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Leptospirose Animal: Estudos para o Desenvolvimento de Vacinas Recombinantes

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“... Nunca tive pretensões
De mestre nem professor
Mas chambão cantando -flor-
Jamais me rouba o sossego,
Pois tirei, quando borrego,
Meu diploma de carpeta
Debaixo de uma carreta
Sobre um carnal de pelego...”

- Jogando truco
Jayme C. Braun

Resumo

FELIX, Samuel Rodrigues. **Leptospirose Animal: Estudos para o Desenvolvimento de Vacinas Recombinantes.** 2013. 89f Tese (Doutorado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

A leptospirose é uma doença causada por espiroquetas do gênero *Leptospira*. É uma zoonose de ampla distribuição geográfica, sendo um problema de saúde pública e veterinária, principalmente em países subdesenvolvidos e em desenvolvimento de clima tropical e subtropical. Em medicina veterinária, a leptospirose é uma doença importante tanto para a clínica quanto para a produção, devido ao risco à saúde pública, perdas reprodutivas e óbitos. A vacinação animal é realizada como medida de prevenção da enfermidade, entretanto, a proteção conferida pelas vacinas comerciais é limitada e não evita a condição de portador. Os antígenos protéicos da membrana externa parecem ser a melhor alternativa para substituir as vacinas atualmente disponíveis, porém, após diversos estudos, nenhum apresentou resultados satisfatórios. O objetivo deste trabalho foi avaliar antígenos recombinantes e preparações vacinais, capazes de conferir proteção de amplo espectro, em hamsters, contra leptospirose letal. A prevalência da leptospirose em cães da cidade de Pelotas foi aferida em um ensaio de diagnóstico sorológico usando como antígeno cepas dos sorogrupos Icterohaemorrhagie e Canicola. Em uma série de experimentos de prospecção de alvos, grupos de hamsters foram vacinados com diferentes proteínas recombinantes e posteriormente desafiados com cepa virulenta de *Leptospira* sp. Após o desenvolvimento de modelo para avaliação de proteção contra desafios heterólogos, as proteínas rLipL32 e rLigBNI foram avaliadas quando coadministradas com bacterinas (preparações de células inteiras inativadas por calor) em hamsters, sofrendo posterior desafio com quatro cepas de sorogrupos diferentes. Uma soroprevalência de 28,96% foi encontrada nos ensaios de prevalência. Duas de 27 proteínas recombinantes triadas foram identificadas como possíveis imunógenos. Apesar da falta de proteção demonstrada no experimento de coadministração de proteína e bacterina contra desafio homólogo ou heterólogo, a bacterina parece ter ação imunoestimulante.

Palavras-chave: Zoonose. Antígeno recombinante. Imunoproteção. Desafio heterólogo.

Abstract

FELIX, Samuel Rodrigues**. Leptospirose Animal: Estudos para o Desenvolvimento de Vacinas Recombinantes**. 2013. 89f Tese (Doutorado) - Programa de Pós-Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a disease caused by pathogenic spirochetes of the *Leptospira* genus. This zoonosis of worldwide distribution causes veterinarian and public health issues, especially in underdeveloped and developing countries with tropical and subtropical climates. In veterinary medicine, leptospirosis is important both as a clinical problem, causing illness in domestic animals, and an economic problem, causing productive and reproductive losses in commercial herds. Vaccination of these animals is applied, however protection conferred by these conventional vaccines is limited, and the carrier status is not always avoided. Recombinant outer membrane proteins seem to be the most promising antigens to replace the traditional bacterins (whole cell inactivated preparations), but thus far none of the tested proteins have turned satisfactory results. The goal of this study was to assess recombinant antigens and vaccine preparations, regarding their capability of producing protective immunity in hamsters, against lethal leptospirosis. Moreover, heterologous protection was sought, and assessed. The prevalence anti-*Leptospira* antibodies in stray dogs from the city of Pelotas was assessed using serogroups Icterohaemorrhagie and Canicola antigens. Several experiments were conducted to assess the protective potential of previously described leptospiral proteins. Twenty seven proteins were used to immunize hamsters which were then challenged with virulent *Leptospira.* Furthermore, leading vaccine candidates, LipL32 and LigB, were assessed regarding their protective potential when co-administered with traditional bacterins in previously established heterologous challenge experiments. A total of 28.96% of the animals tested were seropositive for the disease in the prevalence assay. Of the 27 antigens tried, two were shown to have some protective potential. Although no protection was demonstrated in the coadministration experiment, leptospiral bacterins seem to have some immunestimulating activity.

Key words: Zoonosis. Recombinant antigen. Immuneprotection. Heterologous challenge.

Sumário

1 INTRODUÇÃO

A leptospirose, doença de distribuição mundial, com ocorrência, em humanos, estimada em mais de 500.000 casos por ano (WHO, 1999; WHO, 2011), é hoje considerada uma das zoonoses mais difundidas do planeta (ADLER; MOCTEZUMA, 2010). A doença é causada por espiroquetas do gênero *Leptospira*, o qual possui 20 espécies e 24 sorogrupos, com mais de 300 sorovares descritos, sendo mais de 250 patogênicos (CERQUEIRA; PICARDEAU, 2009). Apesar de ser uma doença cosmopolita, ela é mais prevalente em países subdesenvolvidos e em desenvolvimento, principalmente devido a fatores ambientais, climáticos, socioeconômicos, de saneamento básico e pela diversidade de hospedeiros suscetíveis domésticos e silvestres (LEVETT, 2001; KO et al., 2009).

No Brasil, assim como em outros países, a doença é amplamente negligenciada e a qualidade do controle varia de estado para estado. Nos últimos 15 anos, estados como o Piauí e Roraima relataram não mais que 20 casos confirmados de leptospirose humana, enquanto estados como São Paulo e Rio Grande do Sul chegam a relatar mais de mil em apenas um ano (Ministério da Saúde, 2012). Dados oficiais referentes à ocorrência da enfermidade em animais no Brasil são escassos. A prevalência sorológica média em bovinos no país é de 36,7%, sendo que 84,1% das propriedades rurais apresentam casos da doença (FAVERO et al., 2001). Em outro estudo que abrangeu vários estados brasileiros, Favero e colaboradores (2002) relataram prevalência média nacional de 17,7%, 29% e 24,5% de soropositivos para caninos, equinos e suínos, respectivamente.

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 subclínica ou clínica, mas dificilmente tornando-se portadores renais crônicos (ADLER; MOCTEZUMA, 2010). Na forma subclínica, 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 74% (BHARTI, et al.,2003; McBRIDE, et al., 2005; GOUVEIA et al., 2008).

A leptospirose canina possui sintomatologia similar à 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, 2010). Assim como os cães, animais de produção podem tornar-se carreadores renais da bactéria, perpetuando a doença dentro dos rebanhos (LEVETT, 2001). Além disso, a leptospirose causa perdas econômicas para a agropecuária, com altos índices de abortos, natimortos, infertilidade e redução na produção de leite. Resultando em prejuízos para os produtores e, consequentemente, para a economia dos países molestados (FAINE et al., 1999). O controle da doença em animais de produção também deveria ocorrer através de vacinação e educação dos proprietários, veterinários e tratadores. Entretanto, como será demonstrado ao longo deste relato, essas medidas, particularmente as vacinas, são, de forma geral, frustradas.

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 patogênicos isolados no mundo (CERQUEIRA; PICARDEAU, 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 frequentes revacinações tanto para humanos quanto para animais (McBRIDEet al., 2005).

