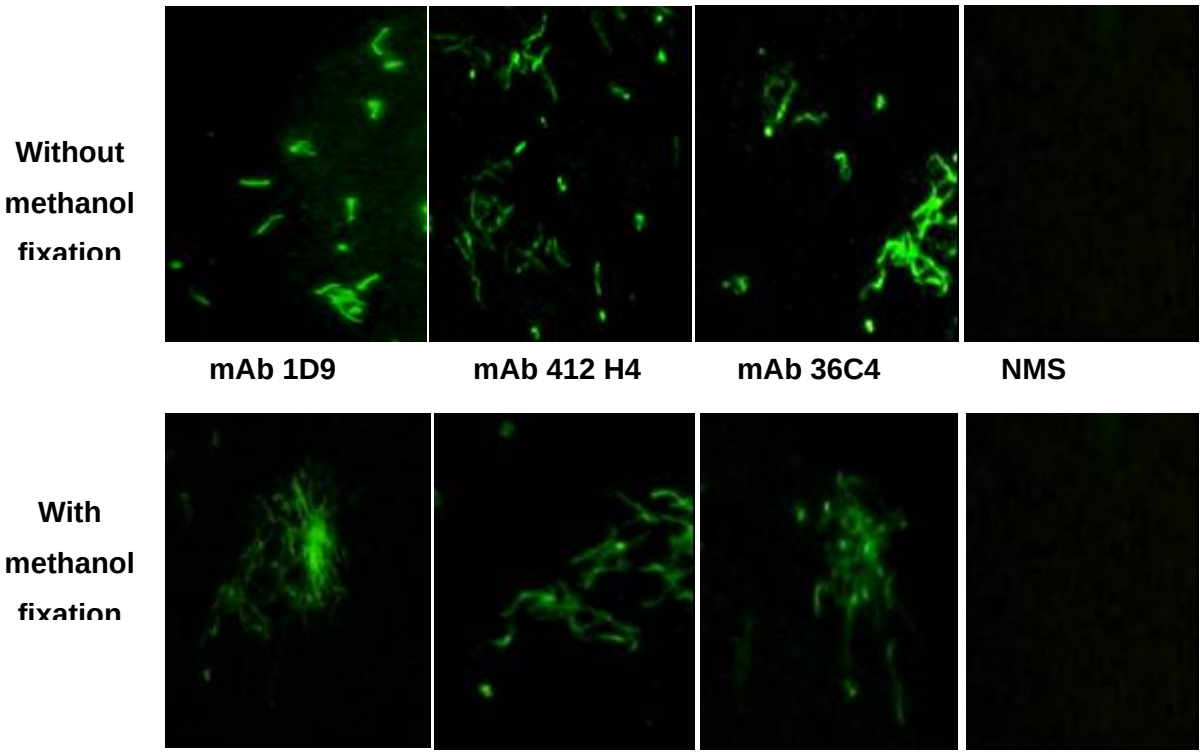

Without methanol fixation
mAb 1D9
mAb 412 H4
mAb 36C4
NMS
With methanol fixation

Figure 3

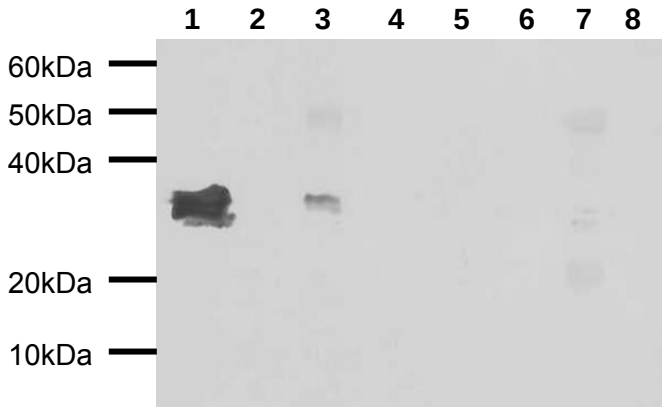
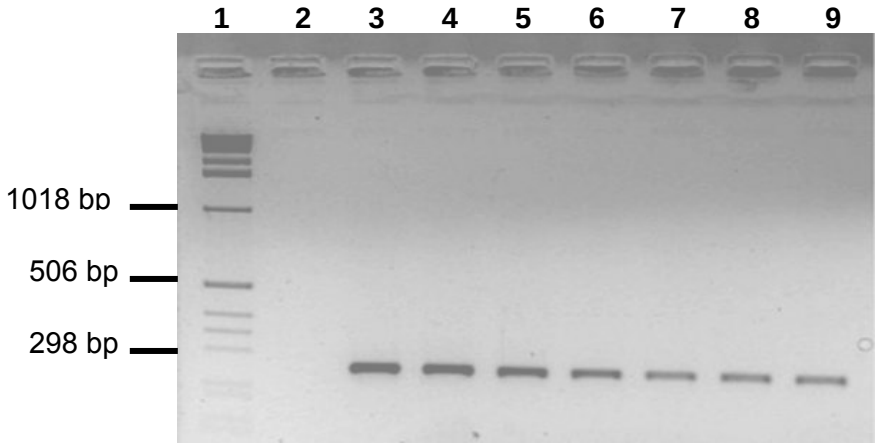


Figure 4



3 ARTIGO 2

AN IMMUNOMAGNETIC SEPARATION-PCR METHOD FOR THE DETECTION OF PATHOGENIC *Leptospira* IN BIOLOGICAL FLUIDS

Artigo a ser submetido ao periódico Microbiology and Imunology

AN IMMUNOMAGNETIC SEPARATION-PCR METHOD FOR THE DETECTION OF PATHOGENIC *Leptospira* IN BIOLOGICAL FLUIDS

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ABSTRACT. Leptospirosis is a zoonotic disease that occurs all over the world and that is caused by pathogenic bacteria of the genus *Leptospira*. Clinical manifestations of leptospirosis are similar to other febrile illnesses and this fact frequently retards beginning of antibiotic therapy. Thus, early and accurate diagnosis is a prerequisite for proper treatment of leptospirosis. Antigen and DNA-based detection tests offer potential advantage over tests based on antibody detection for early diagnosis of leptospirosis since they avoid the window period of the latter. This work describes a method for detection of pathogenic *Leptospira* that associates an immunoseparation step to a PCR assay of the *lipL32* gene and uses an internal amplification control (IAC) to ensure sensitivity and specificity. The immunoseparation was performed with protein A-magnetic beads in house coated with a mAb specific for LipL32, the major outer membrane protein of pathogenic *Leptospira,* and PCR was performed using *lipL32* specific primers. The IMS-PCR method enhanced detection of *Leptospira* in experimentally contaminated human sera and urine when compared to PCR performed alone. IMS-PCR was able to detect 10^2 *Leptospira* cells per mL of human sera and urine, corresponding to 25 genomic copies per PCR reaction.

Key words: Leptospirosis, laboratory diagnosis, monoclonal antibodies, LipL32

Running head: Pathogenic *Leptospira* detection by IMS-PCR on biological fluid samples

INTRODUCTION

Leptospirosis is a zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira* (22). The disease, first considered as an endemic or occupational/recreational hazard to people exposed to contaminated water, now is being recognized as a common cause of febrile illness in tropical environments worldwide, mainly on urban areas lacking basic sanitation (21,22).

Clinical presentation of leptospirosis is common to other febrile illnesses, fact that complicates the diagnosis and prompt treatment, important to avoid renal damage, meningitis, liver failure, and pulmonary hemorrhage (9,12). Unfortunately, limitations in available diagnostic methods such as bacterial isolation, microscopic agglutination test (MAT) and ELISA further complicate timely diagnosis of the disease (12,23,25). Effort is being made to develop novel sensitive and specific diagnostic tests for leptospirosis (22,23).

