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Avaliação do potencial imunoprotetor de antígenos recombinantes de *Leptospira interrogans*

Samuel Rodrigues Felix

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Avaliação do potencial imunoprotetor de antígenos recombinantes de *Leptospira interrogans*

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RESUMO

FELIX, Samuel Rodrigues. **Avaliação do potencial imunoprotetor de antígenos recombinantes de *Leptospira interrogans*** 2009. Dissertação (Mestrado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

A leptospirose é uma doença causada por espiroquetas do gênero *Leptospira*. Ela é a zoonose mais difundida no mundo, sendo um problema de saúde pública e veterinária, principalmente em países em desenvolvimento. Na área de veterinária, a leptospirose é preocupante do ponto de vista clínico e econômico, devido ao risco à saúde pública, perdas reprodutivas e óbitos. A vacinação animal é amplamente realizada como medida de prevenção da enfermidade. Entretanto, a proteção conferida pelas vacinas convencionais é limitada aos sorovares nelas presentes, não evitando o estado portador, mantendo assim o potencial transmissor desses animais, para humanos e outros animais. Por outro lado, vacinas para humanos estão disponíveis e empregadas em alguns países como a China e Cuba. O objetivo deste trabalho foi avaliar, em modelo animal, proteínas recombinantes de *Leptospira interrogans* quanto ao seu potencial imunoprotetor. Para tanto, vinte proteínas foram expressas em *Escherichia coli* e, após a adsorção em hidróxido de alumínio, foram administradas em hamsters através da via intramuscular, em duas doses, com intervalo de 14 dias. Duas semanas após a segunda dose realizou-se o desafio dos animais com 100 leptospiras (~2xDL_{50}). Após o desafio, os animais foram observados diariamente para o aparecimento de sinais clínicos da enfermidade e óbitos, e após 24 dias procedeu-se a eutanásia e necropsia dos animais sobreviventes. Como resultado da avaliação realizada, sete proteínas conferiram proteção ao modelo animal que variou de 16,7% a 50%. Das proteínas que apresentaram maiores níveis de proteção, duas foram indicadas como possíveis candidatas à vacina recombinante contra leptospirose.

Palavras-chave: Leptospirose. Vacina recombinante. Vacina de subunidade. Imunoproteção

ABSTRACT

FELIX, Samuel Rodrigues. **Evaluation of the immune protective potential of *Leptospira interrogans* recombinant antigens**, 2009. Dissertação (Mestrado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a disease caused by pathogenic spirochetes of the *Leptospira* genus. It is the most widespread zoonosis in the world, representing a veterinarian and public health problem especially in developing countries. From a veterinarian point of view, leptospirosis is important both as a medical problem, mainly in dogs, which can suffer the ailment as well as transmit it, and as an economic problem, mainly in cattle and pigs, where it causes direct production and reproductive losses. Vaccination of these animals is largely applied, however protection is limited. Conventional vaccines confer protection restricted to the sorovars present in the preparation and fail to protect against renal infection, allowing seemingly healthy animals to shed bacteria, and produce short term protection requiring frequent revaccination. The vaccines are also available for humans, but are used only in a few countries such as Cuba and China. The goal of this work was to test the immune protective potential of recombinant *Leptospira interrogans* proteins produced in *Escherichia coli*, using the hamster model. Twenty recombinant vaccine candidates were produced and inoculated in hamsters in two doses, fourteen days apart. Two weeks after the second dose all animals were challenged with 100 leptospires (~2xLD_{50}). After the challenge, animals were observed daily for disease. Of all the proteins tested, seven protected animals to some extent, ranging from 16.7% to 50%. Two proteins presented promising results, and were recommended for further studies, as potential leptospira subunit vaccine candidates.

Keywords: Leptospirosis. Recombinant vaccine. Immune protection.

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1. INTRODUÇÃO GERAL

A leptospirose é uma doença de distribuição mundial, acometendo mais de 500.000 pessoas por ano (WHO, 1999), é hoje considerada uma das zoonoses mais difundidas do planeta (ADLER; MOCTEZUMA, 2009). A doença é causada por espiroquetas do gênero *Leptospira*, o qual possui 20 espécies e 24 sorogrupos, com mais de 250 sorovares descritos (CERQUEIRA; PICARDEAU, 2009). Apesar de ser uma doença cosmopolita, ela é mais prevalente em países da África, América Latina e Ásia (LEVETT, 2001), principalmente devido a fatores ambientais, climáticos, socioeconômico, saneamento básico e pela diversidade de hospedeiros suscetíveis domésticos e silvestres (LEVETT, 2001; KO et al., 2009).

No período de 1997 à 2008, o Brasil registrou 23.574 casos de leptospirose humana, com letalidade variando de 8,5 à 12,6%, dependendo do ano (MINISTÉRIO DA SAÚDE, 2009). Dados oficiais referentes à ocorrência da enfermidade em animais no Brasil são escassos. A prevalência sorológica em bovinos é de 36,7%, sendo que 84,1% das propriedades rurais apresentam casos da doença (FAVERO et al., 2001). Em 2000, JOUGLARD e BROD encontraram a prevalência de 2,66% em cães do meio rural de Pelotas. Estudos mais recentes, desenvolvidos pelo nosso grupo, revelaram prevalência de 13,8% em cães não domiciliados do meio urbano de Pelotas (dados não publicados).

A circulação de leptospiras no ambiente é mantida principalmente por espécies ditas “hospedeiras de manutenção”. Essas espécies são adaptadas a determinados sorogrupos, dos quais dificilmente sofrem doença severa, tornando-se portadores renais (KO et al., 2009) e eliminando as bactérias por longos períodos. Os humanos são hospedeiros acidentais da enfermidade, manifestando a doença na forma sub-clínica ou clínica, mas dificilmente tornando-se portadores renais crônicos (ADLER; MOCTEZUMA, 2009). Na forma sub-clínica, cerca de 5 à 10% dos casos tornam-se graves, com hemorragias, icterícia, falência renal e/ou hemorragias pulmonares, nesses casos o risco de morte pode ser de até 50% (BHARTI, et al., 2003; McBRIDE, et al., 2005).