Com o sequenciamento do genoma de diversas espécies de leptospiras, uma gama de antígenos proteicos vem sendo propostos, e avaliados, para uso como vacinas recombinantes, entretanto, poucos deles tem se mostrado eficientes (DELLAGOSTIN et al., 2011). Recentemente, um estudo de prospecção avaliou 238 candidatos vacinais tidos como promissores (MURRAYet al., 2013), mas os autores concluíram que nenhum deles tem potencial para ser usado em vacinas. O nosso grupo demonstrou a imunização de hamsters com um fragmento de LigA (SILVA et al., 2007) e este foi considerado o melhor alvo vacinal descrito até então por revisão de Adler e Moctezuma (2010). Além disso, nosso grupo vem isolando e caracterizando leptospiras patogênicas (SILVA et al., 2008; SILVA et al., 2010; DINIZ et al., 2011) de tal forma a se munir com os insumos necessários para ensaios de imunoproteção contra desafios heterólogos. Nesse trabalho aplicamos os conhecimentos adquiridos e as tecnologias desenvolvidas pelo grupo até este momento, com o intuito de fazer prospecção de novos alvos vacinais, bem como desenvolver modelos de estudo em desafio heterólogo e aplicar esses modelos a preparações vacinais inovadoras.

Inicialmente, apresentamos uma revisão da literatura intitulada "*Controlling animal leptospirosis through vaccination: the Brazilian scenario*", formatada para submissão ao periódico "*Vector-Borne and Zoonotic Diseases".*Nela revisamos a situação da leptospirose animal no Brasil, e inferimos sobre os motivos do controle estar sendo frustrado, bem como o que deve ser feito para melhora-lo.

O segundo artigo, formatado para submissão ao periódico "*Brazilian Journal of Microbiology"* é intitulado "*Canine Leptospirosis: prevalence of serogroups Icterohaemorrhagiae and Canicola in the City of Pelotas, Brazil*". Neste trazemos os resultados de uma investigação sorológica em cães da cidade de Pelotas, com o intuito de demonstrar o risco que esses animais apresentam para humanos e animais domésticos.

O terceiro artigo, publicado no periódico "*Clinical and Vaccine Immunology*" é um trabalho de prospecção de alvos vacinais intitulado "*Subunit approach to evaluation of theimmune protective potential of leptospiral antigens*". Nele relatamos a avaliação de 27 proteínas de membrana externa de leptospiras como alvos vacinas, recomendando duas delas como promissoras.

O quarto artigo está formatado para submissão ao periódico "*Zoonoses and Public Health"* e é intitulado "*A Novel Approach to Leptospirosis Vaccines: Bacterins as Adjuvant in Recombinant Subunit Preparations*". Neste, descrevemos um modelo para ensaios de desafio heterólogo usando cepas de quatro sorogrupos diferentes. Além disso, avaliamos preparações originais de proteínas recombinantes associadas à bacterinas, quanto ao seu potencial imunogênico e imunoprotetor.

2 OBJETIVOS

Objetivo geral

O objetivo deste trabalho foi avaliar a situação da leptospirose canina na cidade de Pelotas, bem como desenvolver novas vacinas efetivas no controle da leptospirose animal.

Objetivos específicos

- Avaliar a situação da leptospirose em cães errantes da cidade de Pelotas.

- Produzir e purificar antígenos recombinantes de leptospiras para uso em ensaios vacinais.

- Avaliar esses antígenos recombinantes em ensaios de desafio em hamsters.

- Desenvolver modelo para ensaios de desafio heterólogo usando vacinas de célula inteira.

- Produzir suspensões de antígenos recombinantes com células inteiras de leptospiras inativadas.

- Avaliar a capacidade imunoprotetora das suspensões em ensaios de desafio homólogo e heterólogo.

- Avaliar a imunogenicidade das suspensões através de ensaios imunoenzimáticos.

3 ARTIGOS

3.1 Artigo 1

Controlling animal leptospirosis through vaccination: the Brazilian scenario

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(Artigo a ser submetido ao o periódico *Vector-Borne and Zoonotic Diseases*)

Controlling animal leptospirosis through vaccination: the Brazilian scenario

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Running title: Leptospirosis vaccines in Brazil

Key words: Leptospirosis, vaccines, veterinary medicine

ABSTRACT

Leptospirosis is one of the most widespread zoonosis in the world. Among other issues, sub-optimal sanitation and characteristic tropical and sub-tropical climates make Brazil particularly vulnerable to the disease. Vaccination of pets and livestock seems to be an important measure to control the disease in animal and human populations and should be applied extensively. Furthermore, with the country's growing livestock and pet markets, veterinarians should be working to minimize losses caused by leptospirosis. However, an apparent lack of awareness by them, and the general population, results in few and ineffective vaccinations. In this manuscript, the authors review the factors undermining the control of animal leptospirosis in Brazil, and infer on how vaccination may minimize them.

Leptospirosis

Leptospirosis is regarded as the most widespread zoonosis in the world (Ko et al., 2009). It is caused by spiraled bacteria of the *Leptospira* genus, and is a major public health concern, particularly in underdeveloped and developing countries (McBride et al., 2005), where tropical and/or sub-tropical climates predominate. The human disease has an estimated 500 thousand cases every year, but these numbers are thought to be grossly underreported (WHO 1999; WHO, 2011). Human leptospirosis usually occurs as a mild and undifferentiated febrile disease (Ko et al., 2009), however, it may evolve to far more severe conditions, with mortality rates ranging from 10% for acute renal failure, to 74% in patients suffering from leptospirosis associated pulmonary hemorrhage (McBride et al., 2005; Gouveia et al., 2008). Humans, and other animals, acquire the disease through direct or indirect contact with contaminated urine or tissues. Considering that human to human transmission is negligible (Adler and Moctezuma, 2010), virtually all cases of human leptospirosis come from infected animals. Therefore, controlling animal leptospirosis is as important for public health agencies as controlling the disease in humans.

Leptospirosis has been reported in more than 150 animal species (Ko et al., 2009), and is considered to occur in all mammals. Rodents, particularly rats and mice, are considered the main urban and rural reservoirs of this zoonosis. They become infected, but do not suffer from the disease, shedding the bacteria for long periods of time in their urine (Adler and Moctezuma, 2010). Furthermore, these rodents live alongside man, especially in settings with less than optimal sanitary conditions, making them even more effective in transmitting the bacteria to humans and domestic animals.

In veterinary medicine, leptospirosis is particularly important where canines, swine, and cattle are concerned. Cattle and swine usually suffer from mild forms of the disease, rarely resulting in death. However, the productive and reproductive losses caused by leptospirosis in these species are a major concern (Adler and Moctezuma, 2010). In breeding herds, leptospirosis causes abortions, stillbirths, and other reproductive shortcomings, in production herds, the disease causes diminished weight gain, weight loss, and reduced milk production (Levett, 2001). Furthermore, veterinarians, animal handlers, slaughterhouse workers, and anyone else that comes in contact with these animals, are at a greater risk of acquiring leptospirosis from them. In dogs, the disease occurs much in the same way as human leptospirosis (Levett, 2001). This species is particularly important for veterinarians due to its key role in the epidemiology of the disease. While people that handle pigs and cattle are, in general, aware of the risks, dog owners are the general population, and this species is second only to synanthropic rodents in the transmission of the bacteria to humans (Bharti et al., 2006; Jimenez-Coello et al., 2010).

Currently there are 20 recognized species belonging to the genus *Leptospira*. Thirteen of these species are potentially pathogenic (8 pathogenic and 5 intermediate), of which over 250 serovars have been described (Cerqueira and Picardeau, 2009). These serovars are differentiated mostly due to minor modifications in the carbohydrate component of the bacterial lipopolysacharide. Immunologically similar serovars are further grouped in serogroups. Immunity against leptospirosis is regarded to be vastly serovar specific and, to a lesser extent, serogroup specific (Levett, 2001). Serovars and serogroups can be, and usually are, associated with specific reservoir hosts, which become asymptomatic carriers, shedding the bacteria in the environment for long periods of time (Bharti et al., 2006).

When these animals are infected with other serovars however, there is a greater chance they will become clinically ill. Serovars can also be associated with geographical distribution, with certain serovars occurring more often, or exclusively, in specific parts of the world. Historically, the serological classification is associated with the epidemiological aspects of the disease and the more recent genotype classification does not seem to hold the same correlations (Cerqueira and Picardeau, 2009).

Considering immunity against leptospirosis as serovar specific, and serovars as host oriented, vaccines against the disease should include the most common serovars for each host species. However, serovar abundance will vary according to the geographical location, therefore vaccines should include the most common serovars in each country, state or region. On the other hand, leptospiral growth is slow and costly, and local isolates are not ready available, leading industries to consider non viable the commercial production of quality local vaccines for different communities. These are the main challenges we are confronted with when trying to control animal leptospirosis through vaccination.

What do we know about immunity?

The development of novel vaccines, or the improvement of those currently available, is unconditionally associated to an enhanced understanding of the immunological response against leptospirosis. Until recently, the protective immune response against the disease was thought to be exclusively humoral. Faine (1999) wrote: "*Immunity is apparently solely humoral, and can be passively transferred via placenta and colostrum or by serum. Intracellular sequestration of leptospires and cellular immunity are not important in resistance to reinfection*". This misconception is

still widespread today. The main reason researchers insist on this theory is that immunity can be passively transferred from one individual to another, or through the use of monoclonal antibodies (Adler and Moctezuma, 2010). Furthermore, these antibodies are capable of agglutinating leptospiras *in vitro*, which is the basis for diagnostic techniques such as the microscopic agglutination test (MAT), the current standard for the diagnosis of leptospirosis. However, the fact that humoral immunity can be conclusively demonstrated does not mean that cellular immunity is not involved. This misconception has not only hampered vaccine development for decades, but it has also fueled the belief that leptospirosis protective immunity is, obligatorily, serovar specific. Although cross-reaction among serovars has been known to occur for a long time (Myers e Coltorti, 1978), the idea that anti-LPS antibodies are responsible for the acquired immunity against leptospirosis led us to believe that only antigenically similar serovars or serogroups could confer cross protection.

These two ideas are slowly losing space, as more and more evidence of cellular involvement in leptospirosis immunity and cross protection arise. Moreover, it is likely that these two phenomenon are related. Cell mediated immunity first became mainstream in vaccine development when it was shown as the principal mechanism for protection in cattle by Naiman and coworkers in 2001 and 2002, and later by Zuerner and coworkers in 2011. These mechanisms have been shown to occur in other species, particularly humans (Klimpel et al., 2003; Barry et al., 2006;) and are likely to be common to all domestic animals. Furthermore, although these may not be the sole mechanisms responsible for protection in these other species (sharing that role with antibodies), unlike cattle, they are undoubtedly involved. Likewise, cross protection seems to be underappreciated. Recent studies have demonstrated an

increased number of cross protecting antigens (Sonrier et al., 2000; Tabata et al., 2002; Srikram et al., 2011). Surprisingly, recombinant immunogens, which received the status of immediate solution after the publication of the leptospiral genome sequences (Ren et al., 2003; Nascimento et al., 2004), are not a majority among the new cross protecting antigens. On the other hand, Sonrier and coworkers (2000) demonstrated that a whole cell protein extract, but not whole cell bacterin, was capable of inducing full cross protection against heterologous challenge in gerbils. This can mean that the LPS, thought to be the source of protective immunity, may actually be "interfering" in the protein antigens' potential for cross protection. This is upheld by the results of Srikram and coworkers (2011), who demonstrated cross protection with a live LPS mutant. In this light, recombinant antigens, or at least protein antigens in general, are still foremost in their potential for cross protection.

It seems that outdated ideas regarding cell mediated immunity and cross protection have hampered the development of modern vaccines. Too much hope has been deposited in recombinant antigens, which should not be set aside, but may share the future with traditional vaccines. Indeed hamsters immunized intramuscularly with two doses of a serogroup canicola bacterin (10^7cells/dose) 14 days apart, survived lethal challenge with three different strains (serogroups Canicola, Iceterohaemorrhagiae, and Ballum) on day 28 (this work). However, these results do not mirror those obtained with commercial vaccines, which are usually underachieving.

Leptospirosis in Brazil

Similarly to the rest of the world, leptospirosis is also neglected in Brazil. In this country of continental proportions, the disease seems to be irregularly diagnosed throughout. While some states reported no more than 20 human cases in the last 15 years, others have reported more than one thousand cases in a single year (Brazilian Ministry of Health, 2012). With an average ~4,000 reports every year, and a case fatality of ~10%, this disease is a serious health issue in the country. Though nationwide prevalence studies are unavailable, seroprevalence seems to vary from 12 to 43% (Homem et al., 2001; Reis et al., 2008) depending on the region.

Animal leptospirosis in Brazil is widespread in domestic herds and pets. In a nationwide study, Favero and co-workers (2002) found that depending on the state, up to 19.7% of dogs, 70% of horses, and 45% of pigs were positive for leptospirosis, with an average of 17.7%, 29%, and 24.5% respectively. Another study, conducted in 21 out of 27 states, shows that 37.9% of Brazilian cattle population is seropositive for leptospirosis. These numbers increase to over 60% in some states (Favero et al., 2001; Homem et al., 2001). While commercial vaccines are available for pets and livestock, little is known regarding the number of vaccinated animals. Apparently as little as 1.6% of livestock are vaccinated in Brazil (Oliveira et al., 2010). While pets seem to have closer veterinarian care, the number of stray dogs in the country makes it impossible to assess the percentage of vaccinated animals, even if such numbers were available.

Commercial vaccines

Commercial vaccines in Brazil are, in general, underachieving. The main reason is the lack of cross protection in livestock vaccines, as well as a lack of

objective studies to assess their effectiveness, and the lack of any protection in canine vaccines (Arduino et al., 2009; Coelho, 2010). Furthermore, these vaccines do not always prevent renal colonization and shedding (Dellagostin et al., 2011). Therefore, animals continue to represent a risk of infection to humans. There are a few reasons this may be happening, and foremost among them is the fact that many commercial vaccines available in Brazil use foreign isolates. This is greatly prejudicial to the vaccine's effectiveness since there are genetic variations, even within a same serovar (ELLIS, 2010; Arent et al., 2012) that may jeopardize the final outcome of protection. Furthermore, a vaccine that does not include local isolates is probably not using the most prevalent local serovars. Another reason for the shortcomings of commercial vaccines is probably the way they are produced, the industrial process requires high yield cultures, for which they use culture adapted strains. These strains may still be virulent, a required trait for potency tests, but their expression patterns, for proteins and/or LPS, may be greatly altered since important outer membrane antigens are down/up regulated after just a few culture passages (Patarakul et al., 2010).

Ruminant livestock vaccines

Cattle vaccines seem to be somewhat effective in Brazil. Although some commercial vaccines have up to 10 serovars, the most common livestock vaccines include Hardjo, Icterohameorrhagiae, Canicola, Grippotyphosa, and Wolfii serovars (Table 1). Although some preparations lack serovar Wolfii, they have been shown to produce cross-reacting antibodies (Arduino et al., 2009). Epidemiological surveys have shown that these are in fact the most important serovars occurring in most of

Brazil (Langoni et al., 2000; Favero et al., 2001) although these authors indicate some regions would benefit with the addition of other serovars, particularly Pyrogenes, Hebdomadis, Djasiman and/or Castellonis, the latter two are particularly important for buffalos (Langoni et al., 1999).

While livestock vaccines do not always protect against renal colonization or shedding (Adler and Moctezuma, 2009), they seem to effectively protect animals against productive and reproductive setbacks (Arduino et al., 2009; Oliveira et al., 2010), which are the most important aspects of the disease as far as the productive chain is concerned. However, there is a lack of challenge studies assessing commercially available vaccines for ruminants in Brazil. Most studies assess only the humoral response to the infecting serovars (Tabata et al., 2002; Nardi et al., 2006; Arduino et al., 2009; Oliveira et al., 2010), which does not say much as to the vaccine effectiveness, since this response does not seem to be involved in the protection of these livestock species (Bolin et al., 1989; Zuerner et al., 2011). In fact, Arduino and co-workers (2009) showed that vaccination lowers a herds general antibody titter over time, making it easier to monitor when actual leptospirosis occurs. Probably the main setback regarding livestock vaccination in Brazil is the lack of mass prevention programs. Most of our bovine population is not vaccinated, particularly beef herds. There are few reports on this matter, but one account regards that no more than 1.6% of the livestock population of the state of Bahia (northeastern Brazil) is vaccinated (Oliveira et al., 2010).

Canine vaccines

Canine anti leptospirosis immunizations in Brazil occur greatly as part of routine veterinary checkups. Vaccines administered are usually coupled with viral antigens such as those for parvovirus, canine distemper, etc. These are administered in three monthly doses at young age, followed by yearly revaccinations. It has been shown that these protocols are effective (Klaasen, 2003, Minke, 2009), and should guarantee protection against the composing serovars. However, there is evidence that the vaccines commercialized in Brazil are far from achieving these goals. In 2010, Coelho assessed the protection conferred by 9 commercial vaccines available in Brazil, using local isolates of serovars Canicola and Copenhageni as challenge (homologous and heterologous challenges respectively). Seven of the 9 vaccines failed to protect even against the homologous challenge and none were capable of avoiding renal colonization. Unfortunately, this is not the only reason why Brazilian dogs are unprotected.

Commercial canine vaccines in Brazil are all comprised of, at least, serogroups Canicola and Icterohaemorrhagiae. Vaccines used are, in their majority, imported, and serovars Pomona and Gripotyphosa are also common, since they are used in North American preparations (Sykes, 2011). These two serovars seem to have been included in a random fashion for the local market, while serogroups Icterohaemorrhagiae and Canicola are among the most prevalent in Brazil (see table 1) (Furtado et al., 1997; Avila et al., 1998, Favero et al., 2001; Castro et al., 2011), serovars Pomona and Gripotyphosa are rarely even cited in epidemiological surveys. Furthermore, Brazilian veterinarians are reluctant to use nationally produced vaccines, due to perceived loose regulations in quality control. This tendency further hinders attempts to include local isolates in commercial vaccines.

While there are no public programs enforcing vaccination, domestic dogs that attend veterinary checkups are generally immunized. However, there is another issue hampering the control of canine leptospirosis in Brazil: stray dogs. Developed countries have stray dog populations under control, and canine vaccination benefits greatly from this (Ellis 2010). Stray dog populations in Brazil are high, especially in urban settings, and these act as reservoirs for the bacteria, jeopardizing what would be the long-term benefits of population wide vaccination.

Other species

Other species receiving anti leptospirosis vaccines in Brazil are pigs and horses. While most, though not all, swine vaccines are the same as those used for cattle, horses have their own preparations, usually including serovar Bratislava, as well as those present in other livestock vaccines (Table 1). It seems vaccination with commercial vaccines, along with educational support, have given positive results for horses in Brazil (Pinna et al., 2008). This is likely due to the fact that commercial vaccines are produced locally and the most prevalent serovars, Bratislava and Icterohameorrhagiae (Lilenbaum, 1998; Favero et al., 2002; Pinna et al., 2008), are included in the vaccine preparations.

Leptospirosis in pigs have experienced much of the same results to vaccination as cattle, reducing abortions and other reproductive setbacks, but not necessarily fully protecting against renal colonization and shedding (Soto et al., 2007; Adler and Moctezuma 2010). However, the success of vaccination in specific locations has been reported (Lobo et al., 2004), resulting in a shift in the most prevalent serovars affecting swine in the region. Serovars included in the livestock

vaccines generally cover the most common causes of swine leptospirosis in Brazil. However, prevalence surveys show that other serovars should be included in certain regions to obtain optimal results, especially Autumnalis and Bratislava (Soto et al., 2007),

Novel and future vaccines

Since the genome of leptospira was first published, several major outer membrane antigens have been identified (reviewed by Dellagostin et al., 2011). Foremost among these are proteins LipL32 and Ligs (Leptospiral immunoglobulin like proteins) (Ko et al., 2009), and the LigA assays conducted by Silva and coworkers (2007) seem to have generated the best results (as reviewed by Adlre and Moctezuma 2010). In parallel, experiments with mutated live antigens (Sikram et al., 2011) and modified bacterins (Sonrier et al., 2000) have demonstrated positive results for cross protection. It is conceivable that these two approaches are not exclusive, and recombinant subunits and modified bacterins may produce improved results if used together.

The scenario for veterinary vaccines seems particularly positive. Since recent studies are changing the paradigm on how the immune system reacts to leptospirosis, the use of alternative adjuvants may greatly enhance vaccine protection, and there is a wide range of adjuvants approved for veterinary use. For further information regarding novel vaccines, see Dellagostin and coworkers (2011).

Final considerations

It seems clear that animal leptospirosis could be better controlled in Brazil, and likewise in many other countries, through appropriate vaccination and education programs. Vaccines available for livestock are, to some extent, effective, but the lack of a systematic vaccination program enforced by the authorities, means that great part of the population is not vaccinated, unprotected, and acting as reservoirs for further transmission to other individuals of the same species, or even to other animal species. Furthermore, while cross-protecting vaccines are not available, local serovars should be included in vaccine composition. On the other hand, vaccines available for pets seem to be largely ineffective, and the fastest way to correct this would be to assure the inclusion of local isolates, or at least locally occurring serovars, in commercial preparations.

Brazil is a country of continental proportions, with a large number of infecting serovars in all domestic species. This is a major setback when attempting to control leptospirosis through vaccination. While effective, cross protecting vaccines are still unavailable, local serovars should compose vaccines for the different regions of the country. However, not only does this seem industrially impractical, but Brazilian small practice veterinarians insist on using imported vaccines. Therefore, before laboratories invest in local serovars, there should be a reeducation of those in charge of vaccination. Future vaccines should be able to protect against heterologous infections, and for such, researchers should focus on enhancing the immune response against the conserved protein antigens of the bacteria.

Author Disclosure Statement

No competing financial interests exist.

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Table 1. Most prevalent serovars occurring in domestic animals in Brazil and most common commercial vaccine compositions.

Animal species	Most frequentinfecting serovars in Brazil[a]	Most common serovars included in vaccines
Cattle	Hardjo; Wolfii; Pyrogenes[1]	Hardjo, Pomona, Canicola, Grippotyphosa, Icterohaemorrhagiae
Canine	Canicola; Australis; Autumnalis; Copenhageni; Icterohamemorrhagiae;[2]	Canicola, Pomona Icterohaemorrhagiae, Grippotyphosa
Swine	Pomona; Grippotyphosa; Icterohaemorrhagiae[3]	Hardjo, Pomona, Canicola, Grippotyphosa, Icterohaemorrhagiae
Horses	Bratislava; Icterohaemorrhagiae[4]	Bratislava; Hardjo, Pomona, Canicola, Grippotyphosa, Icterohaemorrhagiae

[a] Infecting serovars predicted by MAT. Brazil is a country of continental proportions and locally prevalent serovars will vary.