Immunomagnetic separation aided by antibody molecules is a technique that has been used for isolation of specific viable whole organisms and antigens (28). Growth on selective media may be helpful for isolation of several bacterial species however immunomagnetic separation (IMS) techniques assist isolation of strains possessing unique surface antigens. Further separation, cultivation, and identification of the isolate can then be performed by traditional biochemical, immunologic or molecular methods (3,17,27,31).

Several PCR assays for detection of leptospires in clinical samples from animals and humans have been described (1,2,11,24-26). However, despite of the high sensitivity of that assay, inhibitors molecules and presence of heterogeneous bacteria in the samples can affect PCR performance (29).

IMS has been demonstrated to be a useful technique in diagnostic microbiology, and recent studies associating IMS to PCR lead to methods that enhance PCR specificity and sensitivity and provide savings in time compared with traditional diagnostic methods (10,15,16,33,34,35,37). For diagnosis of leptospirosis the IMS-PCR approach was reported to detect *Leptospira* in bovine urine by using monoclonal antibodies against leptospiral lipopolysaccharide (LPS), which makes it a serogroup specific method (32,36).

The aim of this study was to evaluate the potential of IMS associated to PCR as a leptospiral diagnostic test that detect *Leptospira* in human urine and sera. Monoclonal antibody (mAb) against LipL32 (13) was used to in house coat protein A-magnetic beads for capture and concentration of *Leptospira* cells to further detect by amplification of a sequence of the *lipL32* by PCR. This approach provides the ability for detection of all pathogenic *Leptospira* by using a specific mAb and a PCR with primers already characterized (19). Finally, an internal amplification control (IAC) was constructed and its use in the PCR assay was optimized to avoid negative results.

METHODS

Leptospira strains, culture conditions and DNA extraction

L. interrogans serovar Copenhageni strain FIOCRUZ L1-130 used in this study was obtained from Centro de Pesquisas Gonçalo Moniz (FIOCRUZ, Salvador, BA, Brazil). *L. biflexa* serovar patoc strain Patoc I was obtained from the Center for Zoonosis Control (Universidade Federal de Pelotas, Brazil). Leptospires were grown at 30 °C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton, Dickinson Co, MD, USA), supplemented with *Leptospira* Enrichment EMJH (Difco, USA). *Leptospira* numbers were determined with a Petroff-Hauser chamber.

Genomic DNA was extracted from leptospiral cultures according to instructions of the PureGene® DNA purification kit (Gentra Systems, USA). For extraction of bacterial DNA from urine and serum samples, aliquots of 1 mL were centrifuged at 15.000 x g for 10 min, the cells were washed with sterile 0.01 M phosphate-buffered saline (PBS, pH 7.2) and the DNA was extracted by heating at 95 ^{o}C for 15 min in 50 µl of lysis solution (1:1 of 0.125% SDS and 0.05 M NaOH). In the experiments where lysis followed IMS, 20 µl of the lysis solution was directly added to beads in microtubes and then heated at 95 ^{o}C for 15 min.

Coating magnetic beads with mAb

The hybridoma cell line 1D9 (13) secreting a mAb specific for pathogenic leptospires was utilized for production of ascites. The hybridoma cells were removed from liquid nitrogen, cultivated on DMEM with 10% fetal calf serum (FCS), collected by centrifugation, washed five times in DMEM without FCS and injected into pristane-primed BALB/c mice for production of ascites. The mAb was purified from ascitic fluid by affinity chromatography on a protein A-Sepharose CL-4B column (GE Healthcare Company, USA) according to manufacturer instructions. Purification efficacy was evaluated by SDS-PAGE and final concentration was measured by spectrophotometry at 280 nm. Purified mAb was adsorbed onto protein A-coated microspheres (Bangs Laboratories Inc, Fishers, IN, USA) following established procedure (4). Briefly, 100 µl of beads with 1% solids was suspended with 900 µl of 50 mM pH 8.2 borate buffer (BB), washed twice with BB, suspended in 1 ml of BB containing 1.2 mg of mAb and incubated with gentle agitation at 4 °C for 16 h. The mAb-coated beads were washed twice with BB and suspended in stock buffer (100 mM borate pH 8.5, 0.1% BSA, 0.05 Tween 20, 10 mM EDTA e 0.1% NaN_3). An

immunomagnetic separator MPC-S (Invitrogen Corporation, CA, USA) was used in washing steps and the washing buffer was kept for indirect quantification of antibody adsorbed on beads by spectrophotometry at 280 nm. Efficiency of IgG binding was determined indirectly from the amount remaining in the supernatant after the sensitization procedure. Sensitized beads were further observed under microscope to investigate the degree of auto-agglutination and the agglutination behavior with *L. interrogans* L1-130 and *L. biflexa* Patoc I cells.

Oligonucleotide primers and PCR conditions

PCR primers *lipL32* F: 5' CGC TTG TGG TGC TTT CGG TGG T 3' and *lipL32* R: 5' CTC ACC GAT TTC GCC TGT TGG G 3' were used, resulting in a 264 bp amplicon of the *lipl32* coding region (19). For PCR, 5 µL of DNA or DNA-IMS templates were added to a tube with 1 U *Taq* DNA polimerase (Invitrogen) 150 ng of primers, 2.5 µL of 10× reaction buffer containing $MgCl_2$ and 0.2 mM dNTP. For DNA-IMS template optimization volumes of 10 µL, 5 µL, 3 µL, 2 µL and 1 µL of DNA were first tested in the same conditions. For PCR containing IAC 0.5 ρg of TOPO TA ®/IAC plasmid was added to each micro tube. Amplification was carried out in a Peltier Thermal Cycler PTC-100® (MJ Research, USA) with 1 cycle at 94 °C for 5 min, 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and an extension of 7 min at 72 °C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 1% agarose gel with ethidium bromide and visualized under UV transillumination.

IAC development and optimization

An internal PCR control was constructed according to Sachadyn and Kur (30) to amplify a fragment of 545 bp in the same PCR reaction. IAC DNA is a 501 bp fragment of non related *Lepstopira* DNA flanked by target sequences of *lipL*32 primers. Chimera primers (forward 5'**GCTTGTGGTGCTTTCGGTGGT**GCAATAATAGCGGGCGGACG3' and reverse 5'**CTCACCGATTTCGCCTGTTGGG**TGTTCTAGAAAGCTGGCGGC 3') having 5' overhanging ends with identical sequences to *lipL*32 primer sequence (highlighted sequence) and 3' ends complementary to a *Leptospira* non related DNA sequence (underlined sequence) were used to amplify the 545 bp DNA sequence by PCR. PCR product was analyzed on a 1% agarose gel and inserted into TOPO TA® (Invitrogen®, USA) resulting in the TOPO TA ®/IAC plasmid. This plasmid was used to transform *Escherichia coli* TOP10 cells. A recombinant colony was cultivated and the plasmids were extracted according to instructions of GFX^{TM} Micro plasmid prep kit (Amersham Biosciences®, UK) and used as IAC in the *lipL32* PCR assay.

The IAC concentration was estimated spectrophotometrically at 260 nm and the optimal concentration for use in the *lipL*32 PCR was determined by titration studies. The lowest reproducible concentration was determined using decimal dilutions of IAC (50 to 0.05 ρg) as template DNA in a PCR with *lipL*32 primers. The lowest IAC concentration defined from the previous PCR was amplified in the presence of 6, 5, 4, 3, 2, 1 ρg genomic DNA from *L. interrogans* L1-130 per reaction to verify IAC interference on detection limit of genomic DNA by PCR. The same IAC concentration was used in experiments with tenfold dilutions of a 5-day culture of *L. interrogans* L1-130 in urine and serum.

