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Proteína ErpY-like de *Leptospira* spp.: da ciência básica à aplicação no diagnóstico

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“Cada sonho que você deixa para trás é um pedaço do seu futuro que deixa de existir”

(Steve Jobs)

Resumo

PADILHA. Bárbara Couto Roloff. **Proteína ErpY-like de *Leptospira* spp.: da ciência básica à aplicação no diagnóstico.** 2019. 135f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

A leptospirose é uma zoonose causada por bactérias patogênicas do gênero *Leptospira*. Esta doença pode afetar diversos animais, sendo considerada um problema de saúde pública e responsável por perdas na pecuária. O "padrão ouro" para o diagnóstico da leptospirose é a soroaglutinação microscópica (MAT), entretanto apresenta limitações, como elevado número de reações cruzadas, é bastante laboriosa e demorada. Por este motivo, a busca por alternativas para o controle da leptospirose torna-se necessária. A proteína ErpY-like foi reportada como potencial fator de virulência de *Leptospira interrogans*, e assim, possivelmente envolvida no processo de infecção e importante no desenvolvimento de novos testes de diagnóstico e como antígeno vacinal. Desta forma, o objetivo deste trabalho foi a produção da proteína ErpY-like em sua forma recombinante, sua caracterização *in silico* e aplicação no diagnóstico. Para tal, a proteína foi avaliada através de ferramentas de bioinformática e a presença do gene *erpY-like* em diferentes sorovares de *Leptospira* spp. investigada por PCR. A proteína ErpY-like foi produzida na forma recombinante (rErp-like) utilizando sistema heterólogo procarioto (*Escherichia coli*) e sua antigenicidade avaliada em ensaio do tipo ELISA (do inglês *Enzyme-Linked Immunosorbent Assay*) indireto com soro de animais infectados. Camundongos foram imunizados com a proteína rErpY-like para produção de anticorpos policlonais (pAbs), os quais foram titulados em ELISA indireto e testados quanto à reatividade com a proteína nas formas recombinante e nativa de *L. interrogans* em ELISA indireto e imunofluorescência indireta. Por fim, a proteína rErpY-like foi testada no desenvolvimento de um ELISA indireto para o diagnóstico da leptospirose suína. Na PCR, o gene *erpY-like* foi amplificado em todos os sorovares testados. A proteína ErpY-like foi expressa com sucesso utilizando sistema heterólogo, com tamanho esperado de 13 kDa e rendimento de 25 $mg.L^{-1}$. Sua antigenicidade foi confirmada em ensaio de ELISA indireto, onde a proteína recombinante reagiu com soro humano, suíno e canino naturalmente infectados. Os pAbs produzidos ligaram-se a proteína ErpY-like nativa, tanto em células inteiras quanto lisadas de leptospiras. Na imunofluorescência indireta também foi observada reação dos pAbs, marcando com fluorescência a proteína ErpY-like nativa na *Leptospira*. Além disso, o uso da proteína rErpY-like no ELISA indireto mostrou-se sensível e específica para o diagnóstico da leptospirose suína, com sensibilidade de 96,8% e especificidade de 100%. Estes resultados demonstraram que a proteína ErpY-like é um alvo promissor no desenvolvimento de ensaios para o controle da leptospirose.

Palavras-chave: diagnóstico, proteína recombinante, LIC119666, leptospirose, *Leptospira* spp.

Abstract

PADILHA. Bárbara Couto Roloff. **ErpY-like protein of *Leptospira* spp.: from basic science to application in diagnosis.** 2019. 135f. Tese (Doutorado) - Programa de Pós-Graduação em Biotecnologia. Universidade Federal de Pelotas, Pelotas.

Leptospirosis is a zoonosis caused by pathogenic bacteria from the genus *Leptospira*. This disease can affect several animals, being considered a public health problem and responsible for losses in livestock. The "gold standard" for leptospirosis diagnosis is microscopic agglutination test (MAT). However, it has limitations, such as a high number of cross-reactions, it is laborious and time-consuming. For this reason, the search for alternatives to leptospirosis control becomes necessary. The ErpY-like protein was reported as a potential virulence factor of *Leptospira interrogans*, and thus possibly involved in the infection process and important in the development of new diagnostic tests and as a vaccine antigen. In this way, the objective of this work was the ErpY-like protein production in its recombinant form, its *in silico* characterization and application in diagnosis. For this, the protein was evaluated through bioinformatics tools and the presence of the erpY-like gene in different serovars of *Leptospira* spp. investigated by PCR. ErpY-like protein was produced in recombinant (rErp-like) form using a prokaryotic heterologous system (*Escherichia coli*) and its antigenicity assessed in an ELISA (Enzyme-Linked Immunosorbent Assay) indirect assay with serum from infected animals. Mice were immunized with rErpY-like protein to produce polyclonal antibodies (pAbs), which were titrated in indirect ELISA and tested for reactivity with the recombinant and native *L. interrogans* forms in indirect ELISA and indirect immunofluorescence. Finally, the rErpY-like protein was tested in the development of an indirect ELISA for the diagnosis of swine leptospirosis. In PCR, the *erpY-like* gene was amplified in all tested serovars. ErpY-like protein was successfully expressed using heterologous system, expected size of 13 kDa and yield of 25 $mg.L^{-1}$. Its antigenicity was confirmed in an indirect ELISA assay, where the recombinant protein reacted with naturally infected human, swine and canine sera. The produced pAbs bound to native ErpY-like protein, both in whole cells and lysates of leptospires. In indirect immunofluorescence, the pAbs reaction was also observed, fluorescently labeling the native ErpY-like protein in *Leptospira*. In addition, the use of rErpY-like protein in the indirect ELISA was sensitive and specific for the diagnosis of swine leptospirosis, with sensitivity of 96.8% and specificity of 100%. These results demonstrated that ErpY-like protein is a promising target in the development of trials for the control of leptospirosis.

Keywords: diagnosis, recombinat protein, LIC11966, leptospirosis, *Leptospira* spp.

Sumário

1 INTRODUÇÃO GERAL

A leptospirose é uma doença causada por bactérias do gênero *Leptospira* spp. (Faine et al.,1999, Adler & Moctezuma, 2010). Humanos e animais podem ser infectados por contato direto ou indireto com a urina de animais portadores (Faine et al., 1999; Reis et al., 2008). É uma doença de distribuição global, considerada um problema de saúde pública e responsável por perdas econômicas na pecuária (Adler & Moctezuma, 2010; Rocha et al., 2017; Padilha et al., 2019).