1 Faveroet al., 2002

2 Avila et al., 1995; Mascolli et al., 2002; Favero et al., 2002 Blazius et al., 2005; Castro et al., 2011;

3 Favero et al., 2002; Ramos and Lilenbaum, 2002; Soto et al., 2007

4 Favero et al., 2002; Pinna et al., 2008

3.2 Artigo 2

Canine Leptospirosis: prevalence of serogroups Icterohaemorrhagiae and Canicola in the City of Pelotas, Brazil

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Canine Leptospirosis: prevalence of serogroups Icterohaemorrhagiae and Canicola in the City of Pelotas, Brazil

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ABSTRACT: Leptospirosis is a zoonosis of worldwide occurrence. The microscopic agglutination test (MAT) is the gold standard to diagnose and assess the disease´s distribution in a population. Stray canines are important urban reservoirs of leptospirosis and studies regarding their seroreactivity in Brazil are few and far apart. This work reports the seroreactivity against leptospiral antigens of stray dogs in the city of Pelotas, Brazil. Blood samples were collected from 221 female dogs and subjected to the MAT. *Leptospira interrogans* serogroups Icterohaemorrhagie and Canicola were used as the antigens in this assay. Of the 221 tested animals, 64 were positive for agglutinating antibodies, representing a prevalence of 28.96%. This study constitutes an important epidemiological update for the leptospirosis scenario in southern Brazil. Furthermore, this report will aide healthcare agents in controlling both canine and human leptospirosis in the region.

Key-words: Canine leptospirosis. *Leptospira*. MAT. Geoprevalence.

Leptospirosis is a disease of worldwide importance, both from a veterinarian and a public health point of view[1], with reportedly more than 500,000 human cases a year[2]. The disease is caused by spiraled bacteria of the *Leptospira* genus, which comprises 20 species, 24 serogroups and over 250 serovars described thus far[3]. Although it is considered a cosmopolite disease, it is more prevalent in underdeveloped and developing countries, mainly due to environmental factors, climate, and susceptible domestic and wild host diversity[4;5]

Canine leptospirosis has similar symptoms to the disease in humans, such as jaundice, muscle pain, vomiting and, ultimately, death[6]. Clinical manifestations may be acute, involving several organs such as kidneys and liver, leading to jaundice and hemorrhages in the more severe cases[7;8]. Chronic cases may result in asymptomatic carrier animals, which will shed the bacteria in the environment for several months undetected[9]. Leptospirosis is then maintained in the environment by these susceptible reservoir hosts, species that are adapted to determined serogroups, of which they rarely suffer the severe disease, becoming renal carriers and chronic shedders of the bacteria in the environment[5]. Studies have revealed that dogs are a significant risk factor for human leptospirosis, second only to synanthropic rodents[10].

Canine leptospirosis has been studied to some extent in Brazil, however these studies are scarce. In 2001, the isolation of a *Leptospira interrogans* strain of the canicola serovar, revealed that, of 105 canine tested that year, 55 (52.4%) were reactive in the MAT[11]. Other studies carried out in the city of Pelotas both in rural and urban settings revealed prevalence ranging from 2.66%[12] to 34.8%[13].respectively. Several years have passed since the last studies on canine leptospirosis in southern Brazil were published, and new assessments are required to maintain the surveillance on the disease. This study was carried out with the intention of updating the current situation on canine leptospirosis in the city of Pelotas

All animals used in this study were female stray dogs, collected by the Pelotas administration, to be castrated, according to the municipal dog control program. No distinction towards age or race was made. All animal use was previously approved by the comity for animal use and care of the Universidade Federal de Pelotas. Blood samples were drawn using a vaccutiner apparatus, from the cephalic vein, prior to

the ovarian-hysterectomy surgery as part of the standard pre-operatory exam. The blood was immediately stored at 4 ^{o}C for serum separation. Serum was stored at -20 ^{o}C until use. Blood samples were collected from 221 animals.

Leptospira strains used in this study were isolates obtained by our group, described by Silva and co-workers[14], of the Canicola and Icterohemorrhagie serogroups. The strains were grown at 30 ^{o}C in EMJH liquid media supplemented with 10% commercial supplement (DIFCO).The MAT was carried out according to the recommendations of the World Health Organization (WHO) as described byFaine[15]. Briefly, the leptospires were counted in a Petroff-Housser chamber, and the concentration was adjusted to 1x10^{8}leptospires/mL. The serum was diluted 1:25 in sterile PBS, then incubated at 30 ^{o}C with the live antigen (50 µL diluted serum and 50 µL ~10^{8} culture) for a final screening serum dilution of 1:50. After 2 h, the reaction was observed and 50% or more agglutination was considered positive. Positive sera were tittered, using serial dilutions from 1:50 to 1:3200 and repeating the previous procedure.

Of the 221 tested animals, 29 were positive for the Icterohaemorrhagie serogroup and 35 for the Canicola serogroup. Of these, 14 were positive for both antigens, in which case the higher titter was considered. These results are shown in table 1. Furthermore, titers varied from 50 to 3200 (highest titer assayed). The total prevalence was of 28.96%. Of the reactive dogs, 15.83% were positive for Canicola, and 13.12% for Icterohaemorrhagie. Of the 221 animals tested, 96 had information regarding the place of origin, which allowed differences in neighborhood prevalence to be assessed. The results are presented in table2.

Pelotas is a near sea level city, with high annual rainfall and relative humidity[16], which increases the risk of leptospirosis outbreaks. The city has a high number of stray dogs which may be acting as reservoirs, shedding the bacteria, and transmitting the disease to humans and other dogs and domestic animals. Serologic survey of the stray dogs generates an important information for public health authorities. Previous studies have shown comparable serological prevalence in Pelotas, Avila and co-workers (1998)[13] found 34.8% prevalence in 425 animals tested, these results were similar to the 28,96% found in the present study. Although they used six serogroups, over 80% of the positive animals were reactive to the two

used in this study. On the other hand, their assessment did not discriminate housed and stray dogs, therefore vaccine induced agglutinins may have caused false positives. In other studies, Furtado and co-workers (1997)[17] found 28.9% positivity, indicating that little has changed since then, and the city's attempts to control the disease in stray dogs have been, thus far, frustrated. Jouglard and Brod (2000)[12] found 2.66% seroprevalence in dogs from rural settings in the region of Pelotas, however the different setting is most likely responsible for the very different outcome.

Most animals in our study showed a weak reaction, with MAT titer of 50. This may be due to the fact that we used only two strains to screen the sera, and some cross reaction with untested serovars may be occurring. The use of only two serogroups is justified the fact that these are the most prevalent serovars in canine populations in the world and in Pelotas[13]. Our results maintain stray dogs as important reservoir hosts for leptospirosis, once again shining a light on the need to control these dog populations in urban settings.

When the location of capture was considered, our highest seroprevalence was in the São Gonçalo region. This may be explained by the fact that it is a poor area of the city, with some slum settings. It is also a low region, two meters above sea level, as opposed to the average seven meters of the rest of the city[16], with a higher occurrence of flooding. This agrees with previous studies that place terrain height is a risk factor for canine letospirosis[13].

The results described in this study are of concern regarding the strategies, or lack thereof, being undertaken to control leptospirosis in stray dogs. The conclusion is that little has changed in the city of Pelotas in the past ten years. Prevalence and risk factors similar to those described years ago are still the same nowadays. Competent authorities should implement effective control strategies against canine leptospirosis in Pelotas, and researchers should maintain surveillance on the disease in this, and other cities.

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Table 1. Number of positive canine and respective titer

Titer	Icterohaemorrhagie positive	%	Canicola positive	%	Total positive	%
50	21	55.26	20	50	41	52.56
100	7	18.42	3	7.5	10	12.82
200	6	15.79	3	7.5	9	11.54
400	2	5.26	8	20	10	12.82
800	1	2.63	2	5	3	3.85
1600	1	2.63	2	5	3	3.85
3200	0	0	2	5	2	2.56
TOTAL	38	100	40	100	78	100

1 Table 2. Distribution of 96 stray dogs according to the neighborhood they were
2 captured in the city of Pelotas, and their MAT results

	Neighborhood							
	Centro	Fragata	Três Vendas	Areal	Laranjal	São Gonçalo	Capão do Leão	Total
MAT +	1	7	10	4	0	6	3	31
Total	9	21	35	10	2	8	11	96
Positive %	11.1	33.3	28.6	40.0	-	75,0	27.3	32.3

3

3.3 Artigo 3

Subunit approach to evaluation of the immune protective potential of leptospiral antigens

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Subunit approach to evaluation of the immune protective potential of leptospiral antigens

Running headline: Leptospirosis subunit antigen trials

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ABSTRACT

Leptospirosis is the most widespread zoonosis in the world. Current vaccines are based on whole-cell preparations that do not induce satisfactory immunity and cause severe side effects. In light of the recently available leptospiral genome sequences, several studies have sought to identify protective recombinant immunogens, however, few have been successful. The aim of this study was to evaluate 27 recombinant antigens to determine their potential to induce a protective immune response against leptospirosis in the hamster model. Experiments were conducted with groups of female hamsters immunized with individual antigen preparations. Hamsters were then challenged with a lethal dose of *Leptospira interrogans*. Thirteen antigens induced protective immune responses, however, only recombinant proteins LIC10325 and LIC13059 induced significant protection against mortality. These results have important implications for the development of an efficacious recombinant subunit vaccine against leptospirosis.

INTRODUCTION

Leptospirosis is a disease caused by pathogenic spirochetes of the *Leptospira* genus (1). Transmission occurs through direct or indirect exposure to urine of mammalian reservoirs especially during floods, occupational exposure and water sports practice (3). The infection is usually asymptomatic or a self-resolving febrile illness. However, up to 15% of all human infections progress to severe leptospirosis, with complications such as kidney failure and pulmonary hemorrhage and fatality rates of up to 50% (11, 21).Mortality remains high because of delay in diagnosis due to lack of infrastructure and adequate clinical suspicion and other poorly understood reasons, especially in underdeveloped and developing countries (3).

Although vaccines are the recommended method of prevention in at risk settings (17), the currently available vaccines (bacterins), generate most of the immune response against the outer membrane lipopolysaccharide (LPS) component (29). As there are over 250 leptospiral serovars identified thus far (7), with the main antigenic differences attributed to the LPS, these vaccines are limited to short term, serovar-specific immunity. Bacterin-type vaccines have been approved for use in humans in Cuba China, Japan and France. However, bacterins induce adverse reactions and side effects and in general their use has been restricted to animals (21), especially dogs, cattle and pigs (1). Therefore, considerable effort is being made to identify novel leptospiral vaccine candidates, capable of inducing a cross-protective immune response against the pathogenic serovars and with fewer side effects.

In recent years, many potential vaccine candidates have been tested in animal models and several different approaches have been used, including subunit, DNA, Adenovirus and *Mycobacterium bovis* BCG constructs (2, 5, 6, 12, 13, 15, 19, 24, 26, 27). Most of these studies identified their protein targets by screening for antigenicity

using leptospirosis patient sera (14) and/or proteins with predicted surface exposure (10). However, a recent review highlighted the difficulties in evaluating the reports of efficacy for these vaccine candidates due to the different animal models and statistical methods used. The authors reported that when the same statistical analysis of protection against mortality was used, very few candidates were found to offer significant immunoprotection (1). Furthermore, in the majority of reports protection did not induce sterilizing immunity.

Recently, our group used a reverse vaccinology approach to identify eight putative lipoproteins in the *L. interrogans* genome that were subsequently characterized in terms of immunogenicity and antigenicity (16). These eight putative lipoproteins and an additional 19 proteins, predicted to be surface exposed and recognized by sera from convalescent leptospirosis patients (14), were evaluated using the hamster model of lethal leptospirosis. The aim of the study was to identify potential vaccine candidates that could protect hamsters against lethal challenge, the end-points used in the present study included protection against mortality and survival.

MATERIAL AND METHODS

Leptospira *strains.* *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Fiocruz L1-130 was used in this study. Leptospires were cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) liquid medium (Difco Laboratories, USA) at 28 ºC. Growth was monitored by counting leptospires in a Petroff-Hausser chamber (Fisher) and dark-field microscopy as described previously (11). *Escherichia coli* strains TOP10 (Invitrogen) and BL21(DE3) STAR (Novagen) were used in this study. They were grown in Luria-Bertani (LB) or Terrific Broth (TB) media at 37 °C.

Plasmid construction, expression and purification of recombinant proteins. All proteins used in this study were identified from previous studies (14,16,23). The genomic DNA of *L. interrogans* was used as template for amplification of the target sequences. PCR products were cloned into the pAE(25), pQE30 (Qiagen), or pET100-D/TOPO (Invitrogen) plasmid vectors. These vectors contain a 6×His tag which is expressed fused to the recombinant protein. In general, primers were designed to include most of the target genes but not their highly hydrophobic signal sequences. They also included a restriction enzyme site to allow direct cloning of the PCR product. Full primer information is presented in Table 1. Recombinant plasmids were used to transform *E. coli* strains by electroporation and *E. coli* containing the constructs were then cultured at 37 °C. Expression was induced by isopropyl-β-d-thiogalactopyranoside (IPTG), at 1 mM final concentration. Cells were harvested by centrifugation, resuspended in column buffer containing 8 M of urea (no urea was used for soluble protein, see table 1), 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

wash buffer containing 10 mM imidazole. His-tagged proteins were eluted from the column with elution buffer containing 250 mM imidazole. A 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 (4) and BCA (Pierce) methods (Table 1 contains further information regarding solubility, vectors and protein size).

Protein gel electrophoresis. All proteins were submitted to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in order to verify their molecular weight and sample purity. These gels were stained with Coomassie bluesolution to reveal the proteins.

Hamster immune protection studies. Female golden Syrian hamsters, 4–5 weeks of age, were obtained from the animal house facility at the Federal University of Pelotas. The animals were immunized twice in the quadriceps muscle with the recombinant protein in an aluminum hydroxide solution (15%) on day 0 (zero) and day 14. All vaccine doses contained 60 µg of purified recombinant protein, with a standard volume application at 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 200 µL bacterin containing approximately 10^8 leptospires per dose. All hamsters were challenged on day 28 (age 8–9 weeks) with an intraperitoneal inoculum of 100 leptospires of strain Fiocruz L1-130 (~2 × LD_{50}) (28), 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.

Twenty-seven recombinant proteins were tested in eleven individual experiments, each group consisted of six hamsters except where noted otherwise (Table 2). All experiments included negative (PBS) and positive control (bacterin) groups. All animal studies were approved by the Ethics Committee for the Use of Experimental Animals of the Universidade Federal de Pelotas.

Statistical analysis. The log-rank test was used to determine significant differences in survival among the vaccinated and the negative control groups. All *P* values were two-sided and a *P* value <0.05 was considered to indicate statistical significance. 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 6×His tag N-terminus fusion proteins, which allowed purification of the proteins by affinity chromatography. The recombinant proteins required urea-promoted denaturing conditions for purification (except where noted otherwise) followed by prolonged dialysis to obtain soluble protein preparations. The integrity and purity of the recombinant proteins used in this study was verified by SDS-PAGE analysis (Figure 1). 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 onto the aluminum hydroxide overnight.

Protection of hamsters immunized with recombinant proteins against lethal challenge with *L. interrogans.* Immunization with the majority of the proteins did not prevent death among the challenged hamsters. Although immunization with the recombinant proteins LIC11859, LIC12253, LIC10561, LIC10508, LIC10091, LIC13059, LIC10054, LIC11567, LIC20172, LIC10561 and LIC10508 did result in more survivors than the negative control groups, the increase was not significant. Recombinant LIC10325 and LIC13059 significantly increased survival in the vaccinated hamsters ($P<0.05$) (log-rank test). Table 2 shows the days to death timeline for all the experiments. In three experiments one animal survived in the negative control group, whereas animals in the positive control group were fully protected in all experiments.

DISCUSSION

Several studies have employed the recombinant subunit vaccine approach to try to develop a vaccineagainst leptospirosis, however, the results are variable and difficult to interpret (1). Although LigA seems to be the most promising antigen (24, 27), immune protection with this antigen in an adjuvant approved for human use has not been shown. Some authors showed protection induced by LipL32, LigB, and other outer membrane proteins (2, 6, 15, 19, 26), however, these have yet to see practical applicability. In an effort to identify novel vaccine candidates, we evaluated 27 recombinant leptospiral proteins. In our assays a total of 15 recombinant proteins were incapable of inducing a protective immune response against challenge with *L. interrogans*. However, 12 recombinant proteins did improve survival compared to the negative control group. However, only two of these recombinant proteins (rLIC10325 and rLIC13059) induced significant survival ($P<0.05$). These proteins may constitute potential vaccine candidates.

The majority of the proteins evaluated in this study were identified as putative lipoproteins or hypothetical proteins (see Table 2). Of the protective antigens, LIC10325 was annotated as a hemolysin while LIC13059 was a putative lipoprotein (23). A BLAST analysis revealed that these are present in all pathogenic leptospiral genomes described to date and are highly conserved among the pathogenic *Leptospira* spp. These are important features for antigens to be used as vaccines aiming to afford cross-protection against different *Leptospira* spp. and serovars.

Previous reports have shown that vaccine preparations including more than one protein can be more effective than the individual counterparts (8, 15). Several of the leptospiral virulence factors and outer membrane proteins have exhibited redundancy and this was demonstrated by the fact that the knockout of some of these genes did

not reduce virulence (9, 20, 22). Therefore, immunity directed towards one of these proteins may not be effective. Thus, further investigation of the protective proteins identified in this study, fused or co-administered with each other, and/or with proteins described elsewhere, may increase the effectiveness of our preparations.

Although characterization of the immunogenicity and antigenicity of immunogens is an important step in identifying surface-exposed proteins, there is no correlation between the amplitude of the immune response and protection against leptospirosis. Highly immunogenic and antigenic surface proteins such as LipL32 do not induce protective immunity (18). For this reason, we used a challenge assay to screen for protective antigens. This approach produces relatively fast and practical results. Antigens that fail to induce a protective immune response do not need further assessment.

In this study we identified two potentially protective antigens. These, in combination with other leptospiral antigens already described, may result in an effective and cross protective vaccine against human and animal leptospirosis. Studies are being conducted not only to test different immunization protocols and antigen combinations, but also different adjuvants and forms of antigen presentation.

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Figure Legend.

FIGURE 1. 12% SDS-PAGE of purified proteins. Lane M, Molecular weight marker; 1, LIC10501; 2, LIC12555; 3, LIC11087; 4, LIC12253; 5, LIC10645; 6, LIC10021; 7, LIC11184; 8, LIC13006.

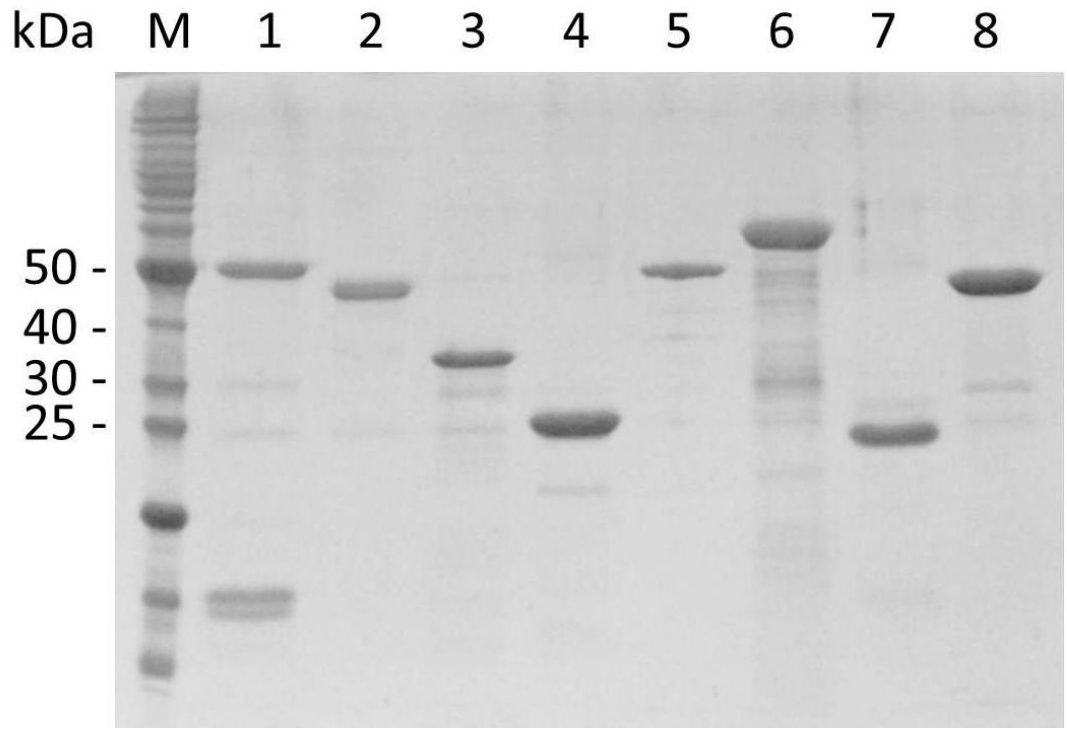

Table 1.Primer and protein information.