Results of PCR/IAC were considered positive when bands of 264 bp and 545 bp were visualized under UV light and negative when only the latter was seen. When neither the IAC nor the target DNA fragments were amplified it was assumed that inhibition of the PCR occurred.

PCR detection limits

To assess the lowest concentration of DNA required to detect the *lipL32* gene sequence by PCR, genomic DNA from *L. interrogans* was diluted with sterile 10 mM Tris, 1 mM EDTA (TE, pH 8.0) to concentrations ranging from 20 to 1 ρg/μL and used in the assay. Also, to assess the number of leptospiral cells required for a positive *lipL32* PCR reaction a 5 days culture of *L. interrogans* L1-130 was tenfold diluted with human serum and urine and assayed. Pellets from 1 mL of each bacterial dilution in serum or urine were washed with PBS and suspended in 50 μL of lysis buffer for DNA extraction. DNA extracted from saprophytic *Leptospira* serovar patoc Patoc I or *E. coli* were used as negative controls. These experiments were repeated three times.

IMS-PCR detection limit

The lowest number of *Leptospira* detected by the IMS-PCR method was determined by the addition of 10 μL of mAb-coated beads to aliquots of 1 mL of decimal dilutions of a 5 days culture of *L. interrogans* L1-130 in sterile media and human sera and urine. Negative controls were prepared with a culture of the saprophytic serovar Patoc. After agitating the sample aliquots gently for 15 min at room temperature the beads were washed twice using the magnetic separator. The

beads were then suspended in 20 µL of lysis buffer (0.02 M Na_2HPO_4; 0.15 M NaCl; pH 7.2), boiled for 15 min and stored at - 20 °C until use in PCR. These experiments were repeated three times.

RESULTS

Coating magnetic beads with mAbs

Purified mAb was successfully adsorbed to magnetic beads. Approximately 50% of the antibody used for adsorption remained bound on beads, and a scanty auto-agglutination and specific agglutination with *L. interrogans* L1-130 cells and no agglutination with *L. biflexa* Patoc I were observed (data not shown).

lipL32 PCR detection limits

The lowest *Leptospira* pure genomic DNA detected was 2 ρg per 25 µL of PCR reaction (Fig1A). PCR detection limit using *L. interrogans L1-130* culture diluted either in urine (data not shown) or serum was 10^3 cells per mL, or approximately 100 genomic copies in the reaction mixture (Fig2B). The fragment of approximately 264 bp was amplified from *L. interrogans* DNA and when DNA from other bacteria or saprophytic strains was used as template no amplification was observed (data not shown).

IAC development and optimization

The optimal concentration of IAC (TOPO TA ®/IAC plasmid) for use in the *lipL32* gene PCR was determined as being 0.5 ρg per 25 µL of reaction volume (data not shown). This IAC concentration did not affect the amplification of *lipL32* gene at

concentrations in the range of 6 to 2 pg (Fig. 1B) and the detection limit of *Leptospira* cells in biological fluids (Fig. 2A).

IMS-PCR detection limit

The IMS-PCR approach reduced the detection limit of *Leptospira* cells to 10^2 per mL, meaning a reduction of ten times either in urine (data not shown) or serum samples (Fig. 3), and detecting approximately 25 genomic copies. No amplification was observed after IMS-PCR performed with saprophytic strains or *E. coli* or when non-sensitized beads were used for IMS with pathogenic strains.

DISCUSSION

The aim of this study was to determine whether the IMS technique could be used to improve detection of pathogenic leptospires in urine and serum samples using *lipL32* PCR. For that purpose in house prepared mAb-coated beads were allowed to capture leptospires from biological fluids. IMS technique has been reported as being efficient to capture spirochaetes from different specimens for further cultivation or PCR amplification (5,7,8).

A PCR using primers derived from the *lipL*32 sequence was previously reported to amplify *Leptospira* DNA from biological samples (19). However, results of this PCR with clinical samples were in that study probably affected by inhibiting substances, detecting approximately 40 genomic copies per reaction in a unique reaction round. A Nested-PCR was then used as an alternative approach to improve on test sensitivity (19). However, Nested-PCR is laborious and susceptible to false positive results. To avoid all these drawbacks we decide to associate IMS using mAb

against LipL32 followed by *lipL32* gene PCR. This method improved PCR detection limit almost two times since approximately 25 genomic copies were amplified.

In order to prevent false-negative results that might be caused by inhibitory substances present in the sample or incorrect PCR reagents manipulation, an internal control was constructed. The presence of IAC in absence of target product allows differentiation between true and false-negative results. IAC reported in this study was developed to carry out PCR with the same pair of primers used for the target DNA. This competitive method between target and IAC for amplification ensures the same conditions for both DNA and avoids undesirable interactions between primers (14). Other advantage of this strategy is that there is no formation of heteroduplexes between amplification products because IAC internal sequence is completely different from target DNA (18,30). The IAC sequence was cloned in a plasmid and transformed in *E. coli* cells for safe conservation (14). The IAC concentration is critical in PCR assays since an excess will compete with the target DNA inhibiting its amplification resulting in diminishing the detection limit or in false-negatives when the concentration of target DNA is low (29). The detection limit of purified DNA by *lipL32* PCR was not influenced by IAC presence on reaction since the same limits were found with and without IAC.

IMS was efficient in concentrating leptospires after a simple washing step prior to PCR and improved detection limit when compared with PCR alone. A method associating immunomagnetic separation and PCR was previously described for *Leptospira* detection in bovine urine (36). However, this method has limited aplication since the antibodies used for coating the beads were generated against LPS from a *Leptospira* serovar. mAb used in our work was generated against a surface exposed outer membrane protein present in all pathogenic leptospires.

In conclusion, the IMS technique was successfully associated to PCR to develop a novel method for detection of pathogenic *Leptospira* in biological fluids. The method was ten times more sensitive than PCR alone for detection of leptospires in human sera and urine artificially contaminated, suggesting that the washing procedure using mAb-coated magnetic beads was more efficient for concentrating the bacterial cells than centrifugation. Also, the IAC used in the PCR reaction is coamplified with target DNA assuring reliability of negative results thus contributing to method accuracy.

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Figures

Figure 1 - Detection limit of genomic DNA by PCR. Agarose gel electrophoresis of different concentrations of standard genomic DNA from *L. interrogans* L1-130 amplified by PCR in presence or absence of IAC. **A**- in absence: Lanes **1**- DNA ladder; **2** to **7**- DNA 6 ρg; 5 ρg; 4 ρg; 3 ρg; 2 ρg and 1 ρg; **8**- IAC only; **9**- DNA only. **B-** in presence of 0.5 ρg IAC DNA: Lanes **1**- DNA ladder; **2** to **7**- DNA 6 ρg; 5 ρg;4 ρg; 3 ρg; 2 ρg and 1 ρg; **8**- DNA only ; **9**- IAC only.

Figure 2 - Detection limit of *Leptospira interrogans* L1-130 in artificially contaminated human sera by PCR. Agarose gel electrophoresis of DNA extracted from different bacterial concentrations amplified by PCR in presence or absence of IAC. **A**- in presence: Lanes **1**- DNA ladder; **2** to **9**- 10^7 cell/mL; 10^6 cell/mL; 10^5 cell/mL; 10^4 cell/mL; 10^3 cell/mL; 10^2 cell/mL; 10^1 cell/mL and 10^0 cell/mL; **10** – DNA only and **11**- IAC only. **B**- in absence. Lanes **1**- DNA ladder; **2** to **9** 10^7 cell/mL; 10^6 cell/mL; 10^5 cell/mL; 10^4 cell/mL; 10^3 cell/mL; 10^2 cell/mL; 10^1 cell/mL and 10^0 cell/mL; **10** – DNA only and **11**- IAC only.

Figure 3 - Detection limit of *Leptospira interrogans* L1-130 in human sera by IMS/PCR. Agarose gel electrophoresis of DNA extracted from different bacterial concentrations after IMS and amplified by PCR in presence or absence of IAC. **A**- in absence. Lanes **1**- DNA ladder; **2** to **9**- 10^7 cell/mL; 10^6 cell/mL; 10^5 cell/mL; 10^4 cell/mL; 10^3 cell/mL; 10^2 cell/mL; 10^1 cell/mL and 10^0 cell/mL; **10** – negative control. **B**- in presence. Lanes **1**- DNA ladder; **2** to **9** - 10^7 cell/mL; 10^6 cell/mL; 10^5 cell/mL; 10^4 cell/mL; 10^3 cell/mL; 10^2 cell/mL; 10^1 cell/mL and 10^0 cell/mL; **10** - IAC only and **11**- DNA only.

Figures

Figure 1-

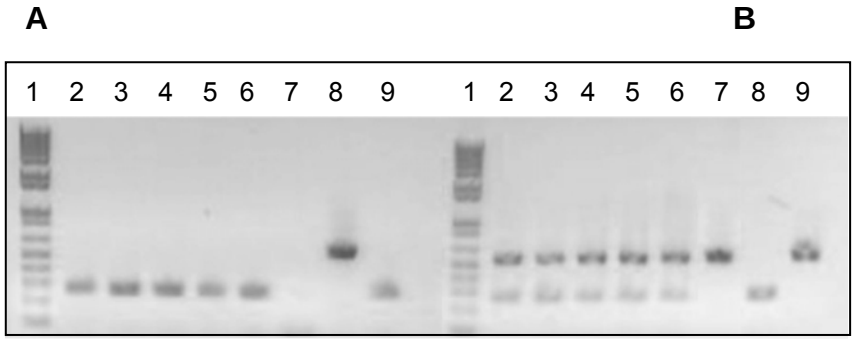


Figure 2 –

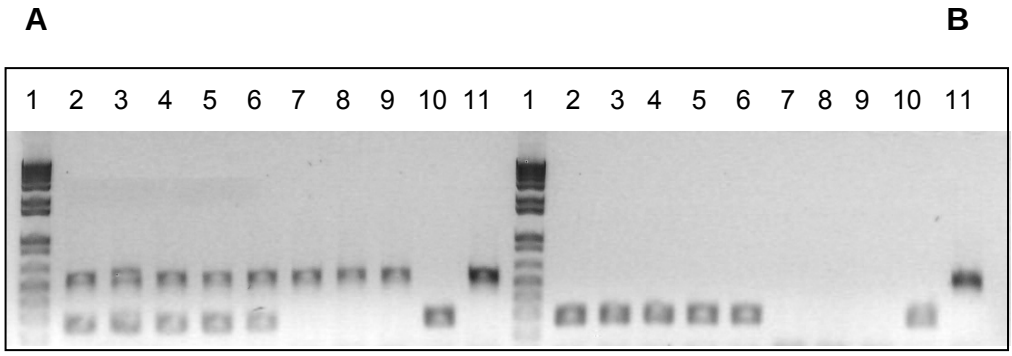
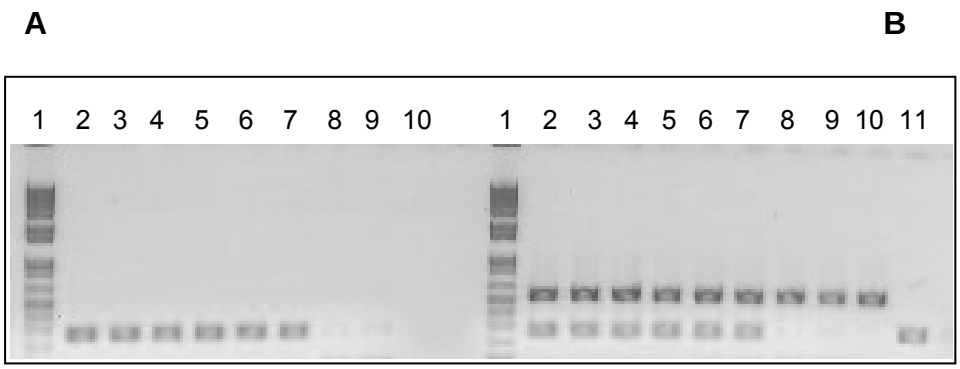


Figure 3 -



4 CONCLUSÕES

- Os anticorpos monoclonais produzidos reagem especificamente com diferentes espécies genômicas de *Leptospira.*
- Os anticorpos monoclonais são capazes de reconhecer determinantes antigênicos expostos na superfície da membrana de leptospiras patogênicas.
- O mAb 1D9 conjugado a enzima peroxidase ou ao fluoróforo FITC manteve reatividade com a proteína exposta na superfície de células íntegras e rompidas, podendo assim ser usado como reagente diagnóstico.
- O reagente desenvolvido para uso em imunoseparação magnética, composto de microesferas magnéticas e mAb 1D9, separa e concentra eficientemente *Leptospira interrogans* a partir de culturas puras e quando diluídas em soro e urina.
- A associação da IMS e da PCR para amplificação gene *lipL32* é uma nova metodologia para detecção de *Leptospira* patogênica que melhorou o limite de detecção da PCR em urina e sangue artificialmente contaminados.

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6 ANEXOS

Anexo 1- Descrição dos anticorpos

Monoclonal Antibodies

MAbs 1D9, 412H4 and 36C against leptospiral major surface protein LipL32

ANTIGEN USED FOR IMMUNIZATION

Recombinant LipL32 *Leptospira interrogans* serovar Copenhageni strain FIOCRUZ L1-130 outer membrane protein produced in *E. coli* BL21 cells.

METHOD OF IMMUNIZATION

Four intraperitoneal injections of BALB/c mice with 100 µg of rLipL32 in Freund's adjuvant complete (initial immunization) or incomplete (subsequent boosts). Mice were boosted IV with 10 µg of antigen in saline 3 days before fusion.

PARENTAL CELL LINE USED FOR FUSION

SP2/O-Ag14

SELECTION AND CLONING PROCEDURE

Hybridomas were selected in HAT medium. Specific antibody secretion was checked by indirect ELISA using rLipL32 antigen. Positive hybridomas were cloned twice by limiting dilution.

HEAVY AND LIGHT CHAINS OF IMMUNOGLOBULIN

Heavy chains are IgG2b (1D9), IgG2a (36C4) and IgG3 (412H4).

SPECIFICITY

Native LipL32 protein through immunoprecipitation, immunoblotting, ELISA and immunofluorescence.

SPECIFIC ANTIGEN IDENTIFIED

Leptospira spp OMP LipL32.