O "padrão ouro" para o diagnóstico da leptospirose é a soroaglutinação microscópica (MAT). Este teste utiliza como antígeno leptospiras vivas de diferentes sorovares, que são confrontados com o soro suspeito, e assim, são estimados os níveis de anticorpos presentes na amostra (Faine et al., 1999). No entanto, a MAT é uma técnica bastante subjetiva, laboriosa e demorada (Adler & Moctezuma, 2010; Caimi et al., 2017), além de apresentar um elevado número de reações cruzadas entre os sorovares (Hartleben et al., 2013; Monte et al., 2011). Devido aos inconvenientes da MAT, técnicas mais sensíveis, como testes moleculares e ELISA (do inglês *Enzyme-Linked Immunosorbent Assay*) com antígenos recombinantes de *Leptospira* spp., têm sido desenvolvidos como métodos alternativos para diagnosticar a infecção (Bomfim et al., 2005; Hartleben et al., 2013; Mariya et al., 2006; Padilha et al., 2019).

Para o desenvolvimento de novos métodos, a ciência básica na busca por alvos que sejam reconhecidos e, assim, capazes de serem utilizados no diagnóstico é determinante. Por isso, a avaliação da proteína ErpY-like de *Leptospira* spp. é uma etapa importante. Esta foi relatada como uma proteína de *Leptospira interrogans*, que possui uma sequência muito semelhante à fatores de virulência de outros agentes patogênicos, como por exemplo, a proteína de membrana externa ErpY de *Borrelia burgdorferi*. Além disso, a expressão desta proteína foi demonstrada durante a infecção *in vivo* (Esghi et al., 2009). Estudos recentes mostraram que quando utilizada como vacina de subunidade, a proteína rErpY-like é protetora contra a infecção por *L. interrogans* (Oliveira et al., 2018). Também, quando testada em ELISA indireto para o diagnóstico da leptospirose em suínos, rErpY-like foi sensível e específica (Padilha et al., 2019). Estes resultados sugerem que esta proteína tem importância no processo de infecção e potencial para ser utilizada como antígeno vacinal e no diagnóstico.

A infecção por leptospiras patogênicas em animais inclui, por exemplo, o aborto e a perda da produção de leite (Guglielmini et al., 2019). Devido a problemática da leptospirose animal, a proteína ErpY-like vem como uma alternativa para uso na análise epidemiológica de rebanhos, reduzindo assim a disseminação da doença e consequente perda econômica.

Assim, a presente tese buscou avaliar a proteína ErpY-like quanto ao seu potencial como insumo biológico, utilizado no desenvolvimento de alternativas para controle da leptospirose animal. Os dados gerados estão apresentados na forma de manuscritos/artigos científicos. Esta forma de apresentação, comparada ao modelo de tese tradicional, visa propiciar uma divulgação objetiva e rápida dos resultados obtidos. Neste contexto, o manuscrito 1 trata de uma revisão de literatura sobre recentes avanços no diagnóstico molecular e sorológico da leptospirose. Esse trabalho está sendo preparado para submissão ao periódico ***American Journal of Tropical Medicine and Hygiene*** **(Impact fator: 2,564)**.

Na sequência, o manuscrito 2 descreve a caracterização *in silico*, *in vitro* e *in vivo* da proteína ErpY-like, quanto ao seu potencial uso como imunobiológico no controle da leptospirose. Este manuscrito foi submetido ao periódico ***Molecular Biotechnology*** **(Impact fator: 1,815)** e está em fase de revisão. Já os resultados obtidos da aplicação desta proteína em sua forma recombinante (rErpY-like), no desenvolvimento de um ensaio sorológico do tipo ELISA indireto, para diagnóstico da leptospirose em suínos, foram publicados na forma de artigo científico no periódico ***Acta Tropica*** **(Impact fator: 2,509).**

Além dos manuscritos/artigos mencionados como produtos da tese, foi solicitado também o depósito de patente intitulado: “Utilização de ErpY-like como imunobiológico no controle da leptospirose”. A solicitação foi enviada ao Instituto Nacional da Propriedade Industrial (INPI) – Número do processo: BR 10 2016 018203 4.

2 HIPÓTESE E OBJETIVOS

2.1 Hipótese

A proteína ErpY-like de *Leptospira* spp. expressa na forma recombinante é antigênica e promissora quando utilizada como antígeno no diagnóstico sorológico da leptospirose.

2.2 Objetivo Geral

Produzir a proteína ErpY-like em sua forma recombinante e avaliá-la *in silico*, *in vitro* e *in vivo* como imunobiológico no diagnóstico sorológico da leptospirose suína.

2.3 Objetivos Específicos

- Avaliar a proteína ErpY-like *in silico*;
- Investigar a presença do gene *erpY-like* nos sorovares de *Leptospira* spp.;
- Expressar e purificar a proteína ErpY-like em sua forma recombinante (rErpY-like);
- Avaliar a antigenicidade da proteína rErpY-like em ELISA indireto utilizando soro de animais naturalmente infectados;
- Imunizar camundongos BALB/c fêmeas com a proteína rErpY-like para produção de pAbs;
- Isotipar o pAb anti-rErpY-like;
- Avaliar a reatividade dos pAbs produzidos com a proteína ErpY-like em sua forma nativa e recombinante através de imunoensaios (ELISA indireto e IFI);
- Utilizar a proteína ErpY-like recombinante como antígeno em um teste de ELISA indireto para o diagnóstico da leptospirose suína;
- Comparar os resultados obtidos com o teste padrão.

3 CAPÍTULOS

3.1 Manuscrito 1 - Diagnóstico molecular e sorológico da leptospirose: uma revisão dos últimos 5 anos (2014-2019)

Manuscrito em fase de preparação para submissão ao periódico *American Journal of Tropical Medicine and Hygiene*.

Diagnóstico molecular e sorológico da leptospirose: uma revisão dos últimos 5 anos (2014-2019)

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A leptospirose é uma zoonose causada por bactérias do gênero *Leptospira*. Esta doença pode afetar diversos animais e o homem. Os sintomas podem variar de leves até graves, podendo levar o indivíduo à morte. Para o diagnóstico da leptospirose, o teste de aglutinação microscópica (MAT) é considerado o “padrão ouro”, entretanto, este apresenta limitações. Devido as limitações encontradas na MAT, estudos estão sendo realizados para o desenvolvimento de métodos mais sensíveis e específicos e menos laboriosos de triagem e diagnóstico para a leptospirose. Os testes sorológicos, que utilizam antígenos recombinantes de *Leptospira*, além de testes moleculares, são os que surgem como alternativa para o diagnóstico da doença. Neste trabalho, relatamos os avanços alcançados nos últimos cinco anos no controle da leptospirose, baseados nos testes de diagnóstico imunológico e molecular.

Palavras-chave: *Leptospira* spp., imunodiagnóstico, proteínas recombinantes, diagnóstico molecular.

Leptospira e leptospirose

Leptospirose é uma doença causada por espiroquetas patogênicas do gênero *Leptospira* spp., família Leptospiraceae, ordem Spirochaetales (Faine et al., 1999, Adler & Moctezuma, 2010). O gênero *Leptospira* é composto por cerca de 35 espécies, divididas em saprófitas, intermediárias e patogênicas (Adler & Moctezuma, 2010, Ko et al., 2009; Guglielmini et al., 2019). Dos mais de 300 sorovares conhecidos, quase 270 são patogênicos (Picardeau, 2017; Thibeaux et al., 2018; Guglielmini et al., 2019). Estas bactérias possuem forma espiralada e helicoidal, com cerca de 6-20μm de comprimento e 0,1-0,2μm de largura (Faine et al., 1999). Além disso, são classificadas em sorovares, de acordo com epítopos expostos na superfície em um mosaico de antígenos, que podem ser proteínas e lipopolissacarídeos (LPS), sendo a especificidade de epítopos dependente da sua composição de carboidratos e orientação (Adler & Moctezuma, 2010).

As espécies de *Leptospira* patogênicas podem infectar humanos e animais. A infecção pode ser contraída de forma direta, através da urina de animais infectados, ou indireta, através de água ou solo contaminado com urina de animais portadores (Faine et al., 1999; Jorge et al., 2018). Os roedores sinantrópicos são os principais reservatórios de leptospiras, eles albergam as bactérias nos rins, sem desenvolver a doença, e as eliminam através da urina. A leptospirose humana constitui uma infecção “sem saída”, ou seja, a transmissão humano-humano é praticamente desconhecida (Victoriano et al., 2009). As condições climáticas influenciam fortemente a transmissão das leptospiras, que exigem condições quentes e úmidas para a sobrevivência. As bactérias persistem por semanas a meses após a sua excreção na água ou solo úmido (Mwachui et al., 2015).

A leptospirose é uma doença zoonótica emergente, com mais de um milhão de casos graves e 60.000 mortes a cada ano em humanos em todo o mundo, principalmente nos países tropicais (Guglielmini et al., 2019). Além disso, é classificada como uma doença negligenciada de distribuição global. Pode acometer uma ampla variedade de animais domésticos, de companhia e de interesse econômico, incluindo cães, bovinos e suínos, além de animais silvestres, peixes e o homem (Bharti et al. 2003; Faine et al. 1999; Mwachui et al. 2015; Vinetz 2001). Em humanos, a leptospirose pode desencadear uma série de sinais clínicos e afetar múltiplos órgãos, incluindo o fígado, rins, pulmões e cérebro, sendo considerada um

problema de saúde pública (Faine et al. 1999; Gouveia et al. 2008; Adler, de la Pena, 2010). Em grandes animais, a leptospirose leva a baixo desempenho reprodutivo, abortos, partos prematuros e natimortos, causando prejuízos econômicos à pecuária (Faine et al., 1999; Levett, 2001; Rocha et al., 2017; Padilha et al., 2019).

O diagnóstico da leptospirose atualmente é realizado através de exame clínico, pesquisa direta e testes sorológicos. Dentre os testes utilizados, a soroaglutinação microscópica (MAT) é considerada o “padrão ouro”. Esta, confronta os soros humanos ou animais com uma bateria de diferentes cepas de leptospiras vivas, e assim, estima os níveis de anticorpos presentes na amostra (Faine et al. 1999). Embora a MAT seja o método de referência para o diagnóstico da leptospirose, apresenta baixa sensibilidade na fase aguda da doença quando os anticorpos são difíceis de detectar e há maior quantidade de IgM, que não é diferenciado do IgG (mais presente nas demais fases da doença) por este método (McBride et al. 2005; Musso & La, 2013; Rajapakse et al. 2015). Além disso, o grande número de variantes sorológicas produz uma alta diversidade de cepas, tornando extremamente difícil rastreá-las através dessa abordagem sorológica, culminando com muitas reações cruzadas. Ainda, a MAT é uma técnica bastante laboriosa e demorada, devido a necessidade da manutenção das bactérias em cultivo (Adler & Moctezuma, 2010; Caimi et al., 2017).

Devido as limitações encontradas no teste padrão, estão sendo desenvolvidos novo métodos de triagem e diagnóstico para a leptospirose. Os testes sorológicos e ensaios imunoquímicos, que utilizam antígenos recombinantes de *Leptospira*, além de testes moleculares, são os que surgem como alternativa para o diagnóstico da doença.

Critérios do estudo

A pesquisa para esta revisão foi realizada no PubMed (http://www.ncbi.nlm.nih.gov/pubmed/) limitando o período de pesquisa aos últimos cinco anos (maio de 2014 a maio de 2019), e usando como palavras-chave os termos: “*Immunodiagnosis leptospirosis*", “*Serological diagnosis leptospirosis*”, “*Detection Leptospira recombinant protein*”, “*Diagnosis leptospirosis recombinant*

protein", "*Immunodiagnosis leptospirosis recombinant protein*" e "*Molecular diagnosis leptospirosis*". Os termos da busca utilizados geraram 156, 102, 12, 31, 9 e 101 artigos, respectivamente. Os resumos desses artigos foram analisados, e todos os estudos com objetivo de desenvolver métodos de diagnóstico molecular e sorológico para leptospirose foram incluídos nesta revisão (n = . Todos os estudos não relevantes para o diagnóstico encontrados nesta pesquisa foram excluídos. Artigos com mais de cinco anos, porém contendo informações relevantes para a história da doença, também são citados neste artigo.

Antígenos de *Leptospira* spp.

Uma variedade de antígenos de *Leptospira* spp. já foram avaliados, muitos deles com potencial vacinal (Conrad et al., 2017; Oliveira et al., 2018; Raja et al., 2018; Cunha et al., 2019; Ghazali-Bina et al., 2019; Oliveira et al., 2019) ou para o diagnóstico (Alizadeh et al., 2014; Deneke et al., 2014; Ye et al., 2014; Nagalingam et al., 2015; Shiokawa et al., 2015; Kumari et al., 2016; Padilha et al., 2019). Como ocorre em outras espiroquetas, o genoma de *Leptospira* spp. codifica para mais lipoproteínas que outras bactérias, contendo aproximadamente 145 genes para prováveis lipoproteínas e *outer membrane proteins* (OMPs) (Setubal et al. 2006; Viratyosin et al. 2008; Yang et al. 2006). Existem diversas proteínas já descritas e comprovadamente presentes na superfície das leptospiras. Dentre essas, recebem destaque as Ligs, Loa22, LipL32, OmpL1, LenA, LenD, e as OMPs, OmpL36, OmpL37, OmpL47 e OmpL54.

Lipoproteínas e OMPs são os principais alvos em pesquisa e desenvolvimento de vacinas e testes de diagnóstico para controle da leptospirose. Todos os antígenos citados foram frequentemente encontrados nos últimos cinco anos em estudos que buscam o desenvolvimento de novas técnicas de diagnóstico, conforme descrito na Tabela 1.

Tabela 1: Antígenos mais frequentemente utilizados no desenvolvimento de testes de diagnóstico para leptospirose.

Antígeno	Localização na bactéria	Sorovar utilizado	Referência

LipL32	Lipoproteína (membrana externa)*	*L. interrogans* sorovar Copenhageni	(Alizadeh et al., 2014; Ye et al., 2014; Shiokawa et al., 2015)
LigB	Proteína de *Leptospira* com domínio semelhante à imunoglobulina (membrana externa)	*L. interrogans* sorovar Pomona; *L. borgpetersenii* sorovar Hardjo	(Deneke et al., 2014; Nagalingam et al., 2015)
LipL21	Lipoproteína (membrana externa)	*L. interrogans* sorovar Pomona; *L. interrogans* sorovar Autumnalis	(Ye et al., 2014; Kumari et al., 2016)
Lsa63	Proteína de adesão (membrana externa)	*L. interrogans* sorovar Copenhageni	(Alizadeh et al., 2014)
Loa22	Lipoproteína (membrana externa)	*L. interrogans* sorovar Pomona	(Ye et al., 2014)
LigA	Proteína de *Leptospira* com domínio semelhante à imunoglobulina (membrana externa)	*L. interrogans* sorovar Pomona	(Ye et al., 2014)
ErpY-like	Proteína de membrana externa**	*L. interrogans* sorovar Copenhageni	(Padilha et al., 2019)

*Localização controversa (Subsuperfície)
**Dados não publicados.

Diagnóstico da leptospirose

Polymerase chain reaction (PCR)

Técnicas baseadas na amplificação do DNA, como a PCR, são importantes ferramentas para detecção de *Leptospira* spp. durante a fase aguda da doença, quando os anticorpos estão abaixo do limite de detecção da maioria dos testes sorológicos (Ahmed et al., 2014; Denipitiya et al., 2016; Waggonera & Pinsky, 2017). A PCR apresenta vantagens por ser uma técnica rápida, sensível e específica. No entanto, ainda costuma ter altos custos (Galloway & Hoffmaster, 2015).

Diversos tipos de PCR já foram aplicados no diagnóstico da leptospirose, conforme descrito na Tabela 2. Dentre eles, PCR convencional (Waggoner et al., 2015; Gökmen et al., 2016), *Real-time* PCR (Nhan et al., 2014; Waggoner et al., 2014; Waggoner et al., 2015; Denipitiya et al., 2016), *Real-time* PCR seguido de análise de fusão de alta resolução (qRT-PCR-HRM) (Esteves et al., 2018),

238 *Recombinase polymerase amplification* (RPA) (Ahmed et al., 2014), PCR seguido de
239 RFLP (16S rRNA-PCR-RFLP) (Gökmen et al., 2016) e método de amplificação
240 isotérmica mediada por *loop* (LAMP) (Suwancharoen et al., 2016). As amostras
241 biológicas utilizadas na maioria destes estudos foram sangue ou suas frações (soro,
242 plasma) (Ahmed et al., 2014; Nhan et al., 2014; Waggoner et al., 2014; Waggoner et
243 al., 2015; Denipitiya et al., 2016; Gökmen et al., 2016; Esteves et al., 2018; Mohd Ali
244 et al., 2018) e urina (Suwancharoen et al., 2016; Esteves et al., 2018).

245

246 **Tabela 2:** Métodos baseados em PCR utilizados para detecção de *Leptospira*
247 spp.

DNA alvo	Tipo de PCR	Hospedeiro	Referência
lipl32	*Recombinase polymerase amplification* (RPA)	Humano (n=63)	(Ahmed et al., 2014)
lipl32	*Real-Time PCR*	Humano (n=33)	(Nhan et al., 2014)
rrs	*Pathogenic* qRT-PCR	Humano (n=65)	(Waggoner et al., 2014)
16S *lipl32*	qRT-PCR; PCR *conventional*	Humano (n= 894)	(Waggoner et al., 2015)
secY	*Real time PCR* (qRT-PCR)	Humano (n=111)	(Denipitiya et al., 2016)
lfb1; *secY*	*Real-Time PCR High Resolution Melting (qRT-PCR-HRM) analysis*	Humano (n=202)	(Esteves et al., 2018)
rrs; *lipl32;* *ompl1*	*16S rRNA-PCR followed by Restriction Fragment Lenght Polymorphism* (16S rRNA-PCR-RFLP); LipL32-PCR; OmpL1-PCR	Humano (n=47) e bovino (n=86)	(Gökmen et al., 2016)
rrs	*Taqman qPCR*	Humano (n=65)	(Mohd Ali et al., 2018)
lipl32 *lipl41*	*Loop-mediated isothermal amplification method (LAMP); qRT-PCR*	Bovino (n=533)	(Suwancharoen et al., 2016)

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Diferentes formatos de PCR são desenvolvidos para serem usados tanto na caracterização, quanto na identificação de *Leptospira* spp. em amostras suspeitas. A detecção molecular de *Leptospira* é comumente baseada em genes marcadores incluindo *16S rRNA*, *flaB*, *lipl32*, *lipl41*, *ligA* e *ligB*. O gene *lipl32* codifica uma lipoproteína de membrana externa que está presente nas espécies patogênicas de *Leptospira*, mas ausente nas espécies não patogênicas (Haake et al. 2000). Por isso, uma vasta gama de estudos utilizam o gene *lipl32* (Waggoner et al., 2015; Gökmen et al., 2016), seja como gene principal do teste, ou como método de comparação com o novo teste desenvolvido (Nhan et al., 2014; Waggoner et al., 2015; Gökmen et al., 2016; Suwancharoen et al., 2016; Monica et al., 2018).

O objetivo da busca por novos testes diagnósticos é aumentar a sensibilidade, especificidade e acurácia, afim de encontrar um método que possa ser utilizado com segurança na rotina laboratorial. Para isso, técnicas como o *Real-time* PCR mostram-se promissoras, são mais rápidas, proporcionam maior sensibilidade e menor manipulação de produtos do que a PCR convencional (Denipitiya et al., 2016). No geral, o *Real-time* PCR é realizado tendo como base o gene *16S*, que será amplificado apenas na presença de DNA de leptospiras (Waggoner et al., 2015). Porém, evidências sugerem que *Leptospira* spp. não patogênicas, como *L. fainei*, pode causar infecção, logo, excluí-las do painel de detecção pode levar a erros de detecção. Por isso, Mohd Ali et al. (2018) desenvolveram e validaram um *Real-time* PCR pan-*Leptospira*, utilizando o gene *rrs*, capaz de detectar a presença de leptospiras patogênicas e não-patogênicas nas amostras. A fim de incrementar os resultados do *Real-time* PCR, Esteves et al. (2018) desenvolveram o *Real-time PCR High Resolution Melting Analysis* - qRT-PCR-HRM (Análise de Fusão de Alta Resolução por PCR em Tempo Real), o qual se mostrou mais rápido, sensível, específico, com sensibilidade de 90% e especificidade de 97%.

Como alternativa ao *Real-time* PCR na rotina, a técnica de RPA (Ahmed et al., 2014), se mostrou precisa, com sensibilidade e especificidade de 94,7% e 97,7%, respectivamente. Técnicas de 16S rRNA-PCR-RFLP (Gökmen et al., 2016) obtiveram resultados promissores, com um diagnóstico precoce e preciso, sendo capaz de distinguir espécies patogênicas e não-patogênicas de *Leptospira*.

Testes sorológicos

A sorologia é a abordagem diagnóstica mais utilizada para a leptospirose. MAT é o teste padrão de referência para o diagnóstico sorológico de leptospiras. Este, detecta anticorpos aglutinantes no soro, mas requer significativa experiência de seus usuários, e a variação interlaboratorial nos resultados é alta (Adler et al., 2010). Resultados são, em geral, considerados positivos quando apresentam título de 400 na presença de sinais clínicos (Faine et al., 1999). Tanto a sensibilidade quanto a especificidade da MAT são muito altas. No entanto, pode apresentar problemas devido à exigência de culturas vivas de diferentes sorovares de *Leptospira* prevalentes em uma área geográfica específica (Bharti et al., 2003). Para atingir confiabilidade e padronização máximas, os laboratórios são incentivados a obter uma coleção de sorovares de um laboratório de referência certificados e a participar de programas de garantia de qualidade, como o esquema de teste de proficiência internacional da Leptospirose, organizado pela Sociedade Internacional de Leptospirose (ILS) (Adler et al., 2010).

Grande parte dos testes sorológicos tem como base a detecção de anticorpos, os quais são produzidos devido ao estímulo da resposta imune humoral do hospedeiro, gerada pela infecção por *Leptospira* spp. (Faine et al. 1999). O período de incubação das bactérias no desenvolvimento da leptospirose varia de 2 a 30 dias, e o início dos sintomas geralmente vem junto com o aparecimento de anticorpos aglutinantes, que aumentam com a progressão da doença (Lessa-Aquino et al., 2013). Este tipo de resposta se dá contra o LPS e contra várias proteínas constitutivamente expressas ou supra-reguladas durante a infecção (Adler & de la Pena 2010; Raja et al., 2016). Anticorpos do isotipo IgM aparecem cerca de 4 dias após o início dos sintomas, que pode variar de acordo com o período de incubação do patógeno. Já os anticorpos do isotipo IgG demoram de 10 a 14 dias, pois são mais específicos (Lessa-Aquino et al., 2013; Raja et al., 2016).

Alguns testes de diagnóstico sorológico visam um resultado precoce, com a detecção de IgM na fase aguda da doença (Alizadeh et al., 2014; Kitashoji et al., 2015; Wynwood et al., 2015; Courdurie et al., 2017). Outros visam uma resposta mais específica, com a detecção de IgG na fase convalescente (Alizadeh et al.,

2014; Brownlow et al., 2014; Ye et al., 2014; Nagalingam et al., 2015; Shiokawa et al., 2015; Wynwood et al., 2015; Kumari et al., 2016; Padilha et al., 2019).

A proteína LipL32 é a lipoproteína expressa em maior quantidade na membrana externa das leptospiras, sendo muito imunogênica. Além disso, LipL32 é conservada dentre as espécies patogênicas, podendo ser utilizada como um marcador distintivo para a leptospirose (Haake & Matsunaga, 2002; Shiokawa et al., 2015). Outra proteína bastante utilizada no desenvolvimento de teses sorológicos é a LipL21, uma importante proteína imunodominante expressa durante a infecção (Nally et al., 2007). Por estes motivos, estas proteínas são eleitas como importantes alvos no diagnóstico sorológico da leptospirose (Tabela 3).

Como mostra a Tabela 3, grande parte dos testes sorológicos que vem sendo desenvolvidos são baseados no ensaio utilizam a técnica de *Enzyme-Linked Immunosorbent Assay* (ELISA) (Alizadeh et al., 2014; Deneke et al., 2014; Ye et al., 2014; Kitashoji et al., 2015; Shiokawa et al., 2015; Courdurie et al., 2017; Padilha et al., 2019) ou no teste de aglutinação em látex (LAT) (Brownlow et al., 2014; Deneke et al., 2014; Nagalingam et al., 2015). Além desses, Wynwood et al. (2015 e 2016) validaram um imunoensaio com microesferas (MIA), que tem a capacidade de testar simultaneamente um grande número de amostras contra um grande número de sorovares, bem como, determinar os títulos individuais de IgG e IgM. Esses fatores, seriam extremamente benéficos nos diagnósticos laboratoriais e nos estudos epidemiológicos da leptospirose. Vanithamani et al. (2015) desenvolveram um ensaio do tipo *lateral flow* baseado em imunocromatografia de LPS (ICG-LFA), para detecção de anticorpos IgM, que se mostrou sensível e específico. Além disso, este teste se mostrou simples, rápido e possibilita o diagnóstico de sorogrupo em regiões endêmicas.

Em outro estudo, Raja et al. (2019) utilizaram nanopartículas de ouro e prata, associadas a diferentes proteínas (algumas bastante conhecidas e outras pouco estudadas), para desenvolver um *dot blot*, o qual se mostrou rápido, confiável e sensível para a detecção precoce da leptospirose.

Tabela 3: Testes sorológicos para o diagnóstico da leptospirose.

Proteína alvo	Tipo de teste	Espécie alvo	Sensibilidade / Especificidade	Referência
rLsa63;	*ELISA* indireto	Humano	96,6% / 83,2%	(Alizadeh et

rLipL32		(n=220)	92,3% / 83,8%	al., 2014)
-	LAT	Humano (n=55)	97,1% / 94,0%	(Brownlow et al., 2014)
rLigB	*ELISA* indireto LAT	Bovino (n=1126)	96,9% / 91,0% 93,6% / 92,3%	(Deneke et al., 2014)
rLipL21; rLoa22; rLipL32; rLigACon4-8	*ELISA* indireto	Canino (n=305)	99,5% / 84,3% 89,7% / 81,4% 92,6% / 84,3% 97,5% / 84,3%	(Ye et al., 2014)
rLigA; Células inteiras	*ELISA* de captura	Humano (n=304)	69.5% / 98,0% 54,3% / 97,0%	(Kitashoji et al., 2015)
rLigB	LAT	Bovino (n=390)	75,65% / 91,27%	(Nagalingam et al., 2015)
rLipL32	*ELISA* indireto	Roedores (n=33)	83,0% / 100%	(Shiokawa et al., 2015)
-	*Lateral flow* baseado em imunocromatografia lipopolissacarideo específica	Humano (n=320)	92,8%-100% / 99,1%-100%	(Vanithamani et al., 2015)
Células inteiras	*MIA*	Humano (n=200)	-	(Wynwood et al., 2015)
rLipL21; rI-LipL21; Células inteiras	IgM *ELISA* e *dotblot*	Humano (n=189)	IgM ELISA-*dotblot* 81,4%-88,8% / 85,7%-84,2% 92,5%-96,3% / 92,8%-92,8% 77,7%-74,0% / 77,1%-81,4%	(Kumari et al., 2016)
Células inteiras	*MIA*	Bovino (n=200)	-	(Wynwood et al., 2016)
rErpY-like	*ELISA* indireto	Suíno (n=72)	96,8% / 100%	(Padilha et al., 2019)
rLipL32; rLigA; rArgC; rRecA; rLruC; rLruD	Imunoensaio com nano-ouro reforçado com prata	Humano (n=112)	95,8% / 93,7% 95,8% / 95,3% 93,7% / 92,1% 93,7% / 92,1% 75,0% / 89,0% 93,7% / 85,9%	(Raja et al., 2019)

Considerações finais

O controle da leptospirose é um desafio, em virtude dos problemas de infrasestrutura, falta de saneamento, enchentes e devido aos reservatórios para esta

enfermidade serem animais de difícil controle. Por isso, torna-se tão importante o desenvolvimento de testes de diagnóstico mais aplicáveis, uma vez que a vacinação é ineficiente, sendo o diagnóstico preconizante para um tratamento adequado dos indivíduos infectados. Métodos sorológicos baseados em antígenos específicos são amplamente estudados, bem como, os métodos moleculares, já que podem ser eficazes desde a fase aguda da doença, antecipando o tratamento e impedindo a evolução e suas complicações.

O grande impacto da leptospirose, tanto na saúde pública, quanto na pecuária, estimula a comunidade científica a buscar métodos que visem melhorar e facilitar o controle da doença. Neste contexto, vários testes foram descritos para melhorar a acurácia diagnóstica, e muitos estudos têm mostrado resultados promissores, trazendo novas perspectivas para o diagnóstico da leptospirose.

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3.2 Manuscrito 2 – ErpY-like protein, a promising antigen to leptospirosis control: *in silico*, *in vitro*, and *in vivo* studies

Manuscrito submetido ao periódico *Molecular Biotechnology*.

ErpY-like protein, a promising antigen to leptospirosis control: *in silico*, *in vitro,* and *in vivo* studies

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Abstract

ErpY-like protein (LIC11966) is a new antigen from *Leptospira interrogans*. We characterized ErpY-like protein in terms of structure, cellular localization, conservation, antigenicity, and immunogenicity. *In silico* analyses using cellular localization predictors indicate ErpY-like protein is extracellular, lacks signal peptide (SP), does not contain transmembrane α-helix, and is not identified as a lipoprotein. The *erpY-like* gene was detected by a polymerase chain reaction in all *Leptospira* pathogenic serovars tested (n = 8) and was absent in saprophytic ones. The rErpY-like protein was recognized by antibodies presents in the sera of humans and animals (swine and canine), suggesting protein expression during natural infection. The polyclonal antibody (pAb) anti-rErpY-like protein was able to recognize the native ErpY-like protein in whole *Leptospira* cells, indicating that the protein is possibly exposed on the surface. In addition, the rErpY-like protein used to immunize mice with Freund's adjuvant stimulated a mixed Th1/Th2 response. In conclusion, rErpY-like protein is a promising candidate for vaccine production or a diagnostic test preparation against leptospirosis.

Keywords: LIC11966; *erpY-like*; *Leptospira* spp.; *in silico* analysis; polyclonal antibodies.

Introduction

Leptospirosis is a zoonosis of global distribution, caused by pathogenic spirochetes from the *Leptospira* genus. Human and animals can be infected by direct contact with the urine of infected carriers or through contaminated water and soil (Faine et al. 1999; Reis et al. 2008). Rodents are the most frequent reservoir of pathogenic leptospires; they harbor the bacteria in their kidneys and consequently disseminate it through urine (Jorge et al. 2015). However, all mammals and birds, amphibians, reptiles, and possibly fish can carry pathogenic *Leptospira* species (Picardeau 2017).

Leptospirosis symptoms include fever, vomiting, and headache, being many times confused with other febrile illnesses. In some cases, the disease may evolve to renal and hepatic failures, uveitis, pulmonary damage with severe pulmonary hemorrhage, and cause death. This disease is considered a public health problem and its economic impact in the agricultural sector is also concerning (Faine et al. 1999; Levett 2001; Ellis 2015). The global burden of human leptospirosis is estimated to be 1 million cases and approximately 60,000 deaths per year (Picardeau 2017). In Brazil, the last decade had about 4,000 confirmed cases per year, with approximately 350 cases leading to death (SINAN 2017).

The *Leptospira* genus comprises 22 species, divided into around 300 serovars, of which more than 270 are pathogenic. This antigenic diversity is attributed to the lipopolysaccharide composition (Bulach et al. 2000; Picardeau 2013; Jorge et al. 2015; Fernandes et al. 2017; Picardeau 2017). Commercial vaccines used to prevent leptospirosis are bacteria-based (killed-whole cells). These vaccines confer short-term immunity, do not provide cross-protection against different serovars, require annual boosters, and usually fail to prevent disease transmission

663 (Ko et al. 2009; Dellagostin et al. 2011). Efforts to develop recombinant vaccines
664 against leptospirosis have focused on conserved outer membrane proteins, bacterial
665 motility factors, lipopolysaccharides, and virulence factors that represent potential
666 targets for immunological defense mechanisms (Dellagostin et al. 2011; Oliveira et
667 al. 2015). Our group has been working on the development of products to control
668 leptospirosis; several proteins have been shown to stimulate a protective immune
669 response against leptospirosis, indicating their potential as vaccine candidates
670 (Haake et al. 1999; Seixas et al. 2007; Felix et al. 2011; Hartwig et al. 2013; Oliveira
671 et al. 2015; Oliveira et al. 2016; Oliveira et al., 2018).

672 In the diagnostics field, the microscopic agglutination test (MAT) is the gold
673 standard technique for leptospirosis. The MAT detects antibody levels in sera from
674 patients with suspected leptospirosis against a panel of live serovars of pathogenic
675 leptospires (Faine et al. 1999). However, this technique presents a high number of
676 cross-reactions among serovars and low sensitivity in the acute phase of the disease
677 (Monte et al. 2011; Hartleben et al. 2013). Because of the drawbacks of the MAT,
678 more sensitive techniques such as enzyme-linked immunosorbent assay (ELISA)
679 and other immunochemical assays using recombinant leptospiral antigens have been
680 developed as alternative methods for leptospirosis diagnosis (Bomfim et al. 2005;
681 Mariya et al. 2006; Hartleben et al. 2013; Lizer et al. 2017).

682 The ErpY-like (LIC11966) protein is an *L. interrogans* antigen, with sequence
683 similarity to virulence factors from other pathogens (such as the outer membrane
684 protein ErpY from *Borrelia burgdorferi*). Furthermore, the protein is expressed during
685 *in vivo* infection (Eshghi et al. 2009) and interacts with the extracellular matrix (ECM)
686 (Oliveira et al. 2018), suggesting that it has importance in infectious process and
687 potential to be used in diagnosis and vaccine development. Recently, we used the

688 rErpY-like protein as a subunit vaccine in protection assays against a virulent strain
689 of *L. interrogans*, demonstrating survival rates of 62.5% in a hamster animal model
690 (Oliveira et al. 2018). This protein was also evaluated in an ELISA for diagnosis of
691 swine leptospirosis, and present sensitivity, specificity and accuracy of 96.8%, 100%,
692 and 99%, respectively (Padilha et al. 2019). Now we characterized the ErpY-like in
693 terms of structure, cellular localization, conservation, antigenicity, and
694 immunogenicity and reported relevant aspects of ErpY-like protein as a new antigen
695 for use in leptospirosis control.

696

697 **Materials and Methods**

698 Bacterial strains and culture conditions

699 *Leptospira* spp. were cultured in Ellinghausen-McCullough-Johnson-Harris
700 (EMJH) liquid medium (BD Difco™) supplemented with *Leptospira* enrichment EMJH
701 (BD Difco™) at 30 °C (Silva et al. 2007). The serovars used in this study included *L.*
702 *borgpetersenii* serovar Javanica and Sejroe, *L. interrogans* serovar Pomona,
703 Canicola, and Copenhageni, *L. kirschneri* serovar Grippotyphosa and Cynopteri, and
704 *L. biflexa* serovar Ranarum. *Escherichia coli* BL21 (DE3) Star (Invitrogen, São Paulo,
705 Brazil) were grown at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast
706 extract, 0.5% NaCl, and 2% agar) with the addition of ampicillin to 100 µg/mL.

707

708 *In silico* analysis and structural modeling

709 A multiple sequence alignment was performed by MUSCLE (Edgar, 2004)
710 using the sequence of ErpY-like protein (UniProt: Q72QY9). Cellular localization was
711 predicted by PSORT 3.0.2 (Nakai and Horton, 1999), CELLO 2.5 (Yu et al. 2010),
712 and GNeg-mPLoc 2.0 (Shen and Chou, 2010). The signal peptide sequence was

713 studied by SignalP 4.1 (Bendtsen et al. 2004), Signal-CF (Chou and Shen, 2007),
714 and PrediSi (Hiller et al. 2004). The Smart (Schultz et al. 1998) and Pfam (Finn et al.
715 2006) web servers were used to search for predicted functional and structural
716 domains within the amino acid sequence. Transmembrane α-helices (TMHs) were
717 predicted using MEMSAT3 (Jones, 2007), TMHMM v. 2.0 (Krogh et al. 2001),
718 HMMTOP version 2.0 (Tusnady and Simon, 2001), and Phobius (Kall et al. 2004).
719 Leptospiral lipoproteins were predicted using LipoP v. 1.0 (Juncker et al. 2003) and
720 SpLip (Setubal et al. 2006).

721 For structural analysis, ErpY-like protein structures were predicted using I-
722 Tasser (Yang et al. 2015) and Raptor X (Källberg et al. 2012) web servers. The
723 structures were then evaluated through DFire (Yang and Zhou, 2008), QMean
724 (Benkert et al. 2008), SolvX (Holm and Sander, 1992), and RAMPAGE (Sheik et al.
725 2002) web suites.

726

727 Presence and conservation of *erpY-like* in *Leptospira* spp.

728 The presence of the *erpY-like* gene among *Leptospira* spp. serovars was
729 performed using bioinformatics tools and the polymerase chain reaction (PCR)
730 assay. BlastP was used to determine the conservation of the *erpY-like* gene in
731 different serovars by *in silico* analyses. The PCR technique was applied to amplify
732 the *erpY-like* gene using genomic deoxyribonucleic acid (DNA) from eight serovars,
733 including *L. borgpetersenii* serovars Javanica and Sejroe, *L. interrogans* serovars
734 Canicola, Pomona, and Copenhageni, *L. kirschneri* serovars Grippotyphosa and
735 Cynopteri, and *L. biflexa* serovar Ranarum. PCR was performed using the following
736 oligonucleotides: *erpY-like* F: 5′-ACTCTCGAGGAAATCATGAACGCT–3′ and *erpY-*
737 *like* R: 5′-ACGGAATTCTTGAGAAGCGTATTC–3′. The primers were designed using

738 VectorNTI 11 software (Invitrogen, USA) according to the *L. interrogans* serovar
739 Copenhageni genome (GenBank: AE016823). PCR reactions followed: 94 °C for 5
740 min, followed by 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min,
741 with a final extension of 72 °C for 7 min. Reactions were performed in a final volume
742 of 25 µL using Taq DNA Polymerase (GoTaq Colorless Master Mix–ProMega, USA)
743 in a Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). The amplification
744 products were visualized by 1% agarose gel electrophoresis.
745
746 Production of rErpY-like protein

747 The gene encoding the ErpY-like protein (approximately 390 bp) was
748 synthesized (GenOne, Rio de Janeiro, Brazil) on basis of the genomic sequence of *L.*
749 *interrogans* serovar Copenhageni strain L1–130 (FIOCRUZ, Bahia, Brazil) available
750 at GenBank (GenBank: AE016823). The *erpY-like* gene was obtained, cloned in the
751 pAE vector, and fused with an N-terminal 6×His-tag. The pAE/*erpY-like* plasmid was
752 used to transform *E. coli* BL21 (DE3) Star cells, which were cultivated in LB medium
753 (tryptone 1.6%, yeast extract 1%, and NaCl 0.5%, pH 7.0) (Sambrook and Green,
754 2012) containing ampicillin (100 µg/mL). When absorbance at 600 nm (OD_{600})
755 reached 0.8, 1 mmol/L isopropyl-β-D-thiogalactopyranoside was added, and the cells
756 were harvested by centrifugation (4000 ×*g* for 15 min at 4 °C) 3 h later. After
757 centrifugation, the cells were lysed by sonication and centrifuged (11,000 × *g* for 30
758 min at 4 °C). For protein purification, cell pellets were suspended in a solubilization
759 buffer (8 M urea, 200 mM NaH_2PO_4, 0.5 M NaCl, and 5 mM imidazole, pH 8.0).
760 Purification was performed by immobilized metal ion affinity chromatography using
761 Ni^{2+} Sepharose HisTrap columns using the ÄKTA prime liquid chromatography
762 system (GE Healthcare, USA). The purified proteins were dialyzed against

763 phosphate-buffered saline (PBS) pH 8.0 with decreasing concentrations of urea in 16
764 steps over five days at 4 °C. The purification was evaluated by 15% SDS-PAGE
765 (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and the gels were
766 stained with Coomassie Blue. The expression of the rErpY-like protein was confirmed
767 by Western Blotting (WB), employing an anti–6× His-tag monoclonal antibody
768 (Sigma-Aldrich, USA). Briefly, the rErpY-like protein was separated by 15% SDS-
769 PAGE and electro-transferred onto nitrocellulose membranes (GE Healthcare,
770 Illinois, USA). The membranes were blocked with PBS-FBS 1%, (phosphate buffered
771 saline with 1% [v/v] fetal bovine serum) and reacted with the anti–6× His-tag
772 monoclonal antibody (Sigma-Aldrich, USA) at 1:100 dilution in PBS. Then, a goat
773 anti-mouse Ig peroxidase conjugate (Sigma-Aldrich, USA) was added at 1:4000
774 dilution. Incubations were performed for 1 h at room temperature in agitation (50 rpm)
775 and washes with PBS-T (PBS with 0.05% [v/v] Tween 20) were performed between
776 all steps. Then, reactions were developed with a chromogen/substrate solution (6 mg
777 diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl, pH 8.0, and 0.03%
778 hydrogen peroxide) for visualization of protein bands. The rErpY-like protein was
779 quantified using a BCA Protein Assay Kit (Thermo Scientific, USA) and stored at –20
780 °C.
781
782 Evaluation of rErpY-like protein antigenicity
783 Antigenicity was evaluated by the capacity of the rErpY-like protein to be
784 recognized by antibodies present in sera. Thus, we performed an indirect ELISA
785 using sera from humans, swine, and canines naturally infected and positive to
786 leptospirosis (reciprocal MAT titer >1:25,000) in pools of 5 samples, anti-*Leptospira*
787 hyperimmune sera of rabbit, and normal mouse serum (as a negative control)

788 provided by the Immunodiagnostic Laboratory of the Federal University of Pelotas,
789 Pelotas, Rio Grande do Sul, Brazil. Briefly, polystyrene plates (Nunc, Polysorp) were
790 coated with rErpY-like protein diluted in a carbonate-bicarbonate buffer, pH 9.6, at a
791 concentration of 200 ng per well, and blocked with PBS-FBS 1%. Sera samples were
792 diluted 1:50 in PBS-T. Then, anti-Ig peroxidase conjugates specific for human, swine,
793 canine, and rabbit (Sigma–Aldrich, USA), diluted 1:10,000, 1:5000, 1:6000 and
794 1:4000, respectively, were added. The presence of antigen-antibody complex was
795 revealed by adding a substrate solution containing *o*-phenylenediamine (0.4 mg/mL
796 in 0.1 M citrate buffer, pH 5.0) and 0.03% hydrogen peroxide. The reaction was
797 stopped by adding 0.1 M sulfuric acid and absorbance was determined at 492 nm
798 (OD_{492}) using the VICTOR^{TM} X5 Multilabel Plate Reader (Perkin Elmer, USA). All
799 assay steps were performed in 100 µL/well at 37 °C for 1 h, and the plates were
800 washed three times with PBS-T in between steps.
801
802 Anti-rErpY-like protein polyclonal antibodies

803 For anti-ErpY-like protein polyclonal antibody (pAb) production, two 6-week-
804 old BALB/c mice were injected intraperitoneally with 100 µg of rErpY-like protein on
805 days 0, 14, 21, and 28. Freund's complete adjuvant (Sigma Aldrich, USA) was used
806 in the first dose and Freund's incomplete adjuvant (Sigma Aldrich, USA) in the
807 subsequent ones. On day 35, blood samples were collected from the retro-orbital
808 venous plexus after the administration of eye anesthetic drops to determine antibody
809 levels. Then, mice were boosted with another dose of 100 µg of rErpY-like protein.
810 The blood was collected, centrifuged, and purified by affinity chromatography using a
811 protein A-Sepharose CL-4B column (GE Healthcare, USA) according to the
812 manufacturer's instructions. Next, an indirect ELISA was performed to determine the

rErpY-like protein antibody titer. For titration, polystyrene ELISA microtiter plates (Nunc, Polysorp, USA) were coated with rErpY-like protein (200 ng/well) and blocked with PBS-FBS 1%. Serial dilutions (1:100–1:512,000) of pAb were added to the wells. Then, goat anti-mouse Ig peroxidase conjugate (Sigma-Aldrich, USA), diluted 1:5000, was added for sera antibody detection. The presence of an antigen-antibody complex was revealed, and the reaction was stopped by adding 0.1 M sulfuric acid and absorbance was determined at 492 nm using VICTOR^{TM} X5 Multilabel Plate Reader (Perkin Elmer, USA). All assay steps were performed in 100 µL/well at 37 °C for 1 h, and the plates were washed three times with PBS-T. Lastly, the pAb was confronted with rErpY-like protein in a WB assay, as previously described, to confirm its ability to react with the recombinant protein.

Animal experiments described in this study were performed in strict accordance with the guidelines of the National Council for Control of Animal Experimentation, Brazil (CONCEA, nº 11794) and approved by the Ethics Committee in Animal Experimentation, Federal University of Pelotas, Brazil (Permit number: 5429).

Isotyping of pAb anti-rErpY-like protein

An indirect ELISA was performed to determine the levels of anti-rErpY-like IgG subclasses in the mice serum, according to Schuch et al. 2017. Polystyrene 96-well plates (Nunc, Polysorp, USA) were coated with rErpY-like protein diluted in carbonate-bicarbonate buffer, pH 9.6, at a concentration of 100 ng/well for 16 h at 4 °C. The plates were blocked with 200 µL of PBS-FBS 5% at 37 °C for 2 h. Then, mice serum samples were added to wells at 1:100 in triplicate followed by incubation for 1 h at 37 °C. Goat anti-mouse primary antibodies anti-isotype IgG1, IgG2a,

838 IgG2b, or IgG3 (Sigma-Aldrich, USA) were added at 1:1000 for 1 h at 37 °C. For
839 reaction development, a rabbit anti-goat IgG peroxidase conjugate (Sigma-Aldrich,
840 USA), was added at 1:5000 for 1 h at 37 °C, followed by the liquid substrate 3,3,5,5-
841 tetramethylbenzidine for 15 min at 37 °C. In between all steps, washes with PBS-T
842 were performed to remove non-bound components. The reaction was stopped by
843 adding 0.1 M sulfuric acid and absorbance was determined at 492 nm using a
844 VICTOR^{TM} X5 Multilabel Plate Reader (Perkin Elmer, USA).
845
846 Detection of native ErpY-like protein in *Leptospira* spp.

847 Indirect ELISA and indirect immunofluorescence (IIF) were performed to
848 evaluate the presence of native ErpY-like protein in *L. interrogans* serovar
849 Copenhageni L1–130. For ELISA, polystyrene 96-well plates (Nunc, Polysorp, USA)
850 were coated with a 0.1% poly-*l*-lysine solution. Seven-day cultures of *Leptospira*
851 were washed once in PBS and suspended to a density of 10^8 cells/mL. Some
852 aliquots were used directly to coat the plates with whole cells; others were heated at
853 100 °C to expose the proteins. Then, the plates were blocked with PBS-FBS 1% and
854 reacted with the pAb at 1:100 dilution in PBS. Afterward, a goat anti-mouse Ig
855 peroxidase conjugate (Sigma-Aldrich, USA), diluted 1:5000, was added for the
856 detection of antibodies in sera. Other steps were followed as described above.
857 Normal mouse serum was used as a negative control.

858 For IIF, slide chambers (ICN Biomedicