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Utilização de ovinos para avaliação de vacinas recombinantes contra leptospirose animal

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Resumo

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As vacinas de uso veterinário disponíveis comercialmente contra leptospirose são compostas por bacterinas, e possuem limitações que prejudicam sua eficácia vacinal, devido ao crescimento fastidioso e oneroso da bactéria e à imunidade conferida ser restrita somente aos sorovares antigenicamente relacionados àqueles presentes na formulação vacinal. Nesse cenário, as vacinas recombinantes tornam-se uma alternativa promissora em relação às bacterinas, uma vez que eliminam o cultivo demorado e oneroso e, em teoria, podem conferir proteção contra mais de um sorovar. As proteínas LigAni, LigBrep e LipL32 têm demonstrado capacidade de promover resposta imune protetora contra desafio homólogo em hamsters. No entanto, diversos são os estudos realizados utilizando hamsters como modelo experimental e ainda não há nenhum estudo utilizando animais de produção para avaliação da resposta imune obtida por meio de vacinas recombinantes. Sendo assim, o objetivo deste trabalho foi avaliar antígenos recombinantes, (LipL32, LigAni e LigBrep) associados a diferentes adjuvantes no desenvolvimento de vacinas contra leptospirose animal, utilizando ovinos como modelo experimental. Para isso, o projeto foi dividido em duas etapas. Primeiramente foi avaliada a patogenia de *Leptospira interrogans* utilizando ovinos experimentalmente infectados. Após a avaliação da infecção experimental, foi avaliada a resposta imune obtida por ovinos quando vacinados com formulações contendo antígenos recombinantes e diferentes adjuvantes. Os resultados referentes à infecção experimental demostraram que, apesar de não ter sido observada sintomatologia clara, foi possível ocasionar a infecção experimental nos ovinos, demostrada pelas técnicas de Reação em Cadeia da Polimerase e (PCR) e Teste de Aglutinação Microscópica (MAT). Ademais, foi possível observar que as proteínas recombinantes LigAni, LigBrep e LipL32 possuem propriedades imunogênicas, gerando altos títulos de anticorpos quando coadministradas com diferentes adjuvantes e utilizando ovinos como modelo experimental. Portanto, conclui-se que os ovinos podem ser utilizados como modelo experimental em estudos relacionados à leptospirose animal.

Palavras chave: Vacina recombinante; proteínas recombinantes, Leptospirose; ovelha; modelo experimental.

Abstract

Da Rosa. Matheus Costa. **The use of sheep for evaluation of recombinant vaccines against animal leptospirosis**. 2019. 76 f. Tese (Doutorado) – Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas – Pelotas / RS.

Commercially available veterinary vaccines are composed of bacterins, and have limitations that impair their vaccine efficacy, since the bacteria exhibit fastidious, burdensome growth and confer immunity only to serovars antigenically related to those present in the vaccine formulation. In this scenario, recombinant vaccines become a promising alternative to bacterins, as they eliminate time-consuming and costly culture and, in theory, may confer protection against more than one serovar. LigAni, LigBrep and LipL32 proteins have been shown to promote protective immune response against homologous challenge in hamsters. However, several studies are carried out using hamsters as an experimental model and there is still no study using experimental animals as an experimental model to evaluate the immune response obtained through recombinant vaccines. Thus, the objective of this work was to evaluate recombinant antigens (LipL32, LigAni e LigBrep) associated with different adjuvants in the development of vaccines against animal leptospirosis, using sheep as an experimental model. For this, the project was divided into two stages. First, the pathogenesis of leptospira interrogans was evaluated using experimentally infected sheep. After evaluation of the experimental infection, the immune response obtained by sheep when vaccinated with formulations containing recombinant antigens and different adjuvants was evaluated. The results regarding the experimental infection showed that, despite the absence of clear symptomatology, it was possible to cause experimental infection in sheep, demonstrated by Polymerase Chain Reaction (PCR) and Microscopic Agglutination Test (MAT). In addition, it was possible to observe that the recombinant proteins LigAni, LigBrep and LipL32 have immunogenic properties, generating high titres of antibodies when coadministered with different adjuvants and using sheep as an experimental model. Therefore, it is concluded that sheep can be used as an experimental model in studies related to animal leptospirosis.

Keywords: Recombinant vaccine; recombinant proteins, Leptospirosis; sheep; experimental model.

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1. INTRODUÇÃO GERAL

A leptospirose, além de ser uma enfermidade de grande importância para saúde pública, por possuir um caráter zoonótico, no setor agropecuário também possui uma grande importância, pois costuma causar significativas perdas econômicas devido aos altos indices de abortos, natimortos, infertilidade e redução na produção de leite.

Os principais métodos de controle se resumem a antibioticoterapia, manejo e vacinação. A imunização é o método de controle mais eficaz e representa uma medida essencial para o controle da leptospirose, sendo sua adoção fortemente recomendada. Com base nisso, o desenvolvimento de vacinas de uso veterinário torna-se de extrema importância para o controle dessa enfermidade. As vacinas disponíveis são baseadas em suspensões celulares mono ou polivalentes, compostas por bacterinas, as quais conferem proteção contra os sorovares presentes na composição vacinal. No entanto, há controversias quanto ao grau de proteção e a colonização renal.

Vacinas recombinantes vêm sendo desenvolvidas a fim de superar as deficiências das vacinas compostas por bacterinas. Para a produção de vacinas recombinantes tem-se utilizado sistemas de expressão em procariotos e em eucariotos. Nosso grupo de pesquisa tem trabalhado com a avaliação de antígenos para compor uma vacina recombinante contra a leptospirose animal, buscando um produto que seja mais eficaz que os disponíveis. Neste sentido, caracterizamos quanto ao seu papel imunoprotetor, alguns alvos tais coo, a lipoproteína LipL32, conhecida como proteína-1 associada a hemolisina (Hap-1), sendo a proteína de membrana externa (OMP) mais abundante exposta na superfície celular, conservada entre as espécies patogênicas, ausente nas saprófitas e com características imunogênicas.

As proteínas Lig (LigANI, LigBrep) também são expostas na superfície de leptospiras patogênicas e os genes que as codificam deixam de ser transcritos em cepas alta passagem, estando ausentes nas saprófitas. O potencial imunoprotetor das proteínas recombinantes LipL32, LigAni e LigBrep tem sido avaliado, onde através de diferentes estratégias, elas têm demonstrado bons resultados quanto a sua capacidade antigênica e imunogênica, utilizando hamsters como modelo experimental. No entanto, estes estudos ainda estão muito limitados ao hamster

como modelo experimental e ainda não há nenhum estudo realizado utilizando animais de produção, pequenos ruminantes, como modelo experimental para avaliação de vacinas recombinantes contra leptospirose. A busca por um novo modelo experimental, que aproxime as pesquisas relacionadas à produção de vacinas contra leptospirose dos reais hospedeiros do agente infeccioso, torna-se de extrema importância para que se possa obter um produto final de qualidade e eficácia. Visto isso, este trabalho visou estabelecer um novo modelo experimental para estudos relacionados à leptospirose animal, além de avaliar a resposta imune induzida por antígenos recombinantes de *Leptospira*, associados a diferentes adjuvantes, neste novo modelo.

2. REVISÃO BIBLIOGRÁFICA

2.1. *Leptospira* e leptospirose

2.1.1. Taxonomia da *Leptospira* spp.

O agente etiológico da leptospirose, as leptospiras, pertencem à família Leptospiraceae, ordem Spirochaetales, gênero *Leptospira* (INADA et al., 1916; KO et la., 2009; ADLER, 2015). O gênero *Leptospira* é dividido em três grupos descritos como Patogênicas, Intermediárias e Saprófitas (Tabela 1). Nesses, existem 13 espécies patogênicas, 11 intermediárias e 11 saprófitas, totalizando 35 espécies (PICARDEAU et al., 2017; THIBEAUX et al., 2018).

Tabela 1. Espécies do gênero *Leptospira*.

Patogênicas	**Intermediárias**	**Saprófitas**
L. kirschneri	*L. fainei*	*L. indonii*
L. noguchii	*L. inadai*	*L. wolbachii*
L. interrogans	*L. broomii*	*L. macculloughii*
L. adleri	*L. perolatti*	*L. brenneri*
L. ellisii	*L. wolffi*	*L. harrisiae*
L. barantonii	*L. venezuelensis*	*L. vanthielii*
L. kmetyi	*L. licerasiae*	*L. terpstrae*
L. alstonii	*L. necaledonica*	*L. meyeri*
L. weilii	*L. hartskeerlii*	*L. yanagawae*
L. satarosai	*L. haakeii*	*L. levetti*
L. borgpetersenii	*L. saintgironsiae*	*L. biflexa*
L. mayottensis		
L. alexanderi		

Ademais, membros do gênero *Leptospira* são classificados também em sorovares com base em diferenças antigênicas nos carboidratos do antígeno O do lipopolissacarídeo (LPS). Os sorovares que apresentam certo grau de identidade são agrupados em sorogrupos. Até o momento, já foram descritos mais de 300 sorovares e mais de 24 sorogrupos patogênicos (CAMERON, 2015). A tabela 2 traz alguns exemplos de sorogrupos e sorovares pertencentes à *Leptospira interrogans*.

Tabela 2. Alguns sorovares pertencentes à *Leptospira interrogans* e sua relação com a classificação em sorogrupos.

Sorogrupo	Sorovar
Australis	Australis, Bratislava
Autumnalis	Autumnalis, Forbragg, Bim
Ballum	Ballum, Arborea
Canicola	Canicola, Portlandvere
Icterohaemorrhagiae	Icterohaemorrhagiae, Copenhageni, Lai
Pomona	Pomona, Kennewicki
Sejroe	Sejroe, Hardjo

2.1.2. Microbiologia

As bactérias do gênero *Leptospira* foram observadas e descritas pela primeira vez em 1907, quando Arthur Stimson evidenciou a presença de espiroquetas nos tubos renais de um paciente o qual a provável causa de morte havia sido Febre Amarela. Devido a sua morfologia, com extremidades em gancho, semelhantes a um ponto de interrogação, Arthur Stimson a chamou de *Leptospira interrogans* (LEVETT, 2001). No entanto, devido a dificuldade em cultivar a bactéria, o primeiro isolamento do agente infeccioso ocorreu anos mais tarde, em 1916, a partir do sangue de pacientes diagnosticados com a enfermidade de Weil, como era anteriormente chamada a leptospirose (INADA et al., 1916).

Membros do gênero *Leptospira* são aeróbios obrigatórios, móveis, com formato espiralado com diâmetro de aproximadamente 100 nm e comprimento de até 20 µm, com suas extremidades em formato de gancho (ADLER, 2015; CAMERON, 2015; PICARDEAU., 2017). A motilidade bacteriana ocorre por meio de dois endoflagelos, localizados em cada polo da célula, os quais seus filamentos estão localizados no espaço periplasmático. A rotação dos flagelos é responsável pelo movimento ao redor do eixo central da célula e pela propulsão celular (ADLER, 2015; TAHARA et al., 2018). Em relação à motilidade celular, é característico das leptospiras possuírem uma velocidade de motilidade elevada, o que prolonga sua sobrevida em meios naturais e facilita a infecção de tecidos do organismo hospedeiro (TAKABE et al., 2013).

As Leptospiras possuem configuração similar a bactérias Gram negativas, com parede celular composta por peptideoglicano intimamente associado à membrana interna e revestido por uma membrana externa de LPS (PICARDEAU, 2017). No entanto, devido ao seu pequeno diâmetro torna-se difícil a utilização de técnicas convencionais de coloração e sua visualização se da unicamente mediante microscopia de campo escuro e por preparação de contraste de prata (ADLER, 2015). Esta camada de peptideoglicano é a principal responsável, juntamente com as proteínas do citoesqueleto, pela forma helicoidal que a bactéria possui (SLAMTI et al., 2011). Outra característica de suma importância é a membrana externa bacteriana a qual apresenta uma densa camada de Lipopolissacarídeo (LPS), quantidade que varia entre espécies patogênicas, intermediárias e saprófitas (LEVETT, 2001), além de conter uma grande quantidade de proteínas de membrana e lipoproteínas, características que não pertencem a outras espiroquetas, tais como *Borrelia* e *Treponema* (HAAKE, 2000; HAAKE & MATSUNAGA, 2010).