AVAILABILITY

Tissue culture supernatant	Yes √	No
Ascitic fluid	Yes	No √
Hybridoma cells	Yes	No √

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Monoclonal Antibodies Against LipL32, the Major Outer Membrane Protein of Pathogenic *Leptospira*: Production, Characterization, and Testing in Diagnostic Applications

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ABSTRACT

Pathogenic serovars of *Leptospira* have a wide antigenic diversity attributed mainly to the lipopolysaccharide present in the outer membrane. In contrast, antigens conserved among pathogenic serovars are mainly represented by outer membrane proteins. Surface exposure of a major and highly conserved outer membrane lipoprotein (LipL32) was recently demonstrated on pathogenic *Leptospira*. LipL32 in its recombinant form (rLipL32) was used to immunize BALB/c mice to develop murine monoclonal antibodies (MAbs). Three MAbs against rLipL32 were produced, isotyped, and evaluated for further use in diagnostic tests of leptospirosis using different approaches. MAbs were conjugated to peroxidase and evaluated in a native protein enzyme-linked immunosorbent assay (ELISA) with intact and heat-treated leptospiral cells, conjugated to fluorescein isothiocyanate (FITC) for indirect immunofluorescence with intact and methanol fixed cells and were used for LipL32 immunoprecipitation from leptospiral cells. rLipL32 MAbs conjugated to peroxidase or used as primary antibody bound to intact and heat-treated cells in ELISA, proving that they could be used in enzyme immunoassays for detection of the native protein. In immunofluorescence assay, MAbs labeled bacterial cells either intact or methanol fixed. Two MAbs were able to immunoprecipitate the native protein from live and motile leptospiral cells and, adsorbed onto magnetic beads, captured intact bacteria from artificially contaminated human sera for detection by polymerase chain reaction (PCR) amplification. Results of this study suggest that the MAbs produced can be useful for the development of diagnostic tests based on detection of LipL32 leptospiral antigen in biological fluids.

INTRODUCTION

LEPTOSPIROSIS, A ZOONOTIC DISEASE widespread in the world, is caused by pathogenic bacteria of the genus *Leptospira*.[1] There are over 230 pathogenic leptospiral serovars identified and this antigenic diversity is mainly attributed to the lipopolysaccharide (LPS) covering bacterial surface.[2] Diagnostic approaches based on detection of antibodies generated during infection or on detection of antigens using antibodies generated against *Leptospira* strains have poor sensitivity because LPS is the dominant antigen and antibody reaction is serovar specific. Because of the LPS diversity, recent research focused on the use of outer membrane proteins (OMPs) conserved among pathogenic *Leptospira* species to increase sensitivity of diagnostic tests.

A small number of leptospiral outer membrane lipoproteins are exposed on the cell surface, among which are LipL32, LipL21, and LipL41.[3] Expression of the major OMP LipL32 has been demonstrated both in culture and in host infections,[4] and its surface exposure on the bacterial membrane has recently been proven.[3] Sera from patients with leptospirosis react strongly with the recombinant form of LipL32[5] and an enzyme assay using this antigen was able to detect human[6] and animal cases of leptospirosis.[7,8]

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In this study we report on the production and characterization of three monoclonal antibodies (MAbs) against recombinant LipL32 (rLipL32) and their use in different approaches to demonstrate their potential for diagnosis of leptospirosis. The MAbs recognized the native protein on the surface of pathogenic *Leptospira* cells and did not react with saprophytic strains or other microorganisms, suggesting they could be a useful diagnostic tool for leptospirosis.

MATERIALS AND METHODS

Leptospira *strains and culture conditions*

L. interrogans serovar Copenhageni strain FIOCRUZ L1 130 used in this study was provided by A.I. Ko (Centro de Pesquisa Gonçalo Moniz, FIOCRUZ, Salvador, BA, Brazil). Other *Leptospira* serovars used were obtained from the Center for Zoonosis Control (Universidade Federal de Pelotas, Brazil). Leptospires were grown at 30°C in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Becton Dickinson Co., MD), supplemented with 8% of bovine serum albumin.[1]

Antigen preparation

The *lipL32* gene, obtained by polymerase chain reaction (PCR) using the DNA from *L. interrogans* L1 130 as template, was cloned into the pAE expression vector[9] that allows fusion of the protein with a 6×His tag. This plasmid was used to transform *Escherichia coli* BL21 (DE3). Purification of the protein was accomplished by affinity chromatography with Ni-NTA resin using the QIA EXPRESSIONIST Kit (Qiagen Corporation, Valencia, CA) following the manufacturer's instructions. The eluate was then dialyzed against phosphate-buffered saline (PBS) and glycine 0.1%, pH 8.0, for approximately 16 hours at 4°C. Protein in the final preparation was quantified by the Bradford method.[10]

Generation and purification of MAbs

Two 6-week-old BALB/c female mice were immunized intraperitoneally on day 1 with 100 μg of the recombinant protein LipL32 (rLipL32) mixed with Freund's complete adjuvant (Sigma Aldrich Co., St. Louis, MO). This was followed by three intraperitoneal injections of rLipL32 mixed with Freund's incomplete adjuvant (Sigma Aldrich) on days 14, 21, and 28. Three days before fusion the mouse with the highest titer of serum antibodies against rLipL32 in an indirect ELISA was boosted with 20 μg of the protein intravenously. Hybridoma cells were obtained by established procedures.[11] Splenic lymphocytes were fused to murine Sp2/O-Ag14 myeloma cells in the presence of PEG 1450 (Sigma Aldrich). Fused cells were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich) containing 20% fetal calf serum (FCS; Cultilab, Campinas, Brazil) and supplemented with hypoxanthine, aminopterin, and thymidine (HAT; Sigma Aldrich). Hybridomas were primarily screened for specific antibodies by indirect ELISA with immunizing antigen. Supernatants showing at least 40% of the positive control (sera from rLipL32 immunized mouse) OD were considered positive and the hybridomas cloned twice by limiting dilution. A secondary screening was performed by immunoblotting and indirect ELISA using supernatant from cloned cells and heat-treated *Leptospira* from different serogroups as antigen. Specific hybrid cell lines identified in this second screening were expanded and stored in liquid nitrogen. For ascites production the hybridomas were removed from liquid nitrogen, cultivated on DMEM with 10% FCS, collected by centrifugation, washed five times in DMEM without FCS, and injected into pristane-primed BALB/c mice. MAbs were purified from ascitic fluid by affinity chromatography on a protein A-Sepharose CL-4B column (GE Healthcare Company, Piscataway, NJ) according to the manufacturer's instructions. Purification efficacy was evaluated by SDS-PAGE and final concentration was measured by spectrophotometry at 280 nm. Purified MAbs were stored at −20°C. The MAbs were isotyped by ELISA with a mouse subisotyping kit following manufacturer instructions (Sigma Aldrich).

Gel electrophoresis and immunoblotting

Proteins immunoprecipitated from outer membrane or heat-treated leptospiral cells were solubilized in final sample buffer 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 0.1% bromophenol blue, 2% SDS, and 20% glycerol and separated in a 12% polyacrylamide gel using a discontinuous buffer system.[12] After electrophoresis, the gels were stained with 0.2% Coomassie brilliant blue R-250 (in 10% acetic acid—45% methanol) or transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) at 25 volts for 60 minutes. The membranes were blocked with 0.1 M PBS containing 0.1% Tween 20 (PBS-T, pH 7.4) and 5% skim milk for 30 minutes, washed twice in PBS-T and incubated for 1 hour with the MAbs diluted 1/1000 in PBS-T. After washing the membrane three times with PBS-T, a rabbit anti-mouse immunoglobulin (Ig) horseradish peroxidase conjugate (Sigma Aldrich) diluted 1:2000 in PBS-T or a MAb anti-LipL32 peroxidase conjugate was added for 1 hour and the membrane was again washed three times with PBS-T. The MAb-peroxidase conjugate was prepared according to established protocol.[13] Bands were visualized after the addition of the substrate/chromogen solution (H_2O_2/4-chloro-1-naphtol).

ELISA with intact and heat-treated leptospiral cells

Seven-day cultures of *L. interrogans* serovar Copenhageni strain Fiocruz L1 130, and of saprophytic *L. biflexa* serovar patoc (strain Patoc I) were harvested by centrifugation (15,000*g*, for 30 minutes) at 4°C and washed once in PBS (0.01 M, pH 7.2). The cells were resuspended in PBS, counted in a Petroff-Hausser chamber and the concentration was adjusted to approximately 10^9 cells per milliliter. For ELISA with intact leptospira cells, microtiter plates (Nunc Polysorp, Nalge Nunc International, Rochester, NY) were first coated overnight at 4°C with 100 μL of a 10 times diluted 0.1% poly L-lysine solution and then with 100 μL of the bacterial suspension in PBS for 2 hours at 30°C. Wells were washed 3 times with leptospira culture medium (LCM) and 100 μL of twofold dilutions of each ascites MAb in LCM was added to the wells for 2 hours at 30°C. Washing was repeated and 100 μL of rabbit anti-mouse Ig-POase conjugated was added for 2 hours at 30°C. After 2

washes with LCM and 3 washes with PBS, 100 μL of enzyme substrate/chromogen solution (H_2O_2/ortophenylenediamine) in citrate-phosphate buffer, pH 5.0, was added and the reaction was allowed to take place in the dark for 10 minutes. Optical density was read at 450 nm in an ELISA reader (Multiskan MCC/340, Titertek Instruments, Huntsville, AL). ELISA with heat-treated leptospiral cells was performed in the same way except that leptospiral cultures were first inactivated overnight at 56°C and stored at −20°C until use. As control of cell integrity, rat serum against cytoplasmatic protein GroEL was included in both tests. To investigate how protein conjugation would affect MAb performance in ELISA, a conjugate of MAb 1D9 and POase was also used in this experiment.

Direct and indirect immunofluorescence

Slide chambers (ICN Biomedicals Inc., Costa Mesa, CA) were coated with a 0.01% Poly L-lysine solution (Sigma Aldrich) and dried for 1 hour at room temperature. A 7-day culture of *L. interrogans* L1-130 was washed once in PBS, resuspended to a density of 10^8 cells per milliliter in PBS, and incubated in the slide chamber for 2 hours at 30°C. The slides were washed twice with LCM and coated with MAb ascites diluted 1:10 in LCM. After incubating for 1 hour at 30°C, the slides were washed again twice with LCM and a 1:100 dilution of rabbit anti-mouse FITC conjugate was added and incubated for 1 hour in a dark humid chamber at 30°C. After washing with LCM a drop of mounting medium was added and a coverslip was sealed with acrylic. In experiments with permeabilized membrane, slides were incubated in 5 mL of methanol for 10 minutes at 4°C followed by washing twice with LCM. The following controls were used in this experiment: (1) MAb against *Salmonella* OMP was used as primary antibody, (2) rabbit anti-mouse FITC conjugate was applied to slides without primary antibody, and (3) normal mouse serum was used as primary antibody. For direct immunofluorescence MAb 1D9 FITC conjugate was produced following established procedures[14] and used together with a conjugate of rabbit antibodies against whole *Leptospira* and FITC (Ames Laboratories, National Veterinary Services Laboratory, Ames, IA) as positive control. Labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm.

Immunoprecipitation of native LipL32 and immunomagnetic separation of leptospiral cells

Surface immunoprecipitation of native LipL32 was performed according to Shang et al.[15] with modifications. A 2-mL volume of each heat-inactivated MAb ascites was mixed with 30 mL of a culture of *L. interrogans* L1-130 containing 3×10^{10} actively motile bacteria. After shaking the suspension gently for 1 hour at 30°C the cells were pelleted at 2000*g* for 15 minutes at 4°C, resuspended in PBS with 5 mM $MgCl_2$, centrifuged again, and resuspended in 9 mL of 10 mM Tris-HCl (pH 8.8), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethyl sulfonyl fluoride (PMSF). A volume of 1 mL of 10% protein grade Triton × 100 (Sigma Aldrich) was added and the suspension was agitated for 30 minutes at 4°C. The insoluble material was removed by centrifugation at 16,000*g* for 20 minutes and 1 mL of 2% deoxycholate, 50 μL of 20% SDS and 500 μL of Sepharose-SpA (Sigma Aldrich) were added to the supernatant. After agitating the mixture gently for 30 minutes at 4°C, the complex of Sepharose SpA-MAb-antigen was washed by centrifugation two times with 0.01% Triton × 100 in 10 mM Tris-HCl (pH 8.8) and resuspended in final sample buffer. The complex was submitted to SDS-PAGE and immunoblotting using MAb 1D9-POase as probe. As negative controls two additional immunoprecipitation experiments were carried out in parallel: in the first one MAb against LipL32 was omitted and in the second one the culture of *L. interrogans* L1-130 was replaced with *L. biflexa* serovar patoc. For the immunomagnetic separation (IMS) procedure, 1D9 MAb was absorbed onto protein A-coated microspheres (Bangs Laboratories Inc., Fishers, IN). Briefly, 100 μL of particles with 1% solids was suspended with 900 μL of 50 mM pH 8.2 borate buffer (BB), washed twice with BB, resuspended in 1 mL of BB containing 1.2 mg of MAb and incubated with gentle agitation at 4°C for 16 hours. The MAb-coated particles were washed twice with BB and resuspended in stock buffer (100 mM borate pH 8.5, 0.1% bovine serum albumin [BSA], 0.05 Tween 20, 10 mM EDTA, 0.1% NaN_3). An immunomagnetic separator (Invitrogen Corporation, Carlsbad, CA) was used in washing steps. Standard IMS was performed with 5 μL of MAb-coated particles per milliliter of different dilutions of a pool of sterile human sera artificially contaminated with pathogenic and saprophytic *Leptospira* species. The serum samples were gently agitated for 15 minutes at room temperature and then washed twice on a magnetic separator. The particles were then resuspended in 20 μL of lysis buffer (0.02 M Na_2HPO_4; 0.15 M NaCl; pH 7.2), boiled for 10 minutes and stored at −20°C until use in polymerase chain reaction (PCR) as described below.

PCR conditions

PCR primers *lipL32* F: 5′ CGC TTG TGG TGC TTT CGG TGG T 3′ and *lipL32* R: 5′ CTC ACC GAT TTC GCC TGT TGG G 3′ were used, resulting in a 264 bp amplicon of the *lipL32* coding region. Briefly, 2 μL of DNA-IMS template was added to a tube with 1 U *Taq* DNA polimerase (Invitrogen) 150 ng of primers, 2.5 μL of 10× reaction buffer containing $MgCl_2$ and 0.2 mM dNTP. For DNA template optimization, volumes of 10 μL, 5 μL, 3 μL, 2 μL, and 1 μL of DNA-IMS were tested in the same conditions. Amplification was carried out in a Perkin Elmer 2400 thermocycler (PE Biosystems, Foster City, CA) with 1 cycle at 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and an extension of 7 minutes at 72°C at the end of the final cycle. Aliquots were analyzed by electrophoresis in 2% agarose gel with ethidium bromide and visualized under UV transillumination.

RESULTS

Generation of MAbs

From a total of 54 hybridomas tested in the primary ELISA, 3, named 1D9, 36C4, and 412H4, have shown a specific reaction in the secondary ELISA screening and were selected for further characterization and testing in diagnostic applications.

TABLE 1. REACTIONS OF ANTI-LIPL32 MABS WITH DIFFERENT STRAINS OF *LEPTOSPIRA* IN IMMUNOBLOTTING

			MAbs		
Serogroup	*Serovar*	*Genomoespecies*	*1D9*	*412H*	*36C*
Australis	Australis	*L. Interrogans*	+	+	+
Australis	Bratislava	*L. Interrogans*	+	+	+
Autumnalis	Autumnalis	*L. Interrogans*	+	+	+
Autumnalis	Butembo	*L. Kirshneri*	+	+	+
Autumnalis	Rachmat	*L. Interrogans*	+	+	+
Bataviae	Bataviae	*L. Interrogans*	+	+	+
Canícola	Canícola	*L. Interrogans*	+	+	+
Hebdomadis	Hebdomadis	*L. Interrogans*	+	+	+
Icterohaemorrhagiae	Copenhageni	*L. Interrogans*	+	+	+
Icterohaemorrhagiae	Icterohaemorrhagiae	*L. Interrogans*	+	+	+
Pomona	Pomona	*L. Interrogans*	+	+	+
Pyrogenes	Pyrogenes	*L. Interrogans*	+	+	+
Sejroe	Wolffi	*L. Interrogans*	+	+	+
Sejroe	Hardjo	*L. Interrogans*	+	+	+
Djasiman	Sentot	*L. Interrogans*	+	+	+
Djasiman	Djasiman	*L. Interrogans*	+	+	+
Ballum	Castellonis	*L. Borgpetersneii*	+	+	+
Ballum	Ballum	*L. Borgpetersneii*	+	+	+
Celledoni	Withcombi	*L. Borgpetersneii*	+	+	+
Javanica	Javanica	*L. Borgpetersneii*	+	+	+
Tarassovi	Tarassovi	*L. Borgpetersneii*	+	+	+
Cynopteri	Cynopteri	*L. Kirshneri*	+	+	+
Grippotyphosa	Grippotyphosa	*L. Kirshneri*	+	+	+
Louisiana	Louisiana	*L. Noguchii*	+	+	+
Panamá	Panama	*L. Noguchii*	+	+	+
Shermani	Shermani	*L. Santarosai*	+	+	+
Celledoni	Celledoni	*L. Weilii*	+	+	+
Andamana	Andamana	*L. Biflexa*	−	−	−
Semaranga	Patoc	*L. Biflexa*	−	−	−
Semaranga	Semaranga	*L. Meyeri*	−	−	−

MAbs, monoclonal antibodies.

The immunoglobulin classes of MAbs 1D9, 36C4, and 412H4 were IgG2b, IgG2a, and IgG3, respectively. All MAbs reacted with a band of an estimated molecular mass of 32 kd and neither reacted with *E. coli* as seen by immunoblotting (Table 1).

ELISA with intact and heat-treated leptospira

To evaluate MAb reactivity against native LipL32 protein ELISA protocols with intact and heat-treated leptospiral cells were used. A decrease in the absorbance of ELISA with intact cells occurred as the concentration of antibodies decreased, demonstrating the specificity of the reaction (Fig. 1A). In addition, antisera against the cytoplasmatic protein GroEL did not react in this ELISA. A conjugate of MAb 1D9-POase reacted with native LipL32 in both intact and heat-treated cell ELISA, but higher absorbance values were observed with heat-treated bacteria (Fig. 1B). In these ELISA experiments MAbs did not react with saprophytic bacteria and sera against GroEL reacted with heat-treated cells (data not shown).

Direct and indirect immunofluorescence

Reaction of MAbs with native LipL32 on the outer membrane of pathogenic *Leptospira* was investigated by indirect immunofluorescence and by an in-house prepared conjugate of MAb 1D9 and FITC. Bacterial cells were applied to slides with and without methanol treatment to evaluate the effect of the fixing method on MAb reactivity. The three MAbs labeled leptospiral cells in both fixing methods as could be seen by the intense fluorescence (Fig. 2). Labeling of leptospiral cells with MAb anti-*Salmonella* or normal mouse serum was not observed (data not shown).

Immunoprecipitation of native LipL32 and use of a MAb on IMS-PCR

The MAbs were used in immunoprecipitation assays to investigate their ability in binding the surface of live and motile leptospiral cells in suspension. Two MAbs were able to precipitate LipL32 from the suspension of live pathogenic bacteria (Fig. 3). In addition, MAb 1D9 was adsorbed onto protein A magnetic beads to explore its potential for use in diagnostic assays, which associates IMS with PCR amplification. With the use of IMS for capturing bacterial cells prior to DNA extraction and PCR amplification, it was possible to detect as low as 10 leptospires per milliliter of artificially contaminated human sera (Fig. 4). Sera contaminated with saprophytic *Leptospira* submitted to IMS and PCR did not show any amplification product (data not shown).

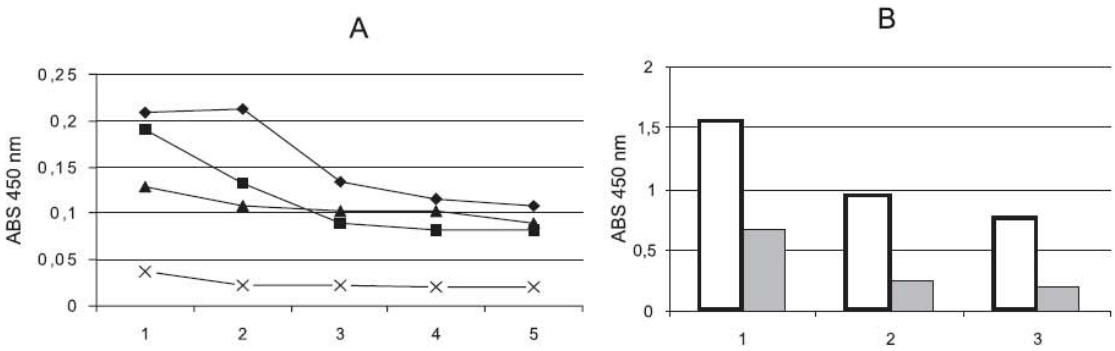

FIG. 1. Enzyme-linked immunosorbent assay (ELISA) reactions of monoclonal antibodies (MAbs) anti-LipL32 using intact and heat-treated *L. interrogans* L1-130 cells. **A**: ELISA using 10^9 intact leptospiral cells per milliliter (100 μl per well) and MAbs 1D9 (◆), 412H4 (■), 36C4 (▲), and GroEL antiserum (×) diluted 1:4 (1), 1:8 (2), 1:16 (3), 1:32 (4), and 1:64 (5). **B**: ELISA reactions with intact (□) and heat-treated cells (■) at 10^9 cell per milliliter (1), 4×10^8 cell per milliliter (2) and 2×10^8 cell per milliliter (3) and 1D9-POase conjugate diluted 1:1000.