A leptospirose canina possui sintomatologia similar a leptospirose humana, como icterícia, mialgia, êmese e morte (BOUTELIER, et al., 2003). De acordo com a

doença clínica e o sorovar causador, são descritas quatro síndromes associadas à leptospirose canina: ictérica, urêmica, hemorrágica e reprodutiva (ADLER; MOCTEZUMA, 2009). A forma amplamente utilizada para o controle da doença em cães é o emprego de vacinas. Entretanto, as vacinas comerciais disponíveis protegem apenas contra um número pequeno de sorovares, e a duração da proteção é limitada (MINKE et al., 2009). Cães que se recuperam naturalmente da leptospirose, mesmo vacinados, muitas vezes tornam-se portadores renais da bactéria, especialmente do sorovar Canicola, passando a disseminar a doença para outros animais e humanos (LEVETT, 2001).

Assim como os cães, animais de produção podem tornar-se carreadores renais da bactéria, perpetuando a doença dentro dos rebanhos (LEVETT, 2001). Por isso, a leptospirose causa grande impacto econômico para a agropecuária, com altos índices de abortos, natimortos, infertilidade e redução na produção de leite (ELLIS, 1994). Resultando em graves prejuízos para os produtores e, conseqüentemente, para a economia dos países acometidos, pois causam transtornos produtivos e reprodutivos (FAINE et al., 1999).

Atualmente, as vacinas disponíveis para humanos e animais são consideradas de baixa eficácia, principalmente por serem limitadas aos sorovares que constituem essas preparações. Isso ocorre devido à existência de mais de 250 sorovares isolados no mundo (ADLER; MOCTEZUMA, 2009), impedindo assim, uma proteção de amplo espectro. Aliado a esse fato, são considerados ainda, os efeitos colaterais relatados como dor no local da aplicação, febre e desconforto causado pelo aumento de volume no local, além da necessidade de freqüentes revacinações tanto para humanos quanto para animais (McBRIDE et al., 2005).

Investimentos foram feitos para gerar conhecimento relacionado à genética e biologia molecular das leptospiras. Tais esforços culminaram com o sequenciamento total do genoma de duas espécies patogênicas, *L. Interrogans* e *L. borgpetersenii* bem como de uma espécie saprófita, a *L. biflexa* (REN et al., 2003; NASCIMENTO et al.,2004; BULACH et al., 2006; PICARDEAU et al., 2008). Estes estudos revelaram um genoma de dois cromossomos de aproximadamente 4 Mb e 300 Kb respectivamente, e um conteúdo GC entre 35 e 41%, a *L. biflexa* possui ainda um terceiro replicon de 74 kb. Estudos genômicos mais recentes, somados a outros trabalhos de comparação de genomas (XUE; PICARDEAU, 2008) revelaram

alguns fatores de virulência e proteínas de membrana que poderão vir a ser usados em estudos de vacinologia e diagnóstico da doença.

Esforços para a identificação de componentes imunogênicos com potencial para o desenvolvimento de vacinas recombinantes resultaram na caracterização de várias proteínas de membrana externa, mas poucas delas foram avaliadas quanto à sua capacidade imunoprotetora (SHANG et al., 1995; HAAKE et al., 1999; BRANGER et al., 2005; FAISAL et al., 2008). Nos últimos anos, proteínas recombinantes de membrana externa (OMPs) de leptospiras como OmpL1, LipL41, LipL32, e *Leptospiral immunoglobulin-Like protein* A (LigA) foram avaliadas como candidatas potenciais à vacinas em modelos animais suscetíveis para a leptospirose (SHANG et al., 1995; HAAKE et al., 1999; FAISAL et al., 2008). Recentemente, nosso grupo demonstrou que a imunização de hamsters com um fragmento de LigA (SILVA et al., 2007) e com BCG expressando LipL32 (SEIXAS et al., 2007), podem representar estratégias potenciais na proteção contra a leptospirose. Além disso, o nosso grupo caracterizou a virulência em modelo experimental suscetível de cinco cepas de *Leptospira*, isoladas no Brasil, e que são pertencentes a sorogrupos causadores de leptospirose no Brasil e no mundo (SILVA et al., 2008).

Nesse trabalho aplicamos os conhecimentos adquiridos e as tecnologias desenvolvidas por nosso grupo até este momento, com o intuito de avaliar 20 antígenos recombinantes adsorvidos em hidróxido de alumínio como adjuvante, quanto à capacidade de induzir proteção contra desafio letal de leptospiras em oito diferentes experimentos de triagem em hamsters, um modelo animal suscetível a leptospirose.

Inicialmente, apresentamos o artigo 1 que foi publicado no periódico Procedia in Vaccinology, onde são relatados dados dos ensaios de imunoproteção de oito imunógenos. Os alvos avaliados neste trabalho foram todos produzidos no Centro de Biotecnologia da UFPel (CENBIOT), Pelotas, Brasil.

Em seguida, o artigo 2 trata dos resultados dos demais alvos triados. Este trabalho traz os resultados dos ensaios de imunoproteção usando como imunógenos os alvos produzidos por nossos colaboradores da Universidade Federal Fluminense e Fundação Oswaldo Cruz. Este artigo está formatado segundo as normas do periódico Clinical and Vaccine Immunology.

Nos trabalhos aqui apresentados, nós descrevemos 7 alvos que foram capazes de conferir algum grau de proteção contra desafio letal com leptospiras

patogênicas. De todos os antígenos recombinantes testados, recomendamos dois para futuros estudos, podendo estes vir a compor uma vacina recombinante contra leptospirose.

2. Artigo 1

Leptospirosis Vaccine: Search for Subunit Candidates

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Leptospirosis Vaccine: Search for Subunit Candidates

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Abstract
Subunit vaccines are a potential intervention strategy against leptospirosis, which is a major public health problem in developing countries. Thus far, several proteins have been evaluated as potential vaccine candidates, but all those tested in aluminum hydroxide adjuvant have failed to protect animals against lethal challenge. Seven new leptospiral lipoproteins were evaluated as vaccine candidates. The coding sequences of these lipoproteins were amplified by PCR from *Leptospira interrogans* serovar Copenhageni, strain Fiocruz L1-130, cloned and expressed in *Escherichia coli*. The purified proteins were adsorbed in aluminum adjuvant and used in the immunization of four to six weeks old hamsters. After two doses of 60 µg of recombinant protein, hamsters were challenged with a lethal dose of *L interrogans*. All seven tested proteins failed to fully protect the animals from disease or death. Further study must be undertaken toward developing an efficient subunit vaccine against leptospirosis for humans, livestock and pets.

Keywords: Leptospira; hamster model; recombinant vaccine; LD 50%

1. Introduction

Leptospirosis is a bacterial disease that affects both humans and animals (29). It is found in a wide variety of environmental contexts, in industrialized and developing countries, and in urban and rural areas (2). The number of human and animal cases worldwide is not well-documented. During outbreaks and in high-risk groups, 100 or more per 100,000 may be infected and for several reasons leptospirosis is overlooked and consequently underreported (28). It is currently considered a neglected tropical bacterial disease (11). In developing countries, leptospirosis has become an urban health problem as slum settlements have grown and outbreaks in these communities have increased (12, 14, 21). In these settings, current control measures have been ineffective in addressing leptospirosis (16). Pathogenic *Leptospira* are highly invasive bacteria capable of infecting a broad range of mammalian hosts (5), and the host infection produces a diverse array of clinical manifestations (2). This infecting agent is transmitted from one animal carrier to another, or to humans, via direct or indirect contact with urine or other body fluids that contain viable leptospires (15).

Current vaccines against leptospirosis target the lipopolysaccharide coat of leptospires, which has over 300 serovars (1), and many different strains with small antigenic differences can be found in some serovars, thus limiting cross-protection (2). The identification of new targets expressed during infection is important for the development of new immunoprotective strategies (9). To date, there is incomplete knowledge on the mechanisms of protective immunity against leptospiral infection, and the current emphasis in research laboratories is to discover cross-species and cross-serovar-conserved protective antigens that may provide longer-term protection from a broad range of *Leptospira* (27)

Several recombinant outer membrane proteins (OMP) have been shown to be protective immunogens in animal models of bacterial diseases (7, 13, 26). Recombinant leptospiral OMPs such as OmpL1, LipL41, LipL32, and Leptospiral immunoglobulin-like protein A (LigA) have been evaluated as potential vaccine candidates (4, 6, 10, 18). Our group has recently demonstrated that vaccination with a LigA fragment (24) or with a recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen (23), may constitute potential intervention strategy against leptospirosis. In the present study we evaluate eight recombinant proteins previously identified as potential subunit vaccine candidates (8).

2. Protein production: cloning, expression and purification

L. interrogans serovar Copenhageni strain Fiocruz L1-130 (14, 17) was cultivated in Ellinghausen- McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories) at 29 °C. Culture growth was monitored by counting in a Petroff-Hausser chamber (Fisher) and dark-field microscopy as previously described (5). *Escherichia coli* strains BL21 (DE3)-RIL and STAR were grown in Luria-Bertani (LB) medium at 37 °C (20). Primers were designed to include most of the target genes but not their highly hydrophobic signal sequences, they also included a restriction enzyme site. Coding sequences were amplified by PCR and cloned into the expression vectors pQE30 (Qiagen) or pAE (19). Recombinant plasmids were used to transform *E. coli* strains by electroporation and *E. coli* transformants containing the constructs were cultured at 37 °C. Expression was induced by isopropyl-β-d-thiogalactopyranoside (IPTG), at 1 mM final concentration. The cells were harvested

by centrifugation, resuspended in column buffer (8 M urea, 100 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8.0), and disrupted by sonication (3× 1 min pulses; Sonics & Material Inc.). His-tagged proteins were purified by affinity chromatography in a nickel (Ni+2) charged Sepharose column. Columns containing bound protein were washed with 10 volumes of column buffer, then 6 × wash buffer (6 M urea, 100 mM Tris, 300 mM NaCl, pH 8.0) containing 5 mM imidazole for the first three washes and increasing to 10 mM imidazole for the remaining washes. His-tagged proteins were eluted from the column with wash buffer containing 250 mM imidazole. Dialysis procedure was used to remove urea and imidazole and to promote refolding of the recombinant proteins. Proteins in the final preparation were quantified by the Bradford method (3).

3. Imunization assays

Female Golden Syrian hamsters 4-6 weeks old were allocated into groups of 6 animals and immunized by intramuscular route (I.M.) with recombinant protein in aluminum hydroxide (15%) on day 0 and day 14. Immunization was performed with two 60 µg doses of recombinant protein. Hamsters were immunized with 250 µL per injection site. A negative control group of hamsters was immunized with aluminum hydroxide adjuvant and PBS, and a positive control group was immunized with killed *L. interrogans*. Immunized hamsters were challenged at age 8-10 weeks, fourteen days after the second immunization, with an intraperitoneal (I.P.) administration of 100 leptospires (~2 × LD50) of strain Fiocruz L1-130 (22). Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared. Surviving hamsters were euthanized on day 24 post-challenge. Experimental animals were maintained at the animal facility of the Biotechnology Center of the Federal University of Pelotas (UFPel). The animals were manipulated in accordance with the University guidelines of the Ethics Committee in Animal Experimentation throughout the experimental period.

4. Results and Discussion

All PCR products were successfully cloned and expressed in *E. coli* as a 6×His tag N-terminus fusion protein, which allows purification of the protein by affinity chromatography. All seven proteins were expressed as insoluble inclusion bodies. They were solubilized and purified using a buffer containing urea. After dialysis and SDS-PAGE, purity was estimated to be over 90%. The immunization assays failed to reveal a fully protective vaccine candidate. Although LIC12099 did fully protect in the first experiment, it failed to protect in a subsequent experiment. Another protein tested twice (in two different experiments), was LIC10508, because, although it did not fully protect, it delayed mortality (Table 1).

Extensive efforts have been made to identify cross-protective antigens of *Leptospira* (25, 27). Lipoproteins and other membrane associated proteins are potential vaccine candidates. However, only a few leptospiral proteins characterized so far have been able to confer protection when used to immunize susceptible animals (10, 18, 23-24) but none of them were able to fully protect, when compared to control groups. In our study, in one case the ~2 × LD50 challenge was not able to kill the entire control group, this did not however mask false protecting proteins, as they also failed to protect.

All experiments were undertaken in the same manner, therefore there would be no reason to attribute the lack of repeatability of the first results obtained with LIC12099, to experimental conditions. However, the same batch of proteins used in

the first experiment was used in the second, and the storage period may have somehow modified the proteins tertiary structure, although no degradation was observed in SDS-PAGE. Other proteins, though not fully protective, were able to delay death in several cases, which may indicate protective potential, if parameters such as dose volume, interval and adjuvant are optimized.

The mechanism of immune protection in leptospirosis remains unknown. There is need for studies directed towards defining how the immunity resulting from recombinant protein immunization can be improved. Determination of the relative contributions of humoral immune mechanisms and cellular-mediated immunity would provide insights about immune response against leptospirosis. Further experiments, evaluating other proteins, and probing leptospirosis associated immune response are currently being performed.

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Table 1. Protection conferred by immunization with recombinant lipoproteins against lethal challenge in the hamster model.

Experiment	Group[a]	Days until death	no. survivors/total
1	LIC12099	-	6/6 [d]
	LIC12730	11,11,12,13,14,14	0/6
	LIC10561	11,11,11,11,13	1/6
	LIC10508	11,13,13,13,18	1/6
	Whole-cell preparation[b]	-	6/6 [d]
	Control immunization[c]	11,11,12,13,13,14	0/6
2	LIC12099	10,13,13,13,14,14	0/6
	LIC10191	10,10,10,10,11,13	0/6
	LIC10508	9,9,9,10,11,12	0/6
	Whole-cell preparation	-	6/6 [d]
	Control immunization	9,9,9,10,11,11	0/6
3	LIC10011	11,12,13,13,14	1/6
	LIC11947	12,12,14,14,16,17	0/6
	LIC12538	11,11,13,13,14	1/6
	Whole-cell preparation	-	6/6[d]
	Control immunization	10,11,11,12,14	1/6

[a] Animals were vaccinated twice (60/60 μg) at 2-week interval and were challenged with Fiocruz L1-130 two week after the last vaccination.

[b] Immunizations were performed with heat-inactivated preparations of *L. interrogans* Fiocruz L1-130.

[c] Control immunizations were performed by administering PBS with aluminum hydroxide adjuvant.

[d] Protection against lethal challenge was statistically significant (P <0.05).

3. ARTIGO 2

Evaluation of the Immune Protective Potential of Leptospiral Antigens: a Subunit Approach

(Formatado segundo as normas do periódico Clinical and Vaccine Immunology)

Evaluation of the Immune Protective Potential of Leptospiral Antigens: a Subunit Approach

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ABSTRACT

Leptospirosis is the most widespread zoonosis in the world. Currently available vaccines are whole-cell preparations, and do not confer satisfactory immunity. In the light of recent leptospiral genome sequencing, several studies have tried to obtain protective recombinant immunogens, few were successful, and even fewer used approved protocols and adjuvants. The aim of this work was to screen 12 recombinant antigens, in aluminum hydroxide, evaluating their immune protective potential. Five experiments were conducted with groups of six female hamsters immunized with the subunit preparations (60 µg of recombinant protein + aluminum hydroxide) in two doses 14 days apart. The hamsters were then challenged with a lethal dose of *Leptospira interrogans*. Five groups presented a number of surviving animals above the control group, however only the group immunized with the protein LIC11325 resulted in statistically significant protection. Therefore, the authors recommend this protein for further studies regarding dose and application dynamics, for the development of a recombinant vaccine against leptospirosis.

INTRODUCTION

Leptospirosis, the most widespread zoonosis in the world, is caused by pathogenic spirochetes of the *Leptospira* genus (1). The transmission occurs through direct or indirect exposure to urine of mammalian reservoirs especially in settings such as floods, occupational exposure, and water sports, among others (2). The infection is usually asymptomatic or a self-resolving febrile illness. However, up to fifteen percent of all human infections may become a severe form of leptospirosis, with complications such as kidney failure and pulmonary hemorrhage, which can have fatality rates of up to 50% (9, 19). Mortality remains high because of delays in diagnosis due to lack of infrastructure and adequate clinical suspicion, and to other poorly understood reasons (2).

Although vaccines are recommended as a prevention method in risk settings (14), current commercially available vaccines are inactivated whole-cell bacterins, generating most of the immune response against the outer membrane lipopolysaccharide (LPS) (25). However, there are over 250 serovars identified so far (6), with the main antigenic differences attributed to the LPS, making the commercial vaccines limited to short term, serovar-specific immunity, as well as several adverse reactions and side effects (9). These vaccines are currently used in humans in Cuba and China, but they are mostly restricted for animal use (19), especially for dogs, cattle and pigs (1). Therefore an effort is being made to produce new leptospiral vaccines, which are cross protective among serovars, and have fewer side effects for animal and human use.

In recent years, many proteins have been tested in animal models as potential vaccine candidates, and several approaches have been used (3, 4, 10, 11, 13, 15, 20, 22, 23). Although most of these studies identified their potential targets based on

antigenicity analysis (12) and/or surface exposure (8), few of them were correlated with immunoprotection; in fact, a recent review revealed that only Silva and co-workers (23) were able to produce a protective antigen (1).

It is imperative that further studies be carried out to identify potential vaccine candidates. In the present study, we tested the immune protection induced by 12 leptospiral antigens in the hamster model.

MATERIALS AND METHODS

Leptospira strains. The *Leptospira* used in this study was *Leptospira interrogans* serogroup Icterohaemorragie serovar Copenhageni strain Fiocruz L1-130. Leptospires were cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories) at 28 ºC. Culture growth was monitored by counting in a Petroff-Hausser chamber (Fisher) and dark-field microscopy as described (Faine et al., 1999). *Escherichia coli* strains BL21 (DE3)-RIL, TOP10 and STAR were used in this study. They were grown in Luria-Bertani (LB) medium at 37 °C whenever cited.

Plasmid constructions, expression and purification of recombinant proteins. Proteins: LIC10325, LIC11859, LIC12253, LIC13006, LIC13306, LIC10021, LIC10645, LIC11184, LIC10087, LIC12555, LIC10501 and LIC12632 were identified from previous studies (18; 12). The genomic DNA of *L. interrogans* was used as template for amplification of the target sequences. PCR products were cloned into the pAE (21) or pQE (Qiagen) plasmid vectors. The pAE and pQE vectors contain a 6× His tag which is expressed fused to recombinant proteins. Primers were designed to include most of the target genes but not their highly hydrophobic signal sequences. They also included a restriction enzyme site each. Recombinant plasmids were used to transform *E. coli* strains by electroporation and *E. coli* transformants containing the constructs were cultured at 37 °C. Expression was induced by isopropyl-β-d-thiogalactopyranoside (IPTG), at 1 mM final concentration. The cells were harvested by centrifugation, resuspended in column buffer, and disrupted by sonication. His-tagged proteins were purified by affinity chromatography in a nickel (Ni^{+2}) charged Sepharose column. Columns containing bound protein were washed with 10 volumes of column buffer, then 4 × wash buffer containing 10

mM imidazole. His-tagged proteins were eluted from the column with elution buffer containing 250 mM imidazole. Dialysis procedure was used to remove urea and imidazole and to promote refolding of the recombinant proteins. Proteins in the final preparation were quantified by the Bradford method (5).

Protein gel electrophoresis and immunoblotting of recombinant proteins. All proteins were submitted to one dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to verify their molecular weight and sample purity. These proteins were also submitted to Western bloting using anti-histidine monoclonal antibody. After the samples were separated by SDS-PAGE, the gel was stained with *Commassie blue* solution to reveal the proteins. Otherwise, SDS-PAGE separated proteins were transferred to nitrocellulose membranes following the manufacturer's instructions (Mini transblotter, Bio-Rad). Membranes were blocked with a non-fat dried milk buffer, washed in TBST and incubated with monoclonal antibody anti-6 × histidines conjugated with alkaline phosphatase (diluted 1:2000 in TBST) (Sigma) for 1 h at room temperature. Membranes were washed again and color development was undertaken using ECL™ WB detection reagents following the manufacturer's instructions (GE Healthcare).

Hamster immune-protection studies. Female Golden Syrian hamsters with 4–5 weeks of age were obtained from the Biotério Central of the Universidade Federal de Pelotas (UFPel). The animals were immunized twice in the quadriceps muscle with the recombinant protein in an aluminum hydroxide solution (15%) on day 0 (zero) and 14. All vaccine doses contained 60 µg of purified recombinant protein, with standard volume application on a single injection site of 200 µL. Negative control groups in all experiments were inoculated with a 200 µL PBS and aluminum hydroxide (15%). Positive control groups were immunized with a 300 µL inactivated

whole-cell bacterin, containing approximately 10^8 leptospiras per dose. All the hamsters were challenged at age 8–9 weeks with an intraperitoneal inoculum of 100 leptospires from strain Fiocruz L1-130 (~2x LD_{50}) (24) 14 days after the last immunization. The inoculum was produced from Log phase cultures, and consisted of a one mL dose. Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared.

Five individual experiments were conducted, all of them comprised of groups of six hamsters, including one negative control (PBS) and one positive control (Bacterin) groups. Experiments were named E1 to E5 and evaluated the following proteins. E1: LIC13306, LIC13006, and LIC12253; E2: LIC11184, LIC10021 and LIC10646; E3: LIC11859 and LIC10325 E4: LIC12555 and LIC11087; E5: LIC10501 and LIC12632.

All animal studies were approved by the Ethics Committee for the Use of Experimental Animals of the Universidade Federal de Pelotas.

Statistical analysis. The Fisher Exact test and log-rank sum test were used to determine significant differences for mortality and survival, respectively, among the groups immunized and the negative control group. All *P* values were two-sided and a *P* value of <0.05 was considered to indicate statistical significance. GraphPad Prism 4 software systems (GraphPad Software) was used to perform the statistical analysis.

RESULTS

Production of recombinant proteins and vaccine preparation. All PCR products were successfully cloned and expressed in *E. coli* as a 6×His tag N-terminus fusion protein, which allowed purification of the proteins by affinity chromatography. The recombinant proteins required urea-promoted denaturing conditions for purification, followed by prolonged dialysis to obtain soluble protein preparations. All proteins used in this study were verified in SDS-PAGE and reacted with the monoclonal antibody. When necessary, proteins were concentrated prior to dialysis, permitting the standardization of vaccine doses to 200 µL. All vaccine doses were prepared at least one day prior to vaccination, and proteins were allowed to adsorb in the aluminum hydroxide overnight.

Protection of hamsters immunized with recombinant proteins against challenge with *L. interrogans.* Immunization with the LIC13006, LIC10021, LIC10645, LIC11184, LIC10087, LIC12555, LIC10501 and LIC12632 proteins did not prevent death among challenged hamsters. Although proteins LIC11859, LIC12253 and LIC13306 did produce more survivors than the negative control groups of their respective experiments, no statistical difference was observed. LIC10325 did prevent death in challenged hamsters ($p<0.05$) (log-rank test). The figure 1 shows the days to death timeline.

DISCUSSION

In the present study, we evaluated the immune protection capabilities of twelve leptospiral proteins. Previous studies have employed subunit vaccine approaches, but few have been able to produce positive results. Although LigA seems to be the most promising target (20, 23), immune protection is still to be shown in adjuvant approved for human use. Although some authors claim protection induced by LipL32, LigB, and other outer membrane proteins (4, 13, 15, 22), according to Adler and Moctezuma (1) only Silva and co-workers (23) were able to produce statistically significant results, however, using an adjuvant not approved for human use (complete Freud's adjuvant), which reduces the practical applicability of these results. Furthermore, there is some level of redundancy in leptospiral virulence factors and OMPs, knockout of genes thought to be essential did not reduce viability (7, 17), therefore, this may explain why single protein preparations did not produce the expected results.

Although it may seem that there were shortcomings in some experiments, where one animal from the negative control group survived, this is due to the fact that we used the minimum described dose to successfully produce death among hamsters (24), and it was expected that some animals could survive. It has been argued that animals that need high challenge inoculations make poor experimental models, not representing a real situation (16).

In this study, seven recombinant proteins were unable to promote significant immunoprotection against challenge with *L. interrogans*. Five proteins did produce survivors beyond the specific experiment control group, but four of them did not protect to the extent of statistical difference. Statistically significant protection was obtained with LIC10325 ($p<0.05$).

The protective LIC10325 protein described in this work may be a potential vaccine candidate, and further studies must be conducted on it. Several proteins have been able to induce protection using one delivery system but not another, such is the case with LipL32 (3, 4, 24) for example. This indicates the need for some adjustments, possibly dose, volume and frequency of application, as well as adjuvant alterations, in order to obtain better results.

In this study we were able to identify a potentially protective antigen. Further studies must be carried out to enhance its immunogenicity, and test its true potential. These studies are being conducted not only to test different immunization protocols, but also different adjuvant and forms of presentation.

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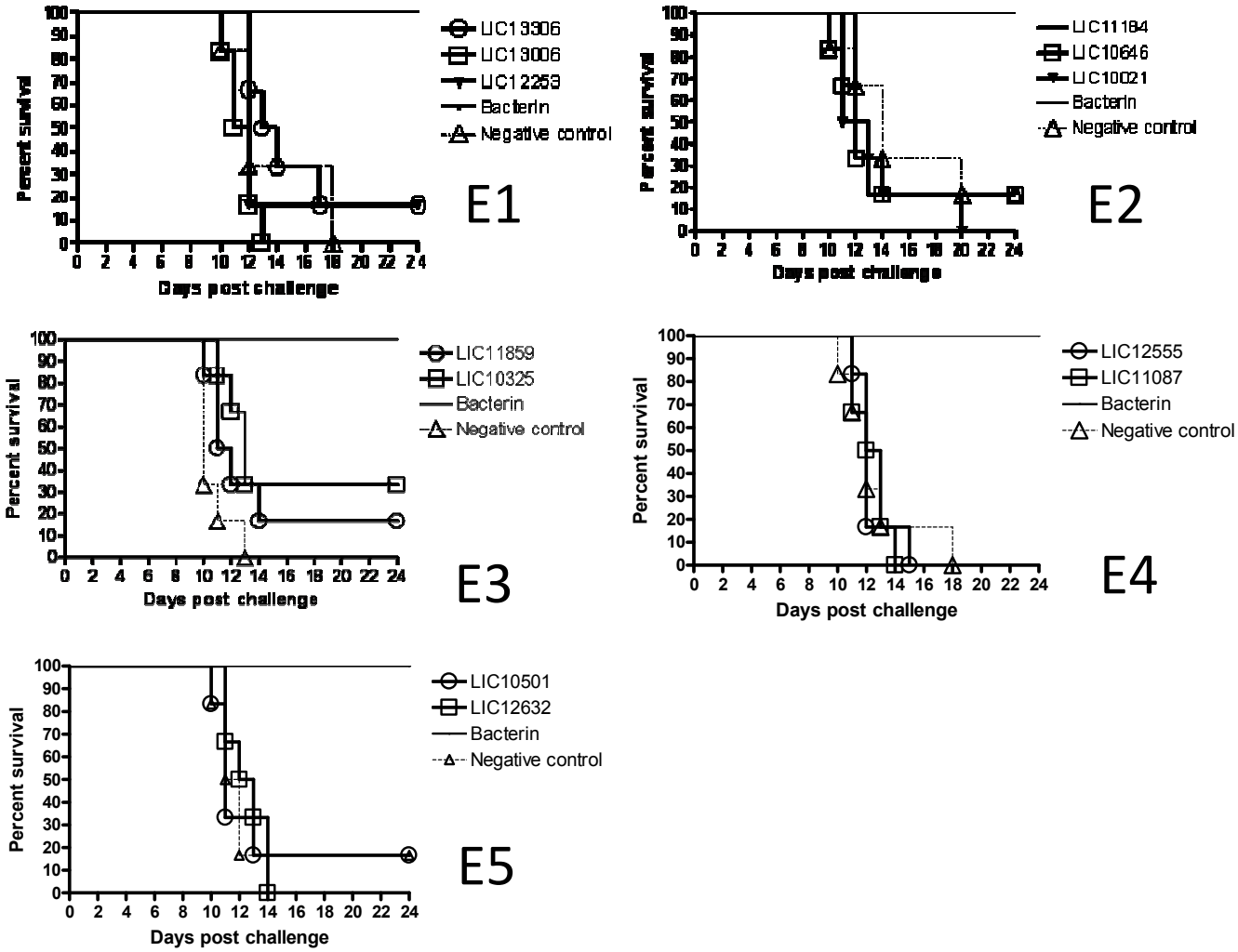

Figure 1. Survival graphs of all 5 experiments (listed E1 to E5). In all experiments the positive control group had 100% survival. In experiment 3 (E3), protection induced by LIC10325 was statistically different from the control group according to the logrank test (GraphPad). No other protein induced statistically significant protection.

4. CONCLUSÕES

- Os antígenos LIC11947, LIC10011, LIC10191, LIC12730, LIC12538, LIC10021, LIC10645, LIC11184, LIC10087, LIC12555, LIC10501 e LIC12632 não são capazes de conferir imunidade protetora quando utilizados como vacinas de subunidade para imunizar hamsters contra desafio letal com leptospiras virulentas.

- Os antígenos LIC12099, LIC10325, LIC10508, LIC10561, LIC13306, LIC11859 e LIC12253 promovem diferentes graus de sobrevivência ao desafio letal com leptospiras virulentas, porém em níveis não estatisticamente significativos quando comparados ao grupo controle negativo.

- Os antígenos LIC12099 e LIC10325 são capazes de induzir proteção quando utilizados para imunizar hamsters, portanto são candidatos para novos estudos visando o desenvolvimento de uma vacina contra a leptospirose.

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6. ANEXO

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Leptospirosis Vaccine: Search for Subunit Candidates

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Abstract

Subunit vaccines are a potential intervention strategy against leptospirosis, which is a major public health problem in developing countries. Thus far, several proteins have been evaluated as potential vaccine candidates, but all those tested in aluminum hydroxide adjuvant have failed to protect animals against lethal challenge. Seven new leptospiral lipoproteins were evaluated as vaccine candidates. The coding sequences of these lipoproteins were amplified by PCR from *Leptospira interrogans* serovar Copenhageni, strain Fiocruz L1-130, cloned and expressed in *Escherichia coli*. The purified proteins were adsorbed in aluminum adjuvant and used in the immunization of four to six weeks old hamsters. After two doses of 60 μg of recombinant protein, hamsters were challenged with a lethal dose of *L. interrogans*. All seven tested proteins failed to fully protect the animals from disease or death. Further study must be undertaken toward developing an efficient subunit vaccine against leptospirosis for humans, livestock and pets.

Keywords: Leptospira; hamster model; recombinant vaccine; LD 50%

1. Introduction

Leptospirosis is a bacterial disease that affects both humans and animals (29). It is found in a wide variety of environmental contexts, in industrialized and developing countries, and in urban and rural areas (2). The number of human and animal cases worldwide is not well-documented. During outbreaks and in high-risk groups, 100 or more per 100,000 may be infected and for several reasons leptospirosis is overlooked and consequently underreported (28). It is currently considered a neglected tropical bacterial disease (11). In developing countries, leptospirosis has become an urban health problem as slum settlements have grown and outbreaks in these communities have increased (12, 14, 21). In these settings, current control measures have been ineffective in addressing leptospirosis (16). Pathogenic *Leptospira* are highly invasive bacteria capable of infecting a broad range of mammalian hosts (5), and the host infection produces a diverse array of clinical manifestations (2). This infecting agent is transmitted from one animal carrier to another, or to humans, via direct or indirect contact with urine or other body fluids that contain viable leptospires (15).

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Current vaccines against leptospirosis target the lipopolysaccharide coat of leptospires, which has over 300 serovars (1), and many different strains with small antigenic differences can be found in some serovars, thus limiting cross-protection (2). The identification of new targets expressed during infection is important for the development of new immunoprotective strategies (9). To date, there is incomplete knowledge on the mechanisms of protective immunity against leptospiral infection, and the current emphasis in research laboratories is to discover cross-species and cross-serovar-conserved protective antigens that may provide longer-term protection from a broad range of *Leptospira* (27)

Several recombinant outer membrane proteins (OMP) have been shown to be protective immunogens in animal models of bacterial diseases (7, 13, 26). Recombinant leptospiral OMPs such as OmpL1, LipL41, LipL32, and Leptospiral immunoglobulin-like protein A (LigA) have been evaluated as potential vaccine candidates (4, 6, 10, 18). Our group has recently demonstrated that vaccination with a LigA fragment (24) or with a recombinant *Mycobacterium bovis* BCG expressing the LipL32 antigen (23), may constitute potential intervention strategy against leptospirosis. In the present study we evaluate eight recombinant proteins previously identified as potential subunit vaccine candidates (8).

2. Protein production: cloning, expression and purification

L. interrogans serovar Copenhageni strain Fiocruz L1-130 (14, 17) was cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories) at 29 °C. Culture growth was monitored by counting in a Petroff-Hausser chamber (Fisher) and dark-field microscopy as previously described (5). *Escherichia coli* strains BL21 (DE3)-RIL and STAR were grown in Luria-Bertani (LB) medium at 37 °C (20). Primers were designed to include most of the target genes but not their highly hydrophobic signal sequences, they also included a restriction enzyme site. Coding sequences were amplified by PCR and cloned into the expression vectors pQE30 (Qiagen) or pAE (19). Recombinant plasmids were used to transform *E. coli* strains by electroporation and *E. coli* transformants containing the constructs were cultured at 37 °C. Expression was induced by isopropyl-β-d-thiogalactopyranoside (IPTG), at 1 mM final concentration. The cells were harvested by centrifugation, resuspended in column buffer (8 M urea, 100 mM Tris, 300 mM NaCl, 5 mM imidazole, pH 8.0), and disrupted by sonication (3× 1 min pulses; Sonics & Material Inc.). His-tagged proteins were purified by affinity chromatography in a nickel (Ni^{+2}) charged Sepharose column. Columns containing bound protein were washed with 10 volumes of column buffer, then 6 × wash buffer (6 M urea, 100 mM Tris, 300 mM NaCl, pH 8.0) containing 5 mM imidazole for the first three washes and increasing to 10 mM imidazole for the remaining washes. His-tagged proteins were eluted from the column with wash buffer containing 250 mM imidazole. Dialysis procedure was used to remove urea and imidazole and to promote refolding of the recombinant proteins. Proteins in the final preparation were quantified by the Bradford method (3).

3. Imunization assays

Female Golden Syrian hamsters 4-6 weeks old were allocated into groups of 6 animals and immunized by intramuscular route (I.M.) with recombinant protein in aluminum hydroxide (15%) on day 0 and day 14. Immunization was performed with two 60 μg doses of recombinant protein. Hamsters were immunized with 250 μL per injection site. A negative control group of hamsters was immunized with aluminum hydroxide adjuvant and PBS, and a positive control group was immunized with killed *L. interrogans*. Immunized hamsters were challenged at age 8-10 weeks, fourteen days after the second immunization, with an intraperitoneal (I.P.) administration of 100 leptospires (~2 × LD_{50}) of strain Fiocruz L1-130 (22). Hamsters were monitored daily for clinical signs of leptospirosis and euthanized when clinical signs of terminal disease appeared. Surviving hamsters were euthanized on day 24 post-challenge. Experimental animals were maintained at the animal facility of the Biotechnology Center of the Federal University of Pelotas (UFPel). The animals were manipulated in accordance with the University guidelines of the Ethics Committee in Animal Experimentation throughout the experimental period.

4. Results and Discussion

All PCR products were successfully cloned and expressed in *E. coli* as a 6×His tag N-terminus fusion protein, which allows purification of the protein by affinity chromatography. All seven proteins were expressed as insoluble inclusion bodies. They were solubilized and purified using a buffer containing urea. After dialysis and SDS-PAGE, purity was estimated to be over 90%. The immunization assays failed to reveal a fully protective vaccine candidate. Although LIC12099 did fully protect in the first experiment, it failed to protect in a subsequent experiment. Another protein tested twice (in two different experiments), was LIC10508, because, although it did not fully protect, it delayed mortality (Table 1).

Table 1. Protection conferred by immunization with recombinant lipoproteins against lethal challenge in the hamster model.

Experiment	Group[a]	Days until death	no. survivors/total
1	LIC12099	-	6/6 [d]
	LIC12730	11,11,12,13,14,14	0/6
	LIC10561	11,11,11,11,13	1/6
	LIC10508	11,13,13,13,18	1/6
	Whole-cell preparation[b]	-	6/6 [d]
	Control immunization[c]	11,11,12,13,13,14	0/6
2	LIC12099	10,13,13,13,14,14	0/6
	LIC10191	10,10,10,10,11,13	0/6
	LIC10508	9,9,9,10,11,12	0/6
	Whole-cell preparation	-	6/6 [d]
	Control immunization	9,9,9,10,11,11	0/6
3	LIC10011	11,12,13,13,14	1/6
	LIC11947	12,12,14,14,16,17	0/6
	LIC12538	11,11,13,13,14	1/6
	Whole-cell preparation	-	6/6[d]
	Control immunization	10,11,11,12,14	1/6

[a] Animals were vaccinated twice (60/60 µg) at 2-week interval and were challenged with Fiocruz L1-130 two week after the last vaccination.

[b] Immunizations were performed with heat-inactivated preparations of *L. interrogans* Fiocruz L1-130.

[c] Control immunizations were performed by administering PBS with aluminum hydroxide adjuvant.

[d] Protection against lethal challenge was statistically significant ($P < 0.05$).

Extensive efforts have been made to identify cross-protective antigens of *Leptospira* (25, 27). Lipoproteins and other membrane associated proteins are potential vaccine candidates. However, only a few leptospiral proteins

characterized so far have been able to confer protection when used to immunize susceptible animals (10, 18, 23-24) but none of them were able to fully protect, when compared to control groups. In our study, in one case the ~2 × LD_{50} challenge was not able to kill the entire control group, this did not however mask false protecting proteins, as they also failed to protect.

All experiments were undertaken in the same manner, therefore there would be no reason to attribute the lack of repeatability of the first results obtained with LIC12099, to experimental conditions. However, the same batch of proteins used in the first experiment was used in the second, and the storage period may have somehow modified the proteins tertiary structure, although no degradation was observed in SDS-PAGE. Other proteins, though not fully protective, were able to delay death in several cases, which may indicate protective potential, if parameters such as dose volume, interval and adjuvant are optimized.

The mechanism of immune protection in leptospirosis remains unknown. There is need for studies directed towards defining how the immunity resulting from recombinant protein immunization can be improved. Determination of the relative contributions of humoral immune mechanisms and cellular-mediated immunity would provide insights about immune response against leptospirosis. Further experiments, evaluating other proteins, and probing leptospirosis associated immune response are currently being performed.

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