As *Leptospiras* são bactérias aeróbias e microaerofilicas, tanto oxidase quanto catalase positiva. Apresentam temperatura ótima de crescimento entre 28 – 30 °C, em um pH de 7.2 – 7.6, e tempo de duplicação, *in vitro*, em torno de 6 – 18 h., sendo que as espécies patogênicas apresentam tempo de duplicação mais elevado que as espécies saprófitas (FAINE et al., 1999; ADLER, 2015). As bactérias cultivadas podem ser armazenadas por longos períodos em meio semissólido a temperatura ambiente, podendo, no entanto, perder viabilidades e fatores de virulência. Entretanto, existe a possibilidade de armazenamento em nitrogênio liquido, que permite um armazenamento em longo prazo sem que ocorra perda dos fatores de virulência, podendo reestabelecer infecções experimentais em hospedeiros apropriados (ADLER & de LA PEÑA MOCTEZUMA, 2010).

2.1.3. Infecção e sintomatologia

A leptospirose em animais de produção está diretamente relacionada com o manejo inadequado e a negligência relacionada às medidas de imunoprevenção (MARTINS & LILENBAUM, 2017). A leptospirose apresenta um amplo espectro de sintomas, principalmente em pequenos animais, desde febre alta, icterícia, insuficiência renal e hemorragia, e a Síndrome Hemorrágica Pulmonar Aguda, quando há hemorragia massiva nos pulmões. Ademais, formas assintomáticas

podem ser observadas frequentemente, em grandes animais (ADLER, 2015; SCHULLER et al., 2015). Tanto animais de produção como domésticos e selvagens podem atuar como portadores e disseminadores do patógeno, principalmente roedores, sendo o homem considerado um hospedeiro acidental (KO et al., 2009; ADLER, 2015).

A leptospirose pode apresentar-se tanto de forma anictérica como de forma ictérica (FAINE et al., 1999). Na forma anictérica, a manifestação clínica divide-se em duas fases. Inicialmente, há dores de cabeça, musculares, calafrios, febre alta e bacteremia, podendo ocorrer a evolução da doença para suas formas mais graves, afetando rins, fígado e pulmões, causando falência desses órgãos e hemorragia. Essa fase costuma durar aproximadamente uma semana e é seguida pela segunda fase, denominada fase imune, caracterizada pela diminuição da febre e dos sintomas e, naturalmente, produção de anticorpos (LEVETT, 2001; BHARTI et al., 2003; RICALDI & VINETZ, 2006). A forma ictérica da doença é mais severa, afetando até 15% do total de casos (COSTA et al., 2015); até 70% dos casos podem evoluir para a forma pulmonar da doença, apresentando elevadas taxas de morte (GOUVEIA et al., 2008; GULATI & GULATI, 2012); 60% dos pacientes com formas graves de leptospirose apresentam dano renal (VISITH & KEARKIAT, 2005). A sintomatologia pode variar de acordo com a virulência, dose da cepa infectante, bem como com o estado de saúde do paciente ou com a espécie do organismo infectado, uma vez que a leptospirose pode ocorrer em diferentes espécies de animais (ADLER, 2015).

2.1.4. Leptospirose em pequenos e grandes ruminantes

A leptospirose bovina é causada por uma grande variedade de sorovares. No entanto, os bovinos são epidemiologicamente mais suscetíveis ao sorovar Hardjo, que tem uma distribuição quase que global. A *Leptospira borgpetersenii* sorovar Hardjo (Hardjobovis, HB) é a cepa mais comum deste sorovar mantida por bovinos, mas a *Leptospira interrogans* serovar Hardjo (Hardjoprajitno, HP) também ocorre em bovinos. Ambas as cepas têm a capacidade de colonizar e persistir no trato uro-genital de bovinos infectados, sugerindo que a disseminação sexual pode ser um fator na transmissão (ADLER 2015).

No setor da bovinocultura, casos de abortos, natimorto, parto prematuro, nascimento de bezerros fracos e peso ao nascer reduzido são os aspectos econômicos mais importantes da leptospirose crônica em bovinos (SANHUEZA et al., 2013; NDENGU et al., 2017). Em infecções incidentais, ocorre 4-6 semanas após a doença aguda, mas com a infecção por Hardjo o intervalo é maior em 6 a 12 semanas. Leptospiras foram detectadas por até 8 dias em descargas vaginais pós-aborto / parto (ELLIS et al. 1985a,b). A infertilidade, que responde ao antibiótico e/ou vacinação, é descrita na infecção por Hardjo (DHALIWAL et al. 1996a, b).

O padrão de infecção por Hardjo em um rebanho varia com as condições de criação e as cepas de Hardjo presentes no ambiente. Em rebanhos infectados endemicamente, onde o rebanho jovem é exposto à infecção antes da criação, os níveis de prejuízo reprodutivo associado são muito baixos. No entanto, nos sistemas de manejo, encontrados particularmente na pecuária leiteira, mais notavelmente a prática de separar bezerros ao nascimento e somente expô-los ao rebanho adulto infectado após a maturidade sexual, garante assim, o fornecimento regular de animais totalmente suscetíveis (SANHUEZA et al. 2013)

Em comparação a bovinos e suínos, as ovelhas foram consideradas resistentes à infecção por leptospirose, com baixas soroprevalências históricas e apenas um pequeno número de sorogrupos sendo associados à doença clínica, sendo esses nomeados Pomona (VERMUNT et al. 1994), Grippotyphosa (TRAP e GARIN 1988), Icterohaemorrhagiae (LEON et al. 1987), Australis e Sejroe (ELLIS et al. 1983a; MCKEOWN e ELLIS 1986). As infecções pelos quatro primeiros sorogrupos são incidentalmente adquiridas e resultaram em surtos esporádicos de doença aguda caracterizada por hematúria, hemoglobinúria, icterícia e morte, geralmente em cordeiros e abortos ocasionais (ELLIS et al., 1983a, b, c; LEON et al. 1987). Estudos envolvendo biologia molecular sugerem que o aborto em ovinos pode ser mais comum do que se pensava anteriormente, visto que o DNA de leptospiras pode ser encontrado no feto, recém-abortado, de ovelhas, caprinos e bovinos (MOSHKELANI et al. 2011). LILENBAUM et al. (2008) também demostrou que é possível detectar DNA da bactéria no sêmen de carneiros infectados e fluidos vaginais de ovelhas, sugerindo transmissão sexual da doença.

Em contraste, as ovelhas atuam como hospedeiro de manutenção alternativo para o sorovar Hardjo. Essa informação pode estar associada a diferentes motivos, (1) prevalências sorológicas significativas para o sorovar Hardjo em algumas populações de ovelhas (EGAN e YEARSLEY, 1987; CERRI et al., 2003; RIDLER et al., 2005; HERRMANN et al., 2004; MARTINS et al., 2012); (2) estudos que envolvem infecções experimentais (COUSINS et al. 1989; FARINA et al. 1996; GERRITSEN et al. 1994) e (3) ovelhas que foram identificadas como um fator de risco para infecção por Hardjo em bovinos e veados (BENNETT 1994; SUBHARAT et al. 2012a, b). Atualmente, sabe-se que o microrganismo pode persistir por longos períodos de tempo nos rins de animais infectados (COUSINS et al. 1989; FARINA et al. 1996; GERRITSEN et al. 1994) e um estudo mostrou que ele também pode persistir no trato genital de animais infectados (ARENT et al. 2013).

A maioria das infecções em ovinos são subclínicas e a infecção clínica pelo Hardjo é raramente vista em bandos amplamente manejados. Em rebanhos intensivamente manejados, o sorovar Hardjo tem sido relatado como causador de infecção clínica na ovelha peri-parturiente e neonatal, com aborto, natimorto, o nascimento de cordeiros fracos (ELLIS et al., 1983a, b, c; MCKEOWN e ELLIS 1986; MCTAGUE 1997) e também foi associado à infertilidade (LILENBAUM et al. 2009).

2.1.5. Patogenia

A entrada da bactéria no organismo do hospedeiro é o primeiro obstáculo para o desencadeamento da enfermidade, obstáculo que normalmente é superado por meio de pequenos ferimentos no epitélio ou pelas vias de mucosa do hospedeiro (PICARDEAU, 2017). Após, ocorre a infecção sanguínea (leptospiremia) e disseminação do patógeno por todo organismo do hospedeiro (TRUCCOLO et al., 2001; SEGURA et al., 2005). Em animais resistentes, há colonização dos túbulos renais proximais e consequente excreção do patógeno ao ambiente, enquanto que em animais susceptíveis há também a colonização de outros órgãos como fígado, pulmões e baço (HAAKE & ZÜCKERT, 2015).

Para que uma bactéria patogênica possa causar doença, sua atividade celular dependerá do grau de virulência que a *Leptospira* possui (ADLER, 2015). Em leptospiras diversos fatores de virulência são propostos (MURRAY, 2015). Entre os fatores que se destacam, encontra-se uma variedade de proteínas de superfície que

possivelmente estejam associadas à infecção e adesão da bactéria no organismo do hospedeiro, embora o mecanismo de invasão ainda não esteja completamente elucidado (FERNANDES et al., 2016), da mesma forma que as bactérias Gram-negativas, o LPS esta relacionado a toxicidade bacteriana (KO et al., 2009; ADLER, 2015). Outro fator de extrema importância, relacionado à virulência bacteriana, é a motilidade que as leptospiras possuem, motilidade essa que está diretamente relacionada a morfologia bacteriana e seu aparato flagelar, que a confere uma capacidade de natação elevada comparada a outras bactérias (WUNDER Jr. et al., 2016).

2.1.6. Modelo experimental

Uma grande variedade de espécies animais tem sido usada como hospedeira para a leptospirose experimental. Estudos iniciais utilizaram porquinhos da índia como um hospedeiro modelo preferido para estudar infecção aguda (NOGUCHI 1918). No entanto, em meados do século XX, descobriu-se que os hamsters eram particularmente suscetíveis à infecção por *Leptospira* (MORTON, 1942). Devido à sua susceptibilidade, rápido crescimento e menor custo, os hamsters são agora rotineiramente usados como modelo primário para a leptospirose aguda (HAAKE 2006). Os hamsters mantêm a suscetibilidade à infecção aguda com o aumento da idade mais do que muitas outras espécies de animais e imitam infecções agudas que compartilham algumas semelhanças com a doença clínica em humanos. Os hamsters têm sido usados extensivamente para testar a tensão bacteriana infectividade e virulência, e como modelo para testar a eficácia de vacinas (ADLER 2015).

Ratos de laboratório comuns (Mus musculus) e ratos (Rattus novegicus) são geralmente inadequados como hospedeiros para a leptospirose aguda, essas espécies são suscetíveis apenas ao desenvolvimento de leptospirose aguda em uma janela de tempo muito curta após o nascimento (PACKCHANIAN 1940). A infecção de camundongos ou ratos com mais de algumas semanas de idade provavelmente levará ao desenvolvimento de infecções crônicas limitadas à colonização do rim. No entanto, a infecção de murganhos tratados com ciclofosfamida (ADLER E FAINE 1976; ADLER 2015) ou murganhos com deficiências de receptores de tipo toll (TLR) podem resultar em infecção letal

(PEREIRA et al. 1998). O uso de cepas de camundongos knockout genéticos (KO) bem definidos na pesquisa da leptospirose está se tornando mais comum. Os ratos também estão sendo usados como hospedeiros experimentais para estudar a leptospirose crônica (TUCUNDUVA de Faria et al. 2007; ATHANAZIO et al. 2008; MONAHAN et al. 2008).

Estudos utilizando bovinos, caprinos e suínos como modelo experimental têm sido realizados, normalmente, para avaliação de hospedeiros experimentais para estudos de infecção e vacinação para leptospirose. Isto se deve, em grande parte, à preocupação com a transmissão zoonótica entre gado e seres humanos, o impacto da leptospirose nos custos de produção animal e a necessidade de vacinas eficazes em animais de produção (ADLER 2015).

Ovelhas têm sido utilizadas como um bom modelo experimental em diversos estudos, como nos domínios cardiovascular, respiratório, reprodutivo e, principalmente, imunológico (RAMOS et al., 2013). As ovelhas são relativamente pequenas em comparação com outros ruminantes, como o gado bovino, e podem ser facilmente alocadas em galpões e galpões menores, facilitando o gerenciamento da pesquisa e minimizando os custos da experimentação (SCHEERLINCK et al., 2008). Bovinos e ovinos apresentam uma forte homologia cromossômica, o que pode justificar as semelhanças nas respostas imunes que eles exibem (PERUCATTI et al., 2012). Neste contexto, os pequenos ruminantes vêm ganhando espaço como modelo experimental, aproximando a pesquisa dos reais hospedeiros da leptospirose. No entanto, ainda são poucos os estudos que utilizam animais de produção como modelo experimental para estudos relacionado à leptospirose, o que demonstra a extrema importância de projetos sobre essa temática para pesquisa na área da leptospirose animal.