DISCUSSION

Leptospiral outer membrane protein LipL32 is prominent on pathogenic strains and is the major detergent-phase protein extracted by Triton X 114.[4,16] Human sera from cases of leptospirosis reacted with native LipL32 and its recombinant form expressed in *E. coli*.[5] After these findings this antigen has been used for developing diagnostic tests that detect antibodies in human and animal leptospirosis.[6,7,17,18] However, diagnostic tests based on antibody detection suffer from lack of sensitivity in the first 10 days of infection. Antigen detection tests should be preferred when early diagnosis is important for initiating treatment, as is the case of leptospirosis.[1,19] MAbs are important tools for antigen detection tests because of their high specificity and permanent availability. The three MAbs reported in this study reacted with a 32-kd polypeptidic band from dif-

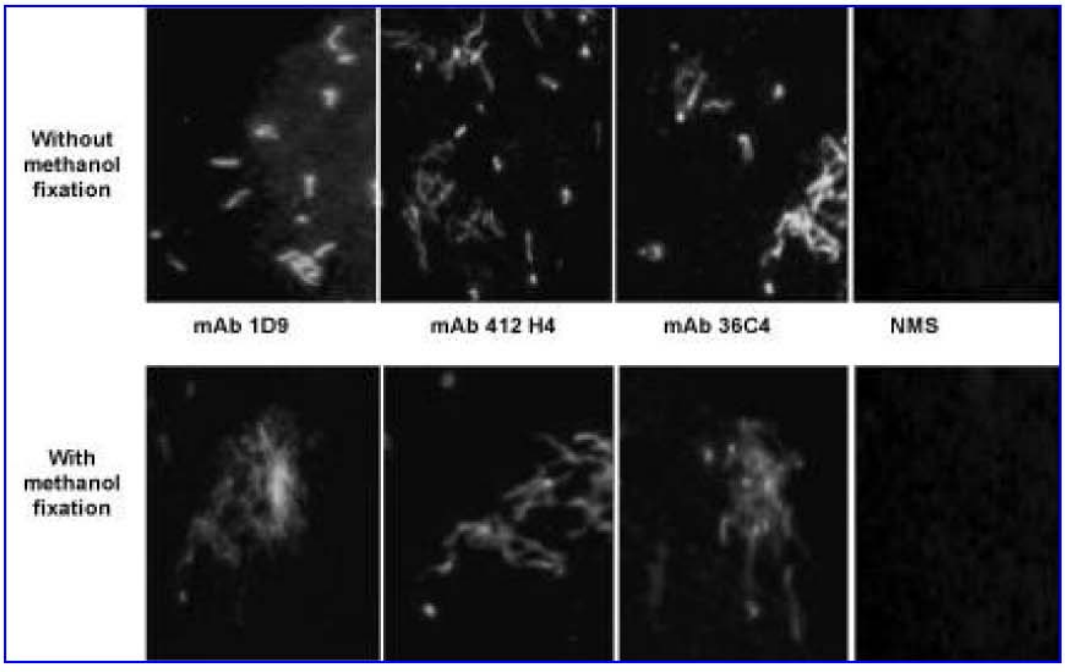

FIG. 2. Staining of LipL32 from *L. interrogans* L1-130 by indirect immunofluorescence. *L. interrogans* L1-130 was fixed to microscope slides with and without methanol and probed with monoclonal antibodies (MAbs). Test control slides were made by reacting fixed bacteria with normal mouse sera and rabbit anti-mouse fluorescein isothiocyanate (FITC).

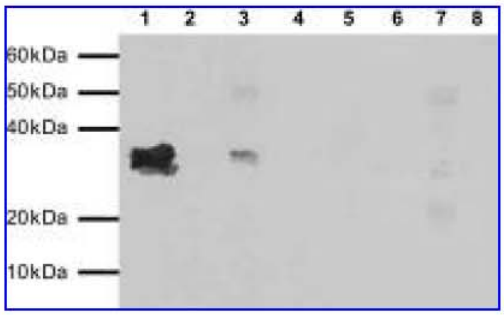

FIG. 3. Immunoprecipitation of native LipL32 on the surface of *L. interrogans* L1-130. Bacterial cells were reacted with monoclonal antibodies (MAbs) before Triton X-100 membrane fractionation and precipitated with protein A-Sepharose. Precipitated material was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blots were probed with MAb 1D9-POase. 1, rLipL32; 2, *L biflexa* Patoc, and MAb 1D9; 3, *L. interrogans* L1-130, and MAb 1D9; 4, *L. biflexa* Patoc and 412H; 5, *L. interrogans* L1-130 and 412H; 6, *L. biflexa* Patoc and 36C; 7, *L. interrogans* L1-130 and 36C; and 8, *L. interrogans* L1-130 treated with protein A-Sepharose only.

ferent leptospiral serovars on immunoblotting, a molecular mass similar to that of LipL32. Moreover, anti-LipL32 MAbs 1D9, 36C4 and 412H4 did not react with the other microorganisms tested. The MAbs were also able to detect native LipL32 antigen when used as primary antibody in indirect ELISA with intact and heat-treated leptospiral cells. Furthermore, MAb 1D9 maintained antibody activity after conjugation with peroxidase suggesting it is suitable for two-site immunoassays such as sandwich ELISA.

Indirect immunofluorescence was used to investigate MAb reaction after fixing leptospiral cells with or without methanol treatment. When anti-LipL32 MAb 1D9 was used conjugated to FITC, it was possible to visualize leptospiral cells by direct fluorescence. MAbs have been successfully used in immunofluorescence based diagnostic tests for Lyme and syphilis, two diseases also caused by spirochetes.[20,21] The antibodies reported here were able to attach to the bacterial outer membrane after the two fixing treatments, providing evidence of their usefulness for immunohistochemical tests.

Surface immunoprecipitation experiments were carried out to explore MAb potential for use in immunoseparation techniques or chromatographic assays. IMS has been used to increase PCR sensitivity and to improve bacterial isolation from clinical samples containing inhibitory substances or contaminating organisms.[22,24] Two of the MAbs obtained in this study were able to form complexes with live leptospiral cells suspended in a liquid phase suggesting that they coud be useful for IMS. One of these MAbs was adsorbed to magnetic particles and used to develop a method for *Leptospira* detection in association with PCR amplification of the *lipL32* gene. Using this novel method we were able to detect approximately 10 leptospiral cells per milliliter of human sera experimentally contaminated. An assay that uses MAb-based magnetic separation coupled to PCR amplification for detection of *L. borgpetersenii* serovar hardjo in urine from experimentally contaminated cattle has been reported.[25] Despite its good sensitivity, the assay consistently detected 10 organisms in urine samples spiked with leptospires, it may have an important limitation in specificity since the mAbs used for IMS are directed to LPS or a non-characterized outer envelope protein.

In conclusion, the results reported in this study demonstrated that MAbs generated against the recombinant form of LipL32 were able to recognize the native protein from pathogenic *Leptospira.* Because LipL32 is a surface protein conserved among pathogenic serovars and is not found on saprophytic strains, the MAbs against this target will be useful in the development of different test formats for diagnosis of human and animal leptospirosis.

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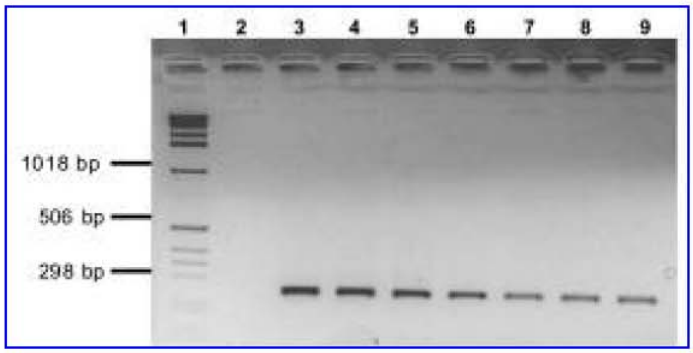

FIG. 4. Polymerase chain reaction (PCR) amplification of the *lipL32* coding region from *L. interrogans* L1-130 after immunomagnetic separation from experimentally contaminated human sera. 1, DNA ladder; 2, *L. biflexa* Patoc 10^7 cells per milliliter; 3, 10^7; 4, 10^6; 5, 10^5; 6, 10^4; 7, 10^3; 8, 10^2; 9, 10^1 cells per milliliter.

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