Antigen	Primers	Molecular weight (kDa)	Vector
rLIC 10191	**F** CGGGATCCTCAACGCAAGAGCA **R** GGGGTACCTTGTTGTGGTGCGGA	22.9	pAE
rLIC 12099	**F** TTGGTACCGCTCAAACGGCAAG **R** GGGAAGCTTTTTATATTTGACGATGA	52.7	pAE
rLIC 11947	**F** CCGGATCCCCTGTGGAAAGAAA **R** GGGAAGCTTTTTTTCTGGAGGAA	17.9	pAE
rLIC 10011	**F** TAGGTACCACGGATGGACTTTTGAA **R** CGGAATTCTTATTGTTTGGAAACCTC	19.8	pAE
rLIC 12730	**F** GCGGATCCATTTTAGTCTTTACCTC **R** CCCAAGCTTGATCAATTCCGTTC	57.8	pAE
rLIC 10561	**F** GCGGATCCTTAATATTTCTGGTCTTTC **R** CCCAAGCTTGATCAATTCCGTTC	30.22	pAE
rLIC 10508	**F** CGGGATCCAATTCAATAACTATG **R** GGGAAGCTTACAACCAGGACCTT	22.99	pAE
rLIC 12538	**F** GGGATCCGCAGACGAAAAGGAAA **R** CCAAGCTTTCAGCTAGTCAGAGTAAAA	24.9	pAE
rLIC 10501	**F** CACCGATAACAAAGAGAAGGGAGG **R** CTACTCCACACATTCGGGACTATTG	48.8	pET100-D/TOPO
rLIC 13006	**F** GACTCGAGAACTCTGCTTTAAGTGGCTTAA **R** GGCCATGGTTATTGTTCTACACAAACTAAA	47	pAE
rLIC 13306 [a]	**F** CACCTCCAAAGAGAAATGTTTATTC **R** TCATTTCCGAACCGGATGACCGT	17.7	pET100-D/TOPO
rLIC12253	**F** TTCTCGAGGAGAAACCGGACGATACTACTT **R** CCCCATGGTTAGGGAAGACTTCTAACAAC	23.4	pAE
rLIC11184	**F** CACCTGTGAAGATGAAAAAAAGGA **R** TTAGTAACCACACTCACTCGCAGC	18.8	pET100-D/TOPO
rLIC 10645	**F** CACCAAAAAAGATAAGGACGATACCTT **R** TTAACGAACTAGTACAGTCGGTAAATG	42.3	pAE
rLIC 10021	**F** GACTCGAGAATTGTTCTGTCAAGCCC **R** CCAAGCTTTCATAAATCCACGGAAGT	63.8	pAE
rLIC11859[a]	**F** CACCGAATTTATGAAGGTCACG **R** TTAAAAAGCACTTAAGGCAGCC	30	pET100-D/TOPO
rLIC 10325[a]	**F** CACCATTCAAGACGAAGATTCCAAAC **R** TCAATCCAATTTTTCGGTTTCTAG	40.6	pET100-D/TOPO
rLIC12555	**F** CATCTCGAGAGCCCAGTACAAATGAAAGT **R** TCCATGGTTAAAGATTTGTAACGCAGATTCC	43	pAE
rLIC 11087	**F** CACCGTTGGAGATTCCAGAAAGGAA **R** TTAAAATAAATTACAACCAGTCTGATATAA	29.9	pET100-D/TOPO
rLIC 12632	**F** GTCTCGAGTGTAAACCTGGCAAACAAAATT **R** GGCCATGGCTAATGATGATAGATTAAATCT	63.8	pAE
rLIC10054	**F** TTTTTTGGATCCGAGTCTAAACGAAG **R** TTTTTTAAGCTTCACCGTATTCTTGTC	32.1	pQE30
rLIC20172	**F** CGGGATCCGATACGGACAAGGACGGG **R** CCCAAGCTTTTCGGAATCCTCGTCCGG	28.1	pQE30
rLIC13059	**F** CGGGATCCGAATCCATGGTATATTAT **R** CCCAAGCTTACTTTGACGAATCAATGC	14.4	pQE30
rLIC11567	**F** CGGGATCCAACAGATTGATTCGTAAA **R** CCCAAGCTTCTTTTTGTATTCCACAAG	13.9	pQE30
rLIC10091	**F** CGGGATCCAAACTATTTTTAGCTCCTTTG **R** CCCAAGCTTGATTTCAAAAGAAGTATG	14.6	pQE30
rLIC10009	**F** TTTTTTTGATCACAAGAAGCGCAGATCT **R** CCCAAGCTTGAACTCATCCTGTTTA	24.8	pQE30
rLIC13305	**F** TTTTTTTGATCAAACTATGATCGTGAC **R** TTTAAGCTTGATATCACCACCCAAA	20.3	pQE30

[a] Expressed as soluble protein and did not require the use of urea on purification.

Table 2.Survival timeline of vaccinated animals.

Antigen	Name or feature	Days to death	Survival / total (%)
rLIC 10191	OmpA-Like	10, 10, 10, 10, 11, 13	0/6 (0)
rLIC 12099	Hypothetical protein	10, 13, 13, 13, 13, 14	0/6 (0)
rLIC 11947	Putative Lipoprotein	12, 12, 14, 14, 16, 17	0/6 (0) [b]
rLIC 10011	LipL21	11, 12, 13, 13, 14	1/6 (16.7) [b]
rLIC 12730	Hypothetical protein	11, 11, 12, 13, 14, 14	0/6 (0)
rLIC 10561	Hypothetical protein	11, 11, 11, 11, 13	1/6 (16.7)
rLIC 10508	Putative lipoprotein	11, 13, 13, 13, 18	1/6 (16.7)
rLIC 12538	SecD	11, 11, 13, 13, 14	1/6 (16.7) [b]
rLIC 10501	Putative lipoprotein	10, 11, 11, 11, 13	1/6 (16.7) [b]
rLIC 13306	Hypothetical protein	12, 12, 13, 14, 17	1/6 (16.7)
rLIC 13006	Putative lipoprotein	10, 11, 11, 12, 12, 13	0/6 (0)
rLIC12253	Putative lipoprotein	12, 12, 12, 12, 12	1/6 (16.7)
rLIC11184	Putative lipoprotein	12, 12, 12, 12, 13	1/6 (16.7) [b]
rLIC 10645	Putative lipoprotein	10, 11, 12, 12, 14	1/6 (16.7) [b]
rLIC 10021	Putative lipoprotein	11, 11, 11, 13, 14, 20	0/6 (0) [b]
rLIC11859	Hypothetical protein	10, 11, 11, 12, 14	1/6 (16.7)
rLIC 10325	Hemolysin	11, 12, 13, 13	2/6 (33.3)*
rLIC12555	Hypothetical protein	11, 12, 12, 12, 12, 15	0/6 (0)
rLIC 11087	Putative lipoprotein	11, 11, 12, 13, 13, 14	0/6 (0)
rLIC 12632	Hemolysin	11, 11, 12, 13, 14, 14	0/6 (0) [b]
rLIC10054	Putative lipoprotein	9, 10, 10, 10, 13	1/6 (16.7)
rLIC20172	Lipoprotein	10, 13, 13, 13, 13, 15, 16	1/8 (12.5) [a]
rLIC13059	Putative lipoprotein	8, 9, 10, 10	2/6 (33.3)*
rLIC11567	Putative lipoprotein	9, 10, 10, 10, 13, 13	2/8 (25) [a]
rLIC10091	Putative lipoprotein	10, 10, 10, 11, 11, 11, 11	1/8 (12.5) [a]
rLIC10009	Putative lipoprotein	9, 10, 10, 10, 10, 13, 14, 15	0/8 (0) [a]
rLIC13305	Putative lipoprotein	10, 10, 10, 11, 11, 11, 11	1/8 (12.5) [a]

[a] Experiment was conducted with eight hamsters per group.
[b] One animal from the PBS control group, of the respective experiment, survived.
* Survival of vaccinated animals was significantly different to the PBS control group (Log-Rank sum test).

3.4 Artigo 4

A Novel Approach to Leptospirosis Vaccine: Bacterins as Adjuvant in Recombinant Subunit Preparations

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7 CONCLUSÃO GERAL

A partir dos dados aqui apresentados pode-se concluir que, positivos para leptospirose (sorogrupos Icterohaemorrhagie e Canicola) representam 28,96% da população de cães errantes na cidade de Pelotas, e este é um valor que tem se mantido estável.Esse estudo sugere que os antígenos recombinantes rLIC10325 e rLIC13059 podem ter efeito protetor contra a doença. Além disso, descrevemos que, nem rLipL32 nem rLigBNI são capazes de gerar imunidade protetora quando associadas à bacterina nas condições aqui apresentadas. Por outro lado essa bacterina parece demonstrar alguma ação adjuvante.

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ANEXOS