2.1.7. Resposta imune e vacinação

Estudos que buscam compreender o mecanismo da resposta imune de um hospedeiro infectado por leptospiras são de extrema importância para o desenvolvimento de vacinas mais eficazes que venham de fato a prevenir e controlar novos surtos de leptospirose. Conforme estudos realizados, a resposta imune humoral é considerada a principal via imunológica para o controle da leptospirose. Isso ocorre devido ao fato das leptospiras apresentarem um grande número de proteínas de superfície e de uma densa camada de LPS, consideradas fatores de virulência, além de serem altamente antigênicas e imunogênicas (CULLEN et al., 2005; ADLER, 2015; GRASSMENN et al., 2017, PICARDEAU, 2017). De fato, camundongos que não possuem células B são susceptíveis à infecção por leptospiras (ADLER & FAINE, 1976). Ao contrário, camundongos que não possuem células T continuam resistentes à leptospirose (ADLER & FAINE, 1977; CHASSIN et al., 2009). A transferência passiva de anticorpos anti-LPS também é capaz de conferir proteção contra leptospirose (JOST et al., 1986; JOST et al., 1989).

Macrófagos desemprenham um importante papel no combate à infecção por leptospiras, tanto por realizar fagocitose como por sintetizar interleucinas pró-inflamatórias, tais como IL-1β, IL-6, IL-8 e TNF-α (XUE et al., 2013). No entanto, estudos descrevem que leptospiras são capazes de sobreviver no interior de macrófagos e de que há diversas proteínas bacterianas responsáveis pela interação com os macrófagos (ESHGHI et al., 2012; ZHANG et al., 2012; TOMA et al., 2014). Apesar de haver associação entre TNF-α e hemorragia pulmonar (KYRIAKIDIS et al., 2011), não há uma relação clara entre níveis de citocinas sintetizadas com severidade ou danos causados por leptospirose (HAAKE & ZÜCKERT, 2015).

A principal diferença entre a resposta imune de hospedeiros suscetíveis e resistentes é que o TLR4 dos hospedeiros suscetíveis não reconhece o LPS de leptospiras devido a uma metilação em resíduo de fosfato, cabendo somente ao TLR2 a sinalização da presença de patógeno, em animais resistentes essa atividade é feita por ambos receptores, acarretando na produção de imunoglobulinas para eliminação das leptospiras do sangue (VIRIYAKOSOL et al., 2006; CHASSIN et al., 2009). Cabe salientar que, espécies saprofíticas morrem na presença de soro com complemento ativado, embora espécies patogênicas sejam capazes de evadir a

ação do sistema complemento (FRAGA et al., 2013; CASTIBLANCO-VALENCIA et al., 2016).

Em relação às medidas de controle, a vacinação é o método mais eficaz para o controle da leptospirose, sendo sua adoção fortemente recomendada (LILENBAUM e MARTINS, 2014). Com base nisso, o desenvolvimento de vacinas para o controle da leptospirose torna-se de extrema importância. As vacinas disponíveis comercialmente são compostas por suspensões celulares (bacterinas) mono ou polivalentes, as quais conferem proteção contra os sorovares presentes na composição vacinal (ANDRE-FONTAINE et al., 2003; KOIZUMI; WATANABE, 2005; GRASSMANN et al., 2017a). No entanto, há controversias quanto a capacidade protetiva e colonização renal. (PETERSEN et al., 200; VERMA; KHANNA; CHAWLA, 2013).

Devido aos argumentos supracitados, diversos estudos têm sido realizados com o intuito de produzir vacinas mais eficazes do que as vacinas compostas por bacterinas e disponíveis comercialmente, destacando-se aqueles estudos que envolvem a produção de vacinas recombinantes. Para isso, os principais alvos para a produção de vacinas recombinantes têm sido as proteínas de membrana externa (*Outer Membrane Proteins* – OMPs) e fatores de virulência que sejam reconhecidos por soro de pacientes naturalmente infectados (GRASSMANN et al., 2017b; DELLAGOSTIN et al., 2017). As proteínas que se destacam, mostrando serem mais promissoras no desenvolvimento de uma vacina recombinante, são as proteínas LigA e LigB. Essas são conservadas em espécies patogênicas (CERQUEIRA et al., 2009; FOUTS et al., 2016) e desempenham importantes funções celulares, tais como adesão e invasão ao hospedeiro e evasão do sistema imune, além de ter sua expressão aumentada durante infecções experimentais (MATSUNAGA et al., 2005; CHOY et al., 2007; CHOY et al., 2011; CASTIBLANCO-VALENCIA et al., 2016; HSIEH et al., 2017). No entanto, mais estudos devem ser realizados, uma vez que esses estudos foram conduzidos utilizando hamsters e estão limitados a esse modelo experimental (SILVA et al., 2007; YAN et al., 2009; EVANGELISTA et al., 2017).

Outra proteína de extrema importância para produção de vacinas recombinantes é lipoproteína LipL32, conhecida como proteína-1 associada a hemolisina (Hap-1) (BRANGER et al., 2001), sendo a proteína de membrana externa (OMP) mais abundante exposta na superfície celular (CULLEN et al., 2005),

conservada entre as espécies patogênicas, ausente nas saprófitas (HAAKE et al., 2004) e com características antigênicas e imunogênicas (HAAKE et al., 2000; GRASSMANN et al., 2012) e que em bovinos, onde a imunidade não é dependente apenas de anticorpos, mas também está correlacionada com a liberação de IFN-γ por células T, o principal antígeno estimulador de IFN-γ é a proteína LipL32 (ADLER, 2015). Entretanto, estudos que utilizaram a proteínas LipL32 não atingiram resultados tão satisfatórios, quando comparados aos resultados obtidos pelas proteínas LigA e LigB. (BRANGER et al., 2005; SEIXAS et al., 2007; HABARTA et al., 2011; LUCAS et al., 2011; GRASSMANN et al., 2012; HUMPHRYES et al., 2014).

2.1.8. Genômica e proteômica da *Leptospira* spp.

As pesquisas relacionadas ao desenvolvimento de vacinas recombinantes contra a leptospirose tiveram uma grande evolução e aprimoramento graças ao maior investimento nos estudos relacionados à área de genômica e proteômica. Os primeiros genomas de *Leptospira* a serem sequenciados foram os da *L. interrogans* sorovar Lai (REN et al., 2003) e *L. interrogans* sorovar Copenhageni (NASCIMENTO et al., 2004), pertencentes ao sorogrupo Icterohaemorrhagiae. Este foi um marco de extrema importância para os estudos envolvendo leptospirose. Ambos os genomas apresentam 95% de identidade a nível nucleotídico e são compostos por dois cromossomas circulares, um com aproximadamente 4.000 kb e outro, de menor tamanho, com aproximadamente 350 kb (NASCIMENTO et al., 2004).

Nos anos subsequentes, diversos estudos relacionados ao sequenciamento do genoma completo de leptospiras foram sendo realizados e a diferença genômica entre as espécies saprófitas, *L. biflexa* (PICARDEAU 2008), espécies intermediárias (RICALDI et al., 20120 PUCHE et al., 2018) e espécies patogênicas (BULACH et al., 2006; CASATE et al., 2015; Nally et al., 2016; JORGE et al., 2018) passaram a ser desvendadas. Os estudos relacionados ao genoma permitiram uma melhor compreensão do mecanismo de virulência das leptospiras e o avanço em outras áreas de extrema importância para a busca de novos antígenos vacinais e a produção de vacinas recombinantes, como por exemplo, áreas voltadas à proteômica.

Com a disponibilidade de quase todos os genomas do gênero *Leptospira* já sequenciados (FOUTS et al., 2016) e com o auxilio da bioinformática, as áreas relacionadas a vacinas recombinantes e vacinologia reversa têm crescido e se destacado nos últimos anos. Inúmeros estudos têm sido realizados com o fito de obter novos antígenos vacinais que permitam o desenvolvimento de vacinas recombinantes e que venham a superar as deficiências das vacinas comercias atualmente disponíveis (HARTWIG et al., 2010; HARTWIG et al., 2011; UMTHONG et al., 2015; CONRAD et al., 2017; EVANGELISTA et al., 2017).

A vacinologia reversa tem por objetivo identificar antígenos vacinais, no caso de leptospiras, OMPs e lipoproteínas expostas, a partir de dados do genoma bacteriano (RAPPUOLI, 2001). Além disso, pode também identificar epítopos para a

confecção de antígenos vacinais (UMAMAHESWARI et al., 2012). De fato, a abordagem da vacinologia reversa já foi aplicada às leptospiras, conforme descrito por Dellagostin et al., (2017). Em recentes estudos realizados, foram identificados 18 OMPs e 8 lipoproteínas como candidatos vacinais (GRASSMANN et al., 2017b) e 191 alvos preditos altamente antigênicos e expostos (ZENG et al., 2017).

Considerando o grande número de alvos identificados, é notável o potencial dos estudos supracitados. Entretanto, mais experimentos são necessários para aprimorar as metodologias para o descobrimento de novos alvos vacinais e avaliação da eficácia desses antígenos vacinais em diferentes modelos experimentais.

2.1.9. As proteínas Lig

As proteínas Lig, *Leptospiral immunoglobulin-like proteins*, foram inicialmente identificadas por meio de soro humano convalescente em biblioteca de antígenos de *L. interrogans* e *L. kirschneri* (MATSUNAGA et al., 2003). Existem três proteínas Lig classificadas como LigA, LigB e LigC. Elas são caracterizadas como proteínas de membrana, estando presentes na superfície bacteriana (GRASSMANN et la., 2017a) e apresentam grande similaridade com proteínas de adesão pertencentes à *Escherichia coli* e *Yersinia pseudotuberculosis* (HAAKE & ZIICKERT, 2015).

As proteínas LigA e LigB possuem características antigênicas e imunogênicas e um alto grau de conservação, 63-99% (MCBRIDE et al., 2005; DELLAGOSTIN et al., 2017). Embora, a LigA esteja presente em somente 3 espécies patogênicas (*L. interrogans, L. kirschneri e L. alstonii*), a LigB, por sua vez, está presente em todas (MATSUNAGA et al., 2003; CERQUEIRA et al., 2009; MCBRIDE et al., 2009; FOUTS et al., 2016). Por outro lado, a proteína Lig C é considerada um pseudogene e não está relacionada com virulência bacteriana e, até então, não foi avaliada como candidata vacinal (MCBRIDE et al., 2009).

Essas proteínas estão diretamente relacionadas a fatores de virulência celular e durante a infecção (*in vitro*) possuem sua expressão aumentada (MATSUNAGA et al., 2005; CAIMANO et al., 2014). Ao fazer uma avaliação comparativa sobre a estrutura proteica, percebe-se que a região N-terminal dessas proteínas possuem um menor grau de identidade, sendo denominadas de LigAni e LigBni (“ni” - não idêntico), enquanto que a região C-terminal dessas proteínas é praticamente

idêntica, sendo designada LigBrep (repetitiva) (CERQUEIRA et al., 2009; MCBRIDE et al., 2009). Diferentes estudos descrevem uma série de funções biológicas dessas proteínas relacionadas à virulência, tais como, funções de adesão e invasão (MATSUNAGA et al., 2005; CHOY et al., 2007), inibidoras de coagulação sanguínea (CHOY et al., 2011; HSIEH et al., 2017) e, quando o assunto é relacionado a imunologia e vacinologia, a importante função de evasão do sistema imune (CHOY, 2012; CASTIBLANCO-VALENCIA et al., 2016).

Estudos têm demostrado que as proteínas LigA e LigB possuem importantes características antigênicas e imunogênicas (HARTWIG et al. 2010; EVANGELISTA et al., 2016; CONRAD et al., 2017) e que, atualmente, são consideradas candidatas promissoras à antígenos vacinais (DELLAGOSTIN et al., 2017). Há uma vasta gama de estudos descrevendo o potencial protetor dessas proteínas, embora muitos destes estudos estejam restritos apenas ao hamster como modelo experimental e também haja algumas discordâncias de resultados entre estudos, conforme previamente revisado (DELLAGOSTIN et al., 2011; DELLAGOSTIN et al., 2017; GRASSMANN et al., 2017a). Ambas proteínas já foram avaliadas como antígenos recombinantes em formulações vacinais, conferindo 100% de proteção (PALANIAPPAN, 2004; SILVA et al., 2007; YAN et al., 2009; CAO et al., 2011; COUTINHO et al., 2011; HARTWIG et al., 2013; CONRAD et al., 2017), e recentemente foi demostrada a capacidade de conferir imunidade esterilizante da proteína LigB, utilizando hamsters como modelo experimental (CONRAD et al., 2017).

2.1.10. A proteína LipL32

A LipL32, conhecida como proteína-1 associada a hemolisina (Hap-1) (BRANGER et al., 2001), é a proteína de membrana externa (OMP) mais abundante em espécies patogênicas de leptospiras, com aproximadamente 40.000 cópias por célula (MALMSTROM et al., 2009). Atualmente, sua localização tem sido alvo de grandes discussões científicas, uma vez que inicialmente a localização da proteína foi demostrada na membrana externa por fracionamento com Triton X-114 (HAAKE et al., 2000) e após mais de 10 anos de estudos, com o avanço de técnicas de biologia molecular, discute-se a possibilidade da proteína LipL32 estar localizada no folheto interno da membrana externa no periplasma celular (PINNE & HAAKE 2013).

Apesar da LipL32 constituir mais de 75% da membrana celular, ainda não foi descoberta a real função que esta proteína possui a nível celular. Pensava-se na possibilidade da LipL32 estar relacionada com a estrutura e estabilização da membrana celular. Mas, Murray et al., (2009), demostrou que o crescimento e a morfologia bacteriana é normal mesmo na ausência dessa proteína, neste mesmo estudo se observou que cepas mutantes carentes de LipL32, foram capazes de unir componentes da matriz extracelular nas mesmas proporções que as cepas não mutantes, assim como também foram capazes de desencadear infecções agudas e crônicas nos hospedeiros. O fato da proteína LipL32 ser abundante na membrana externa da célula e estar presente nas espécies patogênicas e ausente nas espécies saprófitas (HAAKE et al., 2004) faz-se pensar que a função biológica dessa proteína possa estar relacionada à virulência bacteriana. Mas, curiosamente, é demostrado que na sua ausência, outros fatores a suplementam em modelos experimentais de deleção (ADLER, 2015).

No entanto, apesar da função biológica da LipL32, a nível bacteriano, ainda não ter sido descoberta, estudos têm demostrado que essa proteína pode ter importante função na produção de vacinas recombinantes. A proteína LipL32 é uma das mais reativas em soros de pacientes com a enfermidade na fase aguda, assim como na fase crônica da enfermidade (LESSA-AQUINO et al., 2013). Da mesma forma, sua expressão durante a infecção aguda também tem sido evidenciada em modelo animal (HAAKE et al., 2000). A LipL32 tem demostrado possuir características que a tornam um importante alvo de estudos relacionados a vacinas recombinantes (DELLAGOSTIN et al., 2017). Estudos já demonstraram que a proteína possui características antigênicas e imunogênicas (HAAKE et al., 2000; GRASSMANN et al., 2012; DELLAGOSTIN et al., 2017). Numerosos são os estudos relacionados à LipL32, no entanto, pouco ainda se sabe sobre essa abundante proteína pertencente às leptospiras. Adler, (2015) descreve em seu livro que “o papel da LipL32 na imunologia permanece enigmático e merece investigações adicionais”. Sendo assim, tornam-se necessários mais estudos para que se possa obter uma melhor compreensão sobre essa proteína.

3. HIPÓTESE E OBJETIVO

3.1. Hipótese

Ovinos podem ser utilizados como modelo experimental para estudos relacionados à avaliação de vacinas recombinantes. As proteínas recombinantes LigAni, LigBrep e LipL32 associadas com diferentes adjuvantes induzem a produção de altos títulos de anticorpos, utilizando ovinos como modelo experimental.

3.2. Objetivo geral

Avaliar a imunogenicidade de vacinas recombinantes contra leptospirose em ovinos e estabelecer um modelo de infecção experimental.

3.2.1. Objetivos específicos

- Otimizar o processo de expressão das proteínas LigAni e LigBrep em *E. coli*;
- Expressar, purificar e caracterizar as proteínas recombinantes LigAni, LigBrep e LipL32 para utilização como antígenos vacinais;
- Padronizar a metodologia de infecção experimental, para a utilização de ovinos como modelo experimental de leptospirose;
- Avaliar a resposta imune humoral induzida por meio de diferentes formulações vacinais, comparando combinações de antígenos e de adjuvantes, utilizando ovinos como modelo de leptospirose animal.

4. CAPÍTULOS

4.1. Capítulo 1.

Optimization of the expression of the LigAni and LigBrep of *Leptospira interrogans* in *Escherichia coli*

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Abstract

Leptospirosis is a zoonotic disease that is caused by pathogenic bacteria of the *Leptospira* genus. It causes high rates of abortions, stillbirths and infertility and, as such, is a source of significant economic losses in the agricultural sector. Studies that sought to produce more effective vaccines against leptospirosis than those commercially available have focused on the expression of recombinant proteins. However, it is difficult to express some of these proteins, and there is a need to optimize the expression process. The objective of this study was to evaluate the factors that may improve the expression level of the LigAni and LigBrep recombinant proteins in *Escherichia coli*. The relationship between different culture conditions, such as temperature, IPTG concentration, induction time, and different expression strains, and the yield of these proteins was evaluated. The findings revealed that a culture temperature of 37 ºC yielded higher concentrations of both proteins: LigBrep (167 µg/mL) and LigAni (513.5 mg/L). However, no significant difference in the yield of LigBrep or LigAni proteins was observed in response to different concentrations of IPTG, which ranged from 0.25 to 1.5 mM. In addition, the induction period of four hours or 16 hours did not significantly influence the protein yield. Significant difference in LigBrep protein production was observed between the BL21 (DE3) Star (217.3 µg/mL), BL21 (DE3) PlysS (67.6 µg/ml), and *E. coli* Rosetta (62.7 µg/mL) strains. In terms of the recombinant LigAni protein, only *E. coli* BL21 (DE3) Star (218.9 µg/mL) and *E. coli* Rosetta (144.8 µg/mL) were able to express the protein. The findings of this research indicate that culture temperature has a significant influence on the expression levels of recombinant proteins. As such, studies that seek to optimize the process by which recombinant proteins are expressed while also reducing production costs should pay close attention to the relationship between the culturing temperature and level of expression.

Keywords: Optimization, Recombinant protein, Leptospirosis, LigA and LigB

4.1.1. Introduction

The genus *Leptospira* has 35 species and 24 serogroups, with more than 300 described serovars, of which more than 250 are pathogenic [1, 2]. Many proteins, mainly virulence factors and membrane associated or surface exposed, have been studied as possible antigens for diagnostic tests or vaccine antigens [3, 4, 5]. Of these proteins, Leptospiral immunoglobulin-like proteins (Lig), including LigA and LigB are the most prominent. The Lig are exposed on the surface of pathogenic leptospires, and are absent in saprophytes [6]. Lig can interact with multiple proteins of the host extracellular matrix, such as fibronectin, fibrinogen, collagen, and laminin [7, 8]. Lig have a highly conserved amino acid sequence of around 70% to 90% identity among different leptospiral species. LigA and LigB share identical N-terminal portions (LigBrep), and the other regions of the proteins are variable (LigAni and LigBni) [3, 9]. The immunoprotective potential of these proteins has been evaluated and initial results indicate that they offer good antigenic and immunogenic abilities [10, 3, 11, 12] and are, therefore, possible candidates for vaccine antigens [13].

Escherichia coli strains are commonly used in the production of recombinant proteins [14] and have proven to represent a good expression model for the LigBrep and LigAni proteins [15, 12]. However, studies have shown that different culture conditions, such as temperature [16], IPTG concentration, and post-induction culture time [17, 18], may influence the yield of such proteins. Additional elements, such as the type of expression strains [19] and the culture media [20], may also have an influence on yield. Therefore, the aim of this study was to evaluate how variations in the culture conditions influence the level of expression of recombinant LigBrep and LigAni proteins from *L. interrogans* serovar Copenhageni.

4.1.2. Methodology

Study Design

The culture conditions in which LigBrep and LigAni proteins were expressed were varied to assess the relationship between different factors and the expression yield. Four conditions were assessed as follows: temperature (25 ºC, 30 ºC, and 37 ºC); concentration of IPTG (0.25 mM, 0.50 mM, 1.0 mM, and 1.5 mM); induction time (4 h, 8 h, 12 h, and 16 h); and strain of expression (Table 1). Each variable of interest was

separately assessed. With the exception of changes to these variables, the same culture conditions were replicated throughout the experiment.

Table 1: Strains used in the study and their characteristics

Strains	Characteristics
***E. coli* BL21(DE3) Star**	Ability to express high levels of non-toxic recombinant proteins. Expression uses the T7 promoter system.
***E. coli* BL21(DE3) PlysS**	Strain contains the pLysS plasmid, which carries the gene encoding T7 lysozyme. This reduces the background level of target genes following induction by IPTG. Expression uses the T7 promoter system. deficiency in proteases lon and OmpT
***E. coli* Rosetta (DE3) PlysS**	Chloramphenicol-resistant strain that is capable of expressing rare codons such as AGG, AGA, AUA, CUA, and CCC.

Strains and culture conditions

The strains were obtained from the culture collection of the Technology Development Center (CDTec) of the Federal University of Pelotas. Gene fragments of LigBrep and LigAni were cloned into pAE vector that allowed the fusion of a six-amino-acid histidine into the N-terminal portion of the recombinant protein [12]. These plasmids were used to transform the *E. coli* strains used in this study. Strains were cultured in Luria-Bertani medium (LB) and incubated at 37 °C under constant agitation at 180 rpm. BL21 (DE3) PlysS and Rosetta medium was supplemented with 50 µg/mL of the antibiotic Chloramphenicol.

Expression test

To assess the level of expression of the recombinant proteins under standard conditions, the transformed strains were cultured in 50 mL of LB medium supplemented with 100 µg/mL Ampicillin and 50 µg/mL of Chloroanfenicol, when necessary, at a temperature of 37 ºC. A constant agitation of 180 rpm was maintained until the culture reached a OD_{600} 0.6-0.7. Subsequently, 1.0 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the culture to induce the expression of protein of interest, and the culture was then maintained at 37 °C, 180 rpm for 3 h.

Analysis of protein expression and quantification

SDS-PAGE (12% acrylamide) was employed to assess the expression of the recombinant proteins. Culture samples were collected at two periods during the expression: in the pre-induction and post-induction period, with induction by IPTG. They were then evaluated using SDS-PAGE. Western blot technique was used to characterize recombinant proteins using anti-HIS MAB. To quantify the recombinant proteins, a Western blot of samples collected after IPTG induction and the pre-quantified protein sample was performed on a curve at different concentrations. The Western blot result was input into the TotalLab® Quant Software for quantification.

Cell viability

To evaluate the cell viability and microbiological growth, aliquots of 0.1 mL of culture were collected every two hours over a culturing period of 16 hours, and the samples were evaluated by counting colony forming units (CFU). Serial dilutions were performed in phosphate buffered saline (PBS), pH 7.4, and transferred to Petri plates containing 100 µg/ml ampicillin LB agar.

Statistical analyses

The quantitative results were expressed as mean and a two-way analysis of variance (ANOVA) was performed. Post-hoc comparisons were assessed using Tukey test. The significance level was 95%.

4.1.3. Results

Expression of recombinant proteins LigBrep and LigAni

SDS-PAGE analysis demonstrated that the expression of the recombinant proteins showed bands of the expected size. Western blot analysis of the samples using anti-HIS monoclonal antibody confirmed the expression of the target proteins (figure 1).

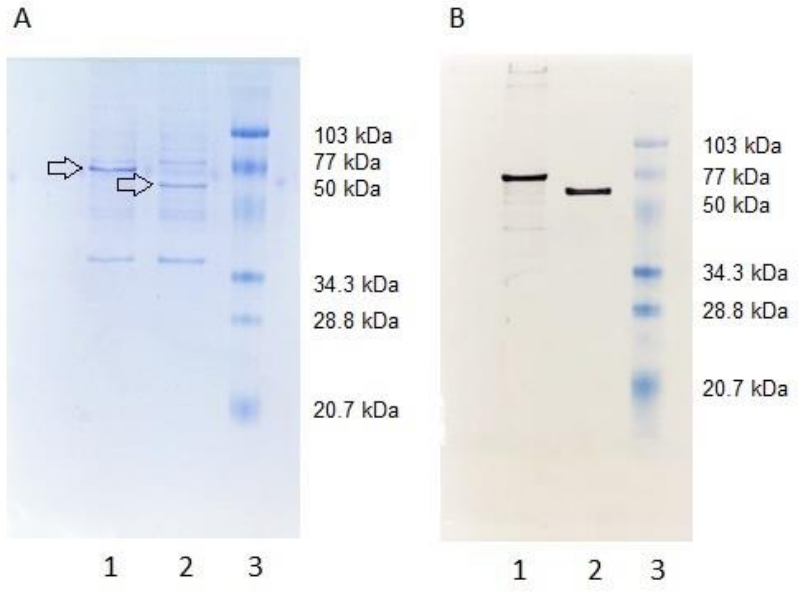

Figure 1. SDS-PAGE and Western blot analyses of recombinant LigBrep and LigAni proteins. A. SDS-PAGE of *E. coli* BL21 (DE3) Star expressing recombinant proteins. 1. LigAni; 2. LigBrep; 3. kDa marker. B. Western blot analysis of *E. coli* BL21 (DE3) Star expressing recombinant proteins. 1. LigAni; 2. LigBrep; 3. kDa marker (Bio-Rad).

Optimization of the temperature expression process

Significantly higher concentrations of the recombinant protein LigBrep were expressed at a temperature of 37 °C (167 µg/mL) than at 25 °C (54.4 µg/mL). However, this difference was not significant in comparison to the concentrations obtained at 30 ºC (95.1 µg/mL). As was the case with LigBrep, the highest yield of LigAni protein was observed at a culture temperature of 37 ºC (513.5 µg/mL). However, this concentration was significantly higher than that observed in the cultures at 30 ºC (9.6 µg/mL) and 25 ºC (4.5 µg/mL) ($p < 0.05$), as shown in Figure 2.

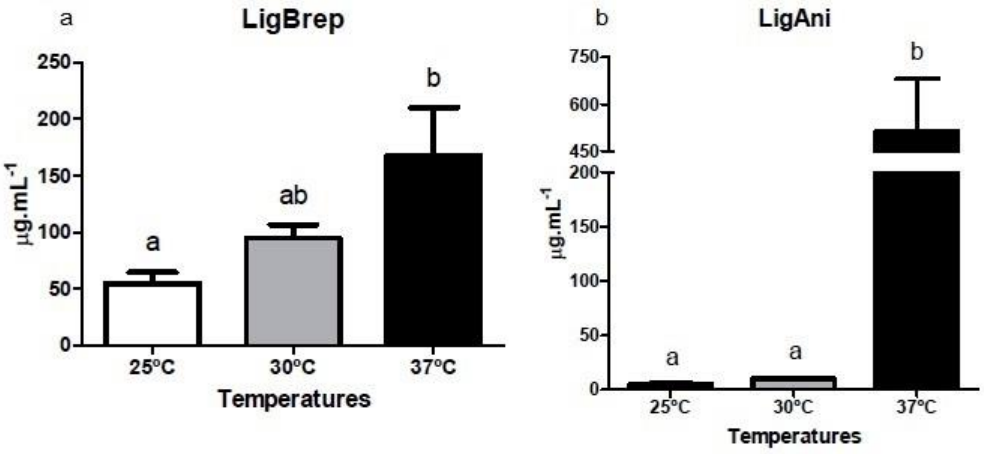

Figure 2. The expression of recombinant proteins in varying culture temperature.

Optimization of IPTG concentration and induction time

Figure 3 presents the levels of expression in response to variations in the concentration of IPTG. No significant difference in the yield of LigBrep protein was observed in response to different concentrations of IPTG: 0.25 mM (228.0 µg/mL), 0.50 mM (231.1 µg/mL), 1.0 mM (188.0 µg/mL), and 1.5 mM (144.3 µg/ml). Similarly, no significant difference in LigAni yield was observed in response to the different concentrations of IPTG: 0.25 mM (389.6 µg/ml), 0.50 mM (377.1 µg/ml), 1.0 mM (490.1 µg/ml), and 1.5 mM (373.8 µg/mL).

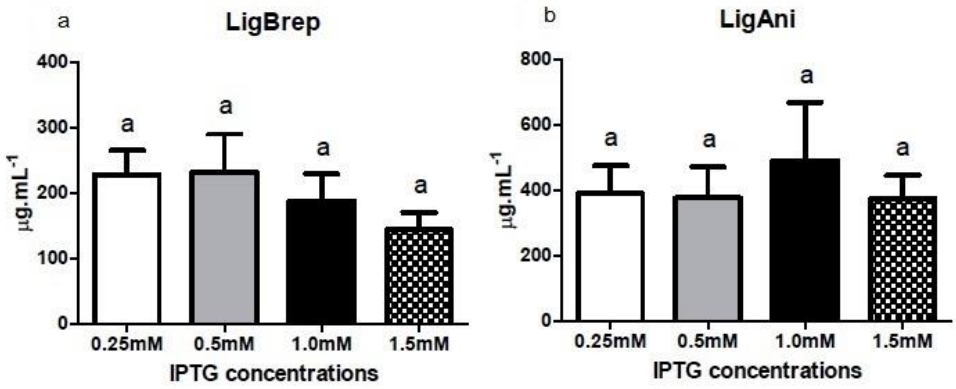

Figure 3. Expression of recombinant proteins at different concentrations of IPTG.

Figure 4 presents the yield in response to variations in the induction time. There was no relationship between the induction time and the protein yield.

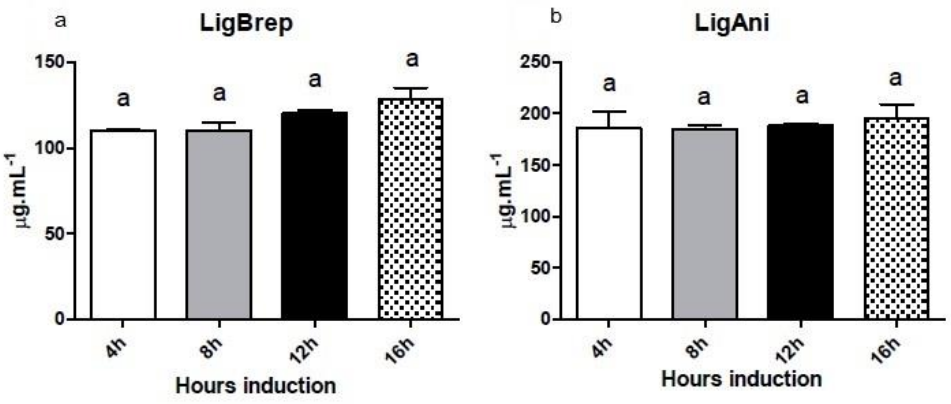

Figure 4. Expression of recombinant proteins at different induction times.

The CFU counts per milliliter (CFU/mL) were assessed to evaluate if an exponential increase in viable cells occurred at different culture times. A cell viability count of 10^7 CFU/mL and 10^8 CFU/mL was obtained from the expression of the LigBrep and LigAni proteins respectively over the full 4-16h period. As such, there was no exponential increase in viable cells. This finding is in line with the results of the assessment of the relationship between the induction period and protein yield. Those variations in the induction period did not influence the yield of recombinant protein obtained.

Optimization of the expression process for the different strains

Figure 5. Presents the levels of expression in response to variations in the strain. Significant difference in LigBrep protein production was observed between strains BL21 (DE3) Star (217.3 µg/mL), BL21 (DE3) PlysS (67.6 µg/mL), and *E. coli* Rosetta (62.7 µg/mL). In terms of the recombinant LigAni protein, only *E. coli* BL21 (DE3) Star (218.9 µg/mL) and *E. coli* Rosetta (143.8 µg/mL) were able to express the protein with statistical significant difference ($p > 0.05$).

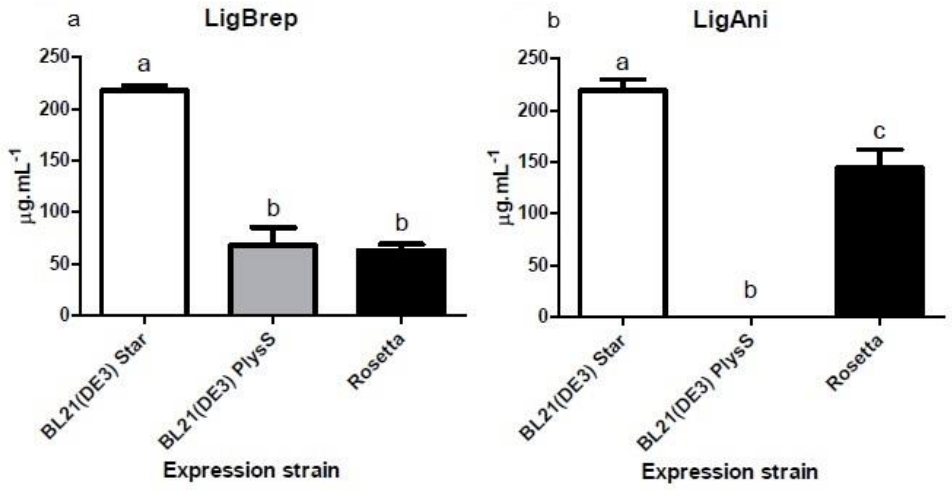

Figure 5. Expression of recombinant LigBrep (A) and LigAni (B) proteins by the strains of *E. coli* BL21 (DE3) Star, BL21 (DE3) PlysS and Rosetta.

4.1.4. Discussion

Four different culture conditions were assessed with the intention of identifying methods of optimizing the process by which LigBrep and LigAni recombinant proteins are expressed. Of these, the temperature was shown to have a strong influence on the expression level of the recombinant proteins, as well as the different expression strains. The fact that the concentration of IPTG and the induction time did not show statistical difference brings the possibility of using a lower concentration of IPTG and during a shorter induction period is of great importance to minimize costs and time, without losing quality and protein yield.

Previous studies have shown that temperature of 37 °C is the ideal culture temperature when using *E. coli* as a model for the expression of recombinant proteins [21, 22]. Additionally, previous research has reported that a culture temperature of 37 °C was responsible for a 40% increase in yield relative to a culture temperature of 28 ºC [16]. However, the researchers in this study were unable to observe a significant difference in the yield of LigB between the two culture temperatures. Based on these findings, it is recommended that *E. coli* be cultured at a temperature of 37 °C to enhance the expression of LigBrep and LigAni proteins.

The evaluation of different concentrations of IPTG is also of significance when attempting to optimize the expression of recombinant proteins [18, 19]. High concentrations of IPTG may impair the development of the culture and reduce protein

yield because IPTG is toxic to cells and can, therefore, lead to death and protease release [23, 24]. In the present study, the concentration of IPTG did not have a significant impact on the yield of LigBrep or LigAni protein expression. This entails that the lowest concentration of IPTG can be used in the culturing process. Interestingly, the concentration of LigBrep was generally inversely proportional to the concentration of IPTG used in the cultures. As such, the concentration of LigBrep was used to denote the lowest concentration of IPTG. Similarly, another study revealed that concentrations of IPTG as low as 0.1 mM were sufficient for the effective induction of LigB expression [16]. It is worth noting that the reduction in the concentration of IPTG is attractive from the industrial point of view because this compound has a high cost [25, 26]. As such, optimizing the IPTG concentration could minimize production costs while also avoiding loss in protein yield, making the results obtained by the present study industrially interesting.

There was no significant relationship between culture time and expression yield of recombinant proteins, suggesting that the 4 h induction period is more indicated. These results are consistent with recent studies of recombinant *Leptospira* protein expression [15, 16]. Minimizing costs and production time without losing product quality are always well-considered strategies, the present study demonstrates that there is no requirement to perform cultivation of the LigBrep and LigAni proteins over long periods, such as 8 or 16 hours, thereby minimizing the production time.

Some researchers have evaluated the different expression strains as a potential strategy by which a higher yield of recombinant protein can be obtained and these studies have shown that different expression strains have a strong influence on the protein yield obtained [27, 19]. In the present study it was possible to observe that with the exception of non-expression of LigAni by strain (DE3) pLysS, all others demonstrate distinct capacities in final protein yields, *E. coli* strain BL21 (DE3) Star being the most indicated for expression of both the recombinant proteins evaluated. However, another study comparing the yield of OprI lipoprotein using two different expression strains, *E. coli* Rosetta (DE3) pLysS and *E. coli* BL21 (DE3), and shows that Rosetta (DE3) pLysS was the strain that obtained a better protein yield [28]. This could be attributed to the fact that strains of *E. coli* Rosetta (DE3) have additional copies of some of the rare-codon tRNA genes and these facilitate expression of genes from other microorganisms with different codon usage, such as AGA (Arg), AGG (Arg), CCC (Pro), ATA (Ile), CTA (Leu), GGA (Gly), and CGG (Arg) [29].

As such, different strains may have a strong impact on the expression of recombinant proteins [27, 19], which is in agreement with the results obtained in the present study, once all strains evaluated show significant differences in the protein yield obtained. Although the strain BL21 (DE3) Star had a better yield on the expression of both recombinant proteins evaluated, the present study shows that the LigBrep protein can also be expressed in the Rosetta (DE3) PlysS and BL21 (DE3) strains pLysS, however with a lower yield. The same occurs with the LigAni protein which can also be expressed by the Rosetta strain (DE3). Thus, the present study shows other possibilities of expression, in the absence of *E. coli* strain BL21 (DE3) Star. Thus, the results show that it was possible to observe that different factors influence the protein yield and that the optimization of the expression process becomes an important strategy to minimize the costs and time of the expression process without losing protein yield.

4.1.5. Conclusion

The different culture conditions evaluated have been shown to have a strong influence on the final yield of the recombinant LigBrep and LigAni proteins of *Leptospira interrogans*. We conclude that the ideal conditions to obtain the highest yield of both proteins are the use of *E. coli* strain BL21 (DE3) Star, cultivated at a temperature of 37 ° C, induced with 0.25 mM IPTG over a period of 4 h. The possibility of using lower concentrations of IPTG and a shorter induction period then standard protocols becomes an interesting strategy to minimize costs to obtain the final product.

4.1.6. Acknowledgements

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4.1.7. References

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4.2. Capítulo 2.

Trial of experimental leptospiral infection in sheep

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ABSTRACT

***Background*:** Leptospirosis is currently a source of significant economic losses in the agribusiness; as such, experimental studies on this infection are required to develop a better understanding of the pathogenesis, treatment, and immunoprophylaxis of the disease. Sheep may represent a good model for ruminants in such models. Despite the extent of the studies that has been conducted thus far, researchers have yet to reach a consensus on the experimental practices to apply for leptospirosis in this animal species, and several gaps in understanding remain. To bridge these gaps, the present study aimed to assess the usage of several tools for the monitoring of experimental leptospirosis in sheep.

***Material, Methods & Results*:** Twelve Santa Ines sheep of different ages were each allocated to one of four groups (A, B, C, and D). The subjects in groups A, B, and C received different doses of *Leptospira interrogans* serogroup Icterohemorrhagiae by intraperitoneal route, $1x10^2$, $1x10^5$, and $1x10^8$ respectively. Group D was the control. Hematological, biochemical and clinical parameters were evaluated daily. Serology by microscopic agglutination test (MAT) and PCR were performed to evaluate the infection status. The most remarkable clinical signs were fever (41 ºC) and dehydration, and acute pain (cub). Two animals from Group C presented leukocytosis. Only those in Group C exhibited positive results according to serology, while positivity in PCR was observed in animals in groups A and C. The results of the experiment indicated that sheep may be experimentally infected and can, therefore, be used as a model for leptospirosis in ruminants. Clinical signs cannot be considered to represent a reliable parameter for evaluating the development of leptospirosis in experimentally infected sheep. We recommend the use of urine PCR and serology to confirm the infection in experimentally infected animals and daily complete blood count (CBC) as a follow-up tool.

***Discussion*:** It was observed that the clinical signs cannot be considered as a reliable parameter to evaluate the pathogenesis in experimentally infected ewes, being important to emphasize that the age of the animals does not seem to alter their susceptibility to the infection. This finding is in agreement with other experimental

studies, which report that leptospirosis infection in ruminants occurs asymptomatic and subclinical. Hematological and biochemical tests proved to be adequate tools to monitor the experimental infection. Studies have shown that the complete blood count has been used to monitor the acute phase of leptospirosis and is effective in detecting anemia and leukocytosis with neutrophilia in ruminants. Despite the lack of clinical signs, the serological and molecular results confirmed the experimental infection. PCR has been used as an important tool in the diagnosis of leptospirosis. In addition, the current study is the first of its kind to use PCR to detect the carrier status in experimentally infected ewes. Despite this limitation, PCR was very effective in confirming the infection and should be considered for use in experimental studies. Sheep have been used as a good experimental model in several studies, sheep are relatively small compared to other ruminants and can be easily allocated in smaller pens and pens, facilitating the management of research and minimizing the costs of experimentation. In this context, we suggest that sheep represent a good model for the study of leptospirosis in ruminants and therefore a reliable protocol for experimental infection by leptospirosis is necessary.

Keywords: Ruminants, *Leptospira*; Experimental infection; Animal model

4.2.1. INTRODUCTION

Leptospirosis is an infectious, zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira* [21]. This disease causes significant economic losses to livestock [24,12], mainly due to abortions, premature births, stillbirths and weak calf syndrome [5]. In ruminants, leptospirosis usually presents as a subclinical and silent infection [23]. Despite the significance of this disease, there is a lack of available information on the relationship between infection of leptospires and their hosts, particularly in small ruminants, and few studies have been conducted with the intention of developing a better understanding of infectious aspects such as pathogenesis, vaccinology and immune response [11, 14].

The pioneers in the experimental sheep infection were Langham et al. (1958), who infected ten sheep of 4-5 months of age with a *L. interrogans* serovar Pomona strain and observed acute signs of infection, such as anorexia, jaundice and hematuria. A similar study conducted at a later date was the first to report the concentration of $1x10^8$ *leptospires* [10]. A few years later, some experimental studies focused on the use of adapted strains, such as the Hardjo [9, 14, 2]. Due to the lack of updating of the few studies already done, particularly in our scenario, in order to have a better understanding of the infectious aspects in small ruminants, several gaps still need to be elucidated, such as the infectious dose, strains, the route of infection and the methods to follow the experimental infection. Considering the

importance of these animals as models, the purpose of the present study was to assess the usage of several tools for the monitoring of experimental leptospirosis in sheep.

4.2.2. MATERIALS AND METHODS

Animals and experimental groups

The clinical trial used 12 sheep of the Santa Ines breed, the most common breed in Brazil. Animals were randomly allocated into four groups of three animals each. Three groups were experimentally infected (A, B, and C) and one remained as the control (D). Each group consisted of one cub (4-6 months old), one young adult (one- year-old), and one mature adult (four-year-old). There was no contact between animals from different groups. Water, food supply, and excreta collection were isolated to avoid cross infection among groups. The experiments were carried out over a period of 14 days. After that, the animals were all intramuscularly treated with streptomycin, 25 mg/kg, and penicillin, 25.000 IU, (both provided by Pendfort PPU, Ouro Fino, Cravinho/SP/Brazil) for three days, independently of the outcomes. After treatment, the animals were released only after exhibiting PCR negative results.

Experimental infection

Prior to the beginning of the experiment, blood (serology) and urine (PCR) samples were collected to confirm the absence of a preexistent infection. Experimental animals received different doses of low-passage *L. interrogans*, serogroup Icterohaemorrhagiae, strain FIOCRUZ L1-130 by intraperitoneal route, according to the method described by Andreani and collaborators (1983). Groups A, B, and C received $1x10^2$, $1x10^5$, and $1x10^8$ of leptospiras, respectively. Group D (the control) received 1 ml of sterile culture medium (EMJH).

Sampling

Blood samples were collected daily for 14 days via puncture of the jugular vein using sterile needles (40 x 12mm) and were stored in two vacuum tubes (Vacutainer®, BD, São Paulo, SP, Brazil). One tube (without anticoagulant) was allowed to clot, and the serum was used for serology and biochemistry, while the other (with EDTA) was used to assess the complete blood count (CBC). Serum aliquots were stored in duplicates in 1.5 ml micro tubes at a temperature of -20 ºC

until processing. Urine samples were collected after the intravenous administration of furosemide 5 mg/kg (MSD, São Paulo, SP, Brazil). Urine was collected at three-day intervals (D0, D3, D6, D9, D12, and D14) and stored in sterile conical tubes (~15 ml). Aliquots were transferred to micro tubes (1.5 ml) with 100 µl of phosphate buffer, identified, and stored at -4°C until processing.

Clinical signs

Pyrexia, prostration, dorsal arches (indicative of pain), jaundice, hematuria, dyspnea, polypnea, dehydration, and color of mucosae were evaluated daily by the same veterinarian.

Serology

To detect anti-*Leptospira* antibodies, a microscopic agglutination test (MAT) was performed using an antigen panel that consisted of 28 reference strains (originated from Institute Pasteur, Paris) in accordance with international standards (OIE, 2014). Sera displaying at least 50% agglutinating activity at a 1:100 dilution were considered reactive.

Urinary PCR

Leptospiral DNA was extracted by the Wizard SV Genomic DNA Purification System® (Promega, Madison, USA). PCR targeting the *lipL32* gene was performed as described (Hamond et al., 2014).

Hematological and biochemistry analyses

Hematological patterns were determined according to the methods proposed by Salviano et al. (2013). Globular volume was determined by the microhematocrit method, and plasma protein and fibrinogen were evaluated as described by Thrall (2006). Blood aliquots were stained with TÜRK's solution, and cells were counted in Neubauer's chamber (Ridpath et al., 2013). Specific leukogram was performed by fast dye staining and counting (InstantProv®, NewProv™, Pinhais, PR, Brazil).

Concentration of urea and creatinine; seric activity of alanine aminotransferase (ALT); alkaline phosphatase (FAL) and gammaglutamyltransferase (GGT); total protein and fractions (albumin and globulin); and total, direct and indirect

bilirubin was analyzed by spectrophotometry in accordance with the manufacturer's instructions (Labtest™, Belo Horizonte, MG, Brazil).

4.2.3. RESULTS

Clinical signs

After D7 post-infection (p.i.), five animals (55.5%) presented hypochromic mucosa, which could be considered to be a mild unspecific clinical sign of infection. The possible anemia of those animals was not confirmed at CBC. The most remarkable clinical signs, which could clearly be related to the experimental infection, were observed in two animals in Group C, a young animal, which presented fever (41ºC) and dehydration, and a cub in pain, as characterized by dorsal arching (Table 1). This cub died on D14 p.i. and was necropsied. At necropsy, mandibular, submandibular, and retropharyngeal lymph nodes were engorged. Fluid was also observed in the thoracic and abdominal cavities as well as hemorrhagic pulmonary lobes and kidneys.

Haematological and biochemical parameters

Two animals (cub and young) from Group C presented leukocytosis (19,400/mm^3 and 15,800/mm^3). However, neutrophilia (72%) was observed in the cub but not the young animal.

Serological and PCR outcomes

Only the three animals from Group C exhibited positivity in MAT. Titres were observed after D6 and reached up to 3,200 (cub), 800 (young), and 100 (mature). Positivity to PCR was observed in two experimental groups, A and C, thus confirming the presence of the infection. The young animal from Group C presented positivity on D9, D12, and D14, while the cub of the same group and the young of Group A only showed positivity on D14

1

	Dose	Age	Clinical Signs	MAT	PCR	Haematobiochemical alterations	Leptospiruria
Group A							
1	10^{a}	4 mo	Hypochromic mucosa (D8-D10)	Neg	Neg	None	Neg
2	10^{a}	12 mo	Hypochromic mucosa (D8-D14)	Neg	Pos D14	None	Pos
3	10^{a}	4-6 yrs	None	Neg	Neg	None	Neg
Group B							Neg
1	10^{b}	4 mo	None	Neg	Neg	None	Neg
2	10^{b}	12 mo	None	Neg	Neg	None	Neg
3	10^{b}	4-6 yrs	None	Neg	Neg	None	Neg
Group C							
1	10^{8}	4 mo	Dorsal arching (D3-D11) and died (D14)	3,200 (D6)	Pos D14	Leukocytosis (19,400/mm^3) and Neutrophilia 72% (D7)	Pos
2	10^{8}	12 mo	Dehydration (D7-D11) and fever (D4)	800 (D6)	Pos D9, D12, D14	Leukocytosis (15,800/mm^3) (D4)	Pos
3	10^{8}	4-6 yrs	None	100 (D6)	Neg	None	Neg
Group D							Neg
1	None	4-6 yrs	None	Neg	Neg	None	Neg
2	None	4-6 yrs	None	Neg	Neg	None	Neg
3	None	4-6 yrs	None	Neg	Neg	None	Neg

Mo: months; Yrs: years; Neg: Negative; Pos: Positive

Pcr - urine

Table 1. Outcomes of experimentally infected sheep, with different ages and doses of *Leptospira* (reference strain FIOCRUZ L1-130).

4.2.4. DISCUSSION

In the present study, experimental infection was successfully achieved in two groups (A and C), as defined by observation of leptospiruria. It was unsurprising that the animals that received the highest dose (10^8) presented more consistent outcomes. These findings corroborate with similar results of studies conducted in New Zealand [14] and Australia [4].

Despite the use of a highly virulent strain, clinical signs of acute leptospirosis were not observed, with the exception of two animals. This was an unexpected and quite frustrating outcome because it is contradictory to other studies that have reported acute clinical signs after experimental infection with highly virulent *Leptospira* strains [10, 11]. Thus, in light of the results of the current study, clinical signs cannot be considered to represent a reliable parameter for evaluating the development of leptospirosis in experimentally infected sheep. It is particularly important to highlight that the age of the animals did not appear to alter their susceptibility to the experimental infection. This finding also contrasts with those of

other experimental studies [4, 13], which suggested younger animals are more susceptible to acute infection than older ones.

Studies have demonstrated that in sheep the acute form of this disease occurs mainly in European breed animals [10, 14]. A rustic Brazilian breed (Santa Ines) was tested in the current study. This breed is adapted to adverse conditions, such as drought and food shortage, and has been reported to be particularly resistant to parasitism [1]. As such, there is a strong possibility that the rusticity of the animals influenced the outcomes of the experiment. Although further studies are required, this may represent an interesting research line regarding the identification of the genetic markers involved in any resistance observed.

Regarding the haemato-biochemical parameters, CBC is the only tool that has proven to be adequate for monitoring experimental infection in sheep. Therefore, since no clinical signs were visible in the current study, it was concluded that daily CBC should be employed as the main tool for assessing infected sheep. CBC has been used to follow the acute phase of leptospirosis, and it is effective in the detection of anemia and leukocytosis with neutrophilia in ruminants [15]. It is important to highlight that alterations to CBC occurred only in animals that received the highest dose of leptospires. Although the majority of the animals did not present leukocytosis in the current study, we did observe a slight variation in this parameter, mainly during the first days of the experiment. However, biochemical alterations were not deemed to be reliable indications of the infectious status, and this finding corroborates with those of other studies [19, 15].

Despite the lack of clinical signs, serological and molecular results confirmed the experimental infection. Seroconversion was observed only in the animals that received the highest dose of leptospires (Group C), similar to the findings of previous research [11, 13, 14]. Additionally, in this group, only animals with high titers also presented leptospiruria. PCR was positive in animals from two experimental groups. PCR has been used as an important diagnostic tool for assessing leptospirosis in different species, including sheep [3, 6]; however, to the best of our knowledge, the current study is the first of its kind to use PCR to follow up the carrier status in experimentally infected sheep. Nevertheless, the limited number of positive reactions in the present study may be related to the intermittent shedding of leptospires in the urine [5]. Despite that limitation, PCR proved to be very effective in the confirmation of the infection, and it should be considered for use in experimental studies.

Sheep have been used as a good experimental model in several studies, such as in cardiovascular, respiratory, reproductive and, especially, immunology domains [18]. Sheep are relatively small in comparison to other ruminants, such as cattle, and can be easily allocated to smaller pens and sheds, facilitating the management of the research and minimizing the costs of experimentation [24]. Cattle and sheep present a strong chromosome homology, which may justify the similarities in the immune responses they exhibit [17]. It has been suggested that sheep and cattle shared an ancestral chromosome that molded the MHC of ruminants and, as such, they potentially share the same induction of immune response [7]. Additionally, Toll-like receptors 1-10 are present in both bovine and ovine tissues during the regulatory stage of the immune response [17]. In this context, we suggest sheep represent a good model for studying leptospirosis in ruminants and, therefore, a reliable protocol for experimental leptospiral infection is required.

4.2.5. CONCLUSION

In conclusion, we demonstrated that sheep may be experimentally infected and used as a model for leptospirosis in ruminants. As clinical signs were not evident, we recommend urine PCR and serology (MAT) for confirmation of the infection and daily CBC as a follow-up tool to assess experimentally infected animals.

4.2.6. ETHICAL APPROVAL

The study was conducted with the full approval of the Ethics Committee of Universidade Federal Fluminense, Brazil (number 814/2016).

4.2.7. DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper

4.2.8. REFERENCES

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4.3. Capítulo 3

Assessment of the immunogenicity of the leptospiral LipL32, LigAni, and LigBrep recombinant proteins in the sheep model

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Abstract

Veterinary leptospirosis vaccines are composed of bacterins and present limitations, for example, the need for bacteriological culture and serovar-dependent immunity. Recombinant antigens represent a promising alternative. LigAni, LigBrep, and LipL32 proteins have been shown to promote a protective immune response against the homologous challenge in hamsters. Therefore, the next step is to evaluate the immunological properties of these immunogens in the actual hosts, as ruminants, which has never been performed before. The objective of this study was to evaluate the immunogenicity and potential adverse effects of the recombinant proteins LigAni, LigBrep, and LipL32 in the ovine model. For this, 16 Santa Inês sheep were allocated into three groups: two experimental (Groups A and B) and one control group (Group C). Group A was inoculated with a formulation containing the recombinant proteins in combination with the aluminum hydroxide adjuvant; Group B was inoculated with a formulation containing the recombinant proteins in combination with the Montanide adjuvant; and Group C was inoculated with adjuvants only. The results revealed that formulations containing the recombinant proteins induced total IgG seroconversion and led to a significant increase in antibody titers in the sheep model. Besides, there were no clinical changes or adverse effects. Thus, rLigAni, rLigBrep, and rLipL32 proteins elicited a significant humoral immune response with elevated serum IgG levels, demonstrating that they possess the immunogenic and safety characteristics necessary to sustain their potential use as leptospirosis vaccines in the ruminant model.

Keywords: Leptospirosis, sheep, recombinant proteins, vaccines

4.3.1. Introduction

Leptospirosis is a zoonotic disease that is caused by pathogenic strains of the bacterium *Leptospira* spp. [1]. In livestock, leptospirosis causes economic losses due to the high rates of reproductive failures, such as abortion, stillbirth, infertility, and reduced milk production [2,3]. Immunization is the cheapest method of control and represents an essential measure for the control of leptospirosis; as such, its adoption is strongly recommended [4]. Vaccines currently available are composed of bacterins (inactivated cultures) and involve fastidious microorganisms. In addition, the immunity is mainly directed to the serovars antigenically related to those present in the vaccine formulation [5,6]. As such, recombinant vaccines often represent a more attractive alternative to bacterins since they eliminate time-consuming and costly cultivation and, in theory, may confer protection against several serovars [7]. To this end, recombinant vaccines have been developed as a means of overcoming the deficiencies of the bacterin-containing vaccines [8,9]. In order to develop these vaccines, researchers are seeking recombinant antigens that offer an optimal antigenic and immunogenic profile.

Previous studies have evaluated the immunoprotective potential of the LipL32, LigAni, and LigBrep recombinant proteins. Studies using hamsters as an experimental model have generated encouraging results regarding the antigenic and immunogenic capacity of these proteins [10,11,12]. However, the behavior of these immunogens in the actual hosts, i.e., the ruminants, is unknown. Thus, this study aimed to bridge that gap by evaluating the immunogenicity of the LipL32, LigAni, and LigBrep recombinant proteins and identifying any possible adverse effects of the use of such antigens in an experimental sheep model.

4.3.2. Methodology

Expression and purification of recombinant proteins

The LigAni, LigBrep, and LipL32 recombinant proteins were produced as described by Hartwig et al. [10], Hartwig et al. [13], and Grassmann et al. [14]. Briefly, pAE vectors containing *ligAni*, *ligBrep*, and *lipL32* genes were used to transform the *E. coli* TOP10F' and BL21 (DE3) Star expression strains respectively. The strains transformed with pAE recombinant vectors were cultured in 500 mL of LB medium until they reached the OD_{600} 0.5 - 0.7 at 37°C. They were then induced with 1 mM isopropyl-β-1-D thiogalactopyranoside (IPTG) for 4 h. The cells were collected by centrifugation (7000 x *g*, 4°C, 15 min), suspended in a solubilization buffer (200 mM NaH_2 PO 4, 0.5 M NaCl, 5 mM imidazole, pH 8.0), and incubated via orbital shaking at 60 rpm for 16 h at room temperature. Purification was performed by affinity chromatography using nickel-loaded HisTrap sepharose columns. To evaluate the expression of the recombinant proteins, the samples were examined using the SDS-PAGE (12% acrylamide) and Western blot using anti-6xHis monoclonal antibody (Sigma-Aldrich, Brazil). The purified recombinant proteins were dialyzed with 1X phosphate buffered saline (PBS) in 5 steps for 5 days at 4°C. The concentration was determined by the BCA Protein Assay kit (Pierce, USA) and with the aid of TotalLab Quant software. The recombinant proteins were stored at -20°C.

Preparation of formulations

Two formulations, composed of three recombinant antigens, LigAni, LigBrep, and LipL32, in combination with two adjuvants, Alhydrogel or Montanide ISA 50V2, were prepared. For this, 100 µg/mL of each recombinant antigen and 2 mg/mL Alhydrogel or the Montanide 50/50 (volume/volume) adjuvant was used. To achieve emulsification of the Alhydrogel-containing formulation, the formulation was lightly stirred for 16 h at 4°C. In the case of the formulation containing Montanide ISA 50V2, an emulsifier was used. Thereafter, the vaccines were fractionated in a 9 mL volume. At the end of the procedure, the product was stored under refrigeration at 4°C.

Experimental groups

Sixteen female, twelve-month-old Santa Inês breed sheep which had not previously been vaccinated against leptospirosis were used. The animals were

randomly allocated into three groups (A, B, and C), with Groups A and B each consisting of five animals and Group C, the control group, of six animals. In addition to the hematobiochemical and clinical analyses, three consecutive days before immunization all the sheep were evaluated by serology (MAT) and urine PCR (targeting the *lipL32* gene), according to the methodology described by Rocha et al. [15]. The experiment was conducted at the Experimental Research Unit in Goats and Sheep (UniPECO-UFF), a Biological Safety Level 2 facility.

Each experimental group was inoculated intramuscularly with the different formulations. The animals in Group A were inoculated with a formulation containing the LigAni, LigBrep, and LipL32 recombinant antigens plus 2 mg/mL Alhydrogel 2%. Those in Group B were inoculated with a formulation containing the LigAni, LigBrep, and LipL32 recombinant antigens plus Montanide ISA 50V2, 50/50 (volume/volume), and Group C animals were inoculated with formulations that contained only adjuvants. Three of the animals in Group C received Alhydrogel (2 mg/mL) and three received Montanide (volume/volume). The study was conducted with the full approval of the Ethics Committee of Universidade Federal Fluminense, Brazil (number 814/2016).

Sampling

To evaluate the humoral immune response and hematobiochemical changes, blood samples were collected by jugular vein puncture at days 0 (day of inoculation), 7, 14, 21, 28, 35, and 42 post-inoculation (p.i.), using sterile needles (40 x 12 mm), and stored in two vacuum tubes (Vacutainer®, BD, São Paulo, SP, Brazil). Anticoagulant (EDTA) was used for hemogram, and no anticoagulant was used for serology and biochemistry. Serum aliquots were stored in duplicate in 1.5 mL microtubes at a temperature of -20°C until processing. Whole blood samples were processed immediately after collection.

Evaluation of the humoral immune response

The humoral immune response of the sheep was evaluated using the indirect Enzyme Linked ImmunonoSorbent Assay (ELISA). Each antigen was tested separately. Therefore, for the recombinant protein, plate sensitization was performed with 100 ng of antigen diluted in Carbonate-Bicarbonate buffer (pH 9.6) for 16 h at 4°C. The plates were washed three times with phosphate-buffered saline pH 7.6 with

0.05% Tween 20 (PBSt) before the serum of the animals was evaluated at a dilution of 1:500 in a volume of 100 µL and incubated at 37°C for 1 h.

After the primary serum was incubated overnight, the plates were washed again with PBSt, and the peroxidase-conjugated sheep anti-IgG antiserum was added at a dilution of 1:10,000 for 1 h at 37°C in a volume of 100 µL. After the incubation time, the plates were again washed with PBSt. Finally, the reaction was developed using Orto Phenyl Diamine (3.4 mg in 10 mL of citrate phosphate buffer) with the addition of 10 µL of 30 vol of hydrogen peroxide in a volume of 100 µL per well. Fifteen minutes after adding the developing solution, an optical density reading was performed at 450 nm using an ELISA reader (Dynatech MR 700, Germany).

Hematobiochemical analysis and clinical signs

The complete blood count was performed according to the instrumental techniques described by Thrall [16], and the values were compared to those previously determined for the Santa Inês breed [17].

Concentration of urea and creatinine; serum alanine aminotransferase (ALT) activity; alkaline phosphatase (FAL) and gammaglutamyltransferase (GGT); protein and total fractions (albumin and globulin); and total, direct, and indirect bilirubin were analyzed spectrophotometrically using commercial kits (Labtest®, Labtest Diagnostica AS, Minas Gerais, Brazil) in an automatic biochemical analyzer (LabMax 240 premium®, Labtest Diagnostica AS, Minas Gerais, Brazil). The experimental animals were clinically evaluated on a daily basis by a team of veterinarians who were suitably qualified and specialized in the area. Different factors that may indicate the side effects of the inoculation, such as pyrexia, prostration, jaundice, hematuria, dyspnea, polypnea, anorexia, dehydration, fecal changes, and adverse reactions at the inoculation site, were evaluated.

Statistical analysis

Optical density data were analyzed using ANOVA for the different proteins and adjuvants using the IBM SPSS 22 software, with a 95% confidence interval. In addition, the Tukey HSD test was used to compare the mean values of the readings for each protein.

4.3.3. Results

Humoral immune response

As shown in Fig. 1, all recombinant proteins (LigAni, LigBrep, and LipL32) elicited the specific antibody titers in the ruminant model. A significant difference was observed in protein comparison ($p = 0.002$). This difference was observed in LigBrep (mean=1.16) and LipL32 (mean=1.37), triggering the humoral immune response. A significant IgG seroconversion was observed against the recombinant antigens between Day 0 and Day 7 p.i. In this period, LipL32 protein generated the highest serum levels of IgG on Day 7, remaining stable thereafter until Day 42. The other proteins started to generate high serum levels of IgG only on Day 14 p.i., and the seroconversion remained constant after the second dose of the formulation on Day 21. No seroconversion was observed in the control group. Furthermore, when proteins were compared in Groups A and B, significant differences were observed in Group A ($p=0.006$) for LigBrep (mean=1.05) and LipL32 (mean=1.38). In contrast, no difference in mean was observed in Group B ($p=0.153$). The results demonstrate that the ruminants in Group A and Group B developed a humoral immune response against the inoculated recombinant antigens.

In terms of the antibody titers generated, the recombinant proteins responded differently to each other, as did the two experimental groups. The animals in Group A that were inoculated with a formulation containing the proteins plus the Alhydrogel adjuvant obtained antibody titers as follows: LigAni (1:51,200), LigBrep (1:25,600), and LipL32 (1:25,600). The animals in Group B, which were vaccinated with a formulation that contained the proteins plus the Montanide ISA 50V2 adjuvant, obtained LigAni (1:25,600), LigBrep (1:51,200), and LipL32 (1:102,400).

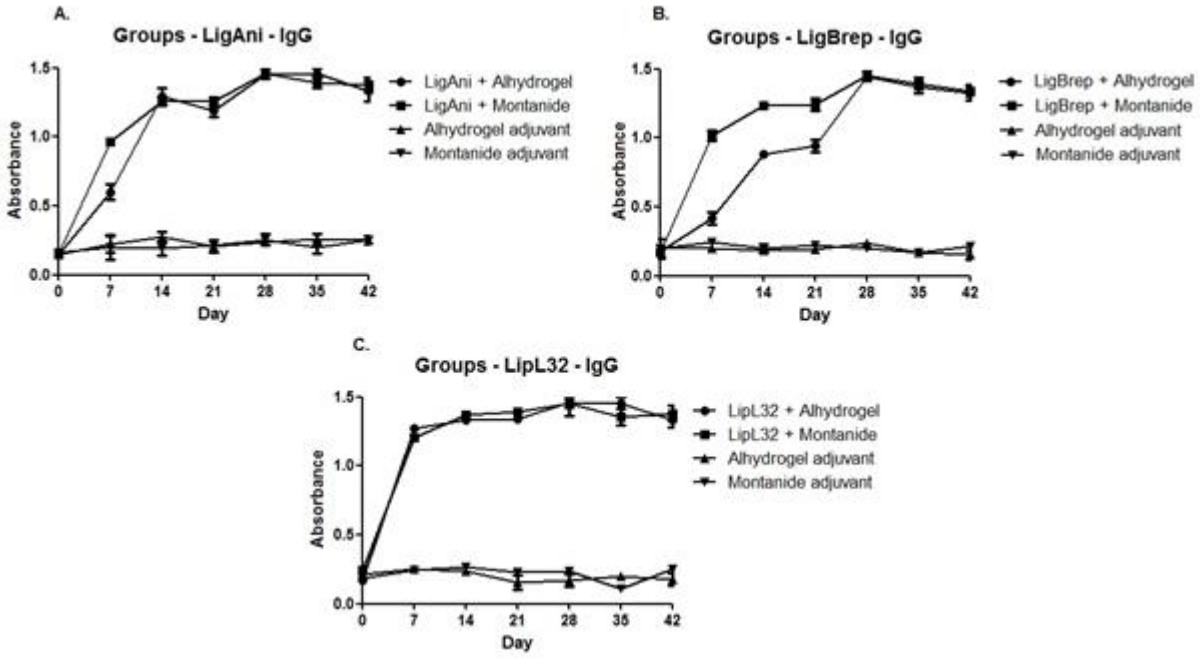

Figure1. Evaluation of the humoral immune response obtained by experimental groups against each recombinant antigen. A. Humoral Immune response against LigAni potein only. B. Humoral immune response against the LigBrep protein only. C. Umoral immune response against LipL32 protein only.

Adjuvant

There was generally no significant difference (p=0.084) observed in terms of the effect of the adjuvant in serum conversion. Nevertheless, a non-significant difference was observed in the LigAni (p=0.705) and LipL32 (p=0.605) protein responses in both groups. The highest titers were observed for LigBrep (p=0.023) in Group B (mean=1.35).

Analysis of clinical signs and hematobiochemical alterations

A small inflammatory reaction followed by swelling at the inoculation site of the formulation was observed in the sheep in Group A. No other clinical signs or hematobiochemical changes were observed in any of the animals in the groups evaluated.

4.3.4. Discussion

This study aimed to analyze the humoral immune profile of sheep inoculated with recombinant proteins belonging to the genus *Leptospira*, which represent promising vaccine candidates for infection control. Several studies have evaluated the antigenic and immunogenic potential of recombinant proteins belonging to the genus *Leptospira* for the development of new vaccines [18,19]. However, the studies have been restricted to the use of the Golden Syrian Hamster experimental model, a vivarium model best suited for experiments involving leptospirosis [20]. Nonetheless, leptospirosis has a high incidence in livestock and leads to significant economic losses in those animals [3,21]. Thus, it is necessary and extremely important to evaluate the immunogenicity of the recombinant proteins in those animals. Although other studies have described how the immune system of ruminants responds to vaccines composed of bacterins [22,23], to the best of the authors' knowledge, the current study represents the first to perform analysis of recombinant vaccines in a ruminant model.

In terms of the adjuvants, the results indicated that there was no statistically significant difference in titer levels between the groups. However, the formulation containing Montanide ISA 50V2 as adjuvant (Group B) maintained the seroconversion of antibodies in a more homogenous manner throughout the experimental period. This result can be justified by taking into account the mechanism of action of the adjuvant. Montanide is considered to represent a deposit adjuvant that slowly releases the antigen at the site of inoculation. This entails that the antigen is exposed to the immune response for a longer period, potentiating the immune response, generating both a humoral (Th2) immune response and a cellular (Th1) immune response, with the most stimulated Th1 immune response [24,25]. In addition, the Montanide adjuvant has been specially developed for use in small and large ruminants (Manufacturer Information). Therefore, when the effect of the Montanide ISA 50V2 adjuvant was evaluated, it was observed that, in addition to a high seroconversion, there was no significant difference between the different recombinant antigens.

Conversely, the aluminum hydroxide adjuvant (Alhydrogel 2%) was also able to potentiate the immune response and aid the seroconversion through high serum titers of antibodies. Studies suggest that the mechanism of action of aluminum

hydroxide can be attributed to the formation of antigen deposit and immunostimulation [26]. However, researchers have yet to develop a working understanding of the actual action of this adjuvant [27,28]. It is known that aluminum hydroxide mainly stimulates an associated humoral immune response (type th2), associated with IL-4, inducing a reduced cellular immune response [29]. However, studies based on ruminant populations have shown that the cellular immune response (Th1 type) is the most incident to a leptospirosis infection [22,23]. These findings reinforce the need for the use of an adjuvant that enhances this immune response.

The only side effect that was observed in the current study was a small inflammatory reaction at the site of application of the formulation, and this only occurred in the experimental animals in Group A. This reaction can be attributed to the mechanism of action of the aluminum hydroxide adjuvant, which induces inflammation and stimulates the local production of granuloma, subcutaneous nodules, and contact hypersensitivity [30,31]. Furthermore, no hematobiochemical changes were observed in any of the evaluated groups. Thus, it can be argued that the formulations studied are safe for use in sheep since no moderate or severe adverse effects were observed.

Sheep have been characterized as being a good experimental model in several studies spanning, for example, cardiovascular, respiratory, reproductive, and mainly immunological domains [23,32]. They are relatively small in comparison to other ruminants, such as cattle, and can be easily allocated in smaller stalls and bays, facilitating the management of research and minimizing the costs of experimentation [33]. Moreover, cattle and sheep have a strong chromosomal homology, which may justify the similarities in the immune responses that they present [34]. This indicates that sheep may be a good experimental model for evaluating the immune response of ruminants.

The results obtained in this study, confirming the immunogenicity and safety of the recombinant antigens, prompt us to continue our studies and to conduct challenge experiments with virulent leptospires. The sheep model of leptospirosis could be the way forward in order to translate the immunoprotection results obtained in the hamster model of leptospirosis and the large ruminant species, particularly bovine, that would benefit from an improved vaccine formulation.

4.3.5. Conclusion

In conclusion, the recombinant proteins evaluated in this study (LipL32, LigAni, and LigBrep) elicited a significant humoral immune response in sheep, generating high serum immunoglobulin (IgG) titers. The encouraging results that were originally observed in hamsters can be successfully reproduced in the ruminant model without side effects, which indicates an important step forward towards the search for a recombinant vaccine for bovine leptospirosis.

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5. Conclusão geral

O presente estudo teve como objetivo avaliar a imunogenicidade das proteínas recombinantes LigAni, LigBrep e LipL32 utilizando ovinos como modelo experimental. Para tanto, tornou-se necessário, desenvolver uma metodologia de infecção experimental em ovinos e avaliar a reação dos animais a uma infecção experimental, uma vez que a literatura que traz uma metodologia para esse tipo de análise é muito desatualizada, além do fato de, até então, nunca ter sido descrito um estudo avaliando a imunogenicidade de proteínas recombinantes em ovinos experimentalmente infectados, o que ressalta o pioneirismo do presente estudo.

Em relação à infecção experimental, foi possível concluir que, apesar da ausência de sinais clínicos e sintomatologia relacionada à leptospirose, foi possível confirmar a infecção experimental em ovinos inoculados com uma dose de $1x10^8$ de *Leptospira interrogans* serogroup Icterohemorrhagiae utilizando técnicas de biologia molecular como diagnóstico confirmatório (PCR de urina). Os resultados demostraram que, os sinais clínicos mais notáveis foram observados em dois animais do grupo C, um animal jovem, que apresentava febre (41 ºC) e desidratação, e um filhote com dor muscular, caracterizado por arco dorsal.

Devido à ausência de sinais clínicos relevantes, a sintomatologia não pode ser considerada como um parâmetro confiável para avaliar o desenvolvimento da leptospirose em ovinos infectados experimentalmente, tão pouco a idade dos animais parece alterar a susceptibilidade a infecção experimental, raça Santa Inês. O hemograma foi a única ferramenta que se mostrou adequada para o monitoramento da infecção. Uma vez que não foi observado sintomatologia, sugerimos que o hemograma seja utilizado como ferramenta diária para avaliação da infecção experimental.

Ao avaliarmos a imunogenicidade das proteínas recombinantes, foi possível concluir que as proteínas LigAni, LigBrep e LipL32 foram capazes de geral altos títulos de anticorpos e demostraram possuir características antigênicas e imunogênicas quando se utilizou ovinos como modelo experimental. Os resultados obtidos neste estudo vão ao encontro de resultados obtidos utilizando hamsters como modelo experimental - modelo considerado padrão em se tratando de estudos relacionados à leptospirose - demostrando que o organismo de ruminantes também

consegue gerar resposta imune robusta contra antígenos recombinantes pertencentes a bactérias patogênicas do gênero *Leptospira*.

Inúmeros são os estudos utilizando hamsters como modelo experimental para estudos relacionados com leptospirose animal, no entanto para obtenção de vacinas recombinantes que venham a superar as deficiências das vacinas compostas por bacterinas e substituir as vacinais disponíveis comercialmente, faz-se necessário a utilização de modelos experimentais que se aproximem das espécies alvo, neste caso os bovinos e outros animais de produção. No presente estudo foi possível demonstrar que ovinos podem vir a se tornar modelos experimentais para estudos relacionados à imunologia e vacinologia contra leptospirose, aproximando a pesquisa e obtenção de produtos vacinais das espécies com maior interesse comercial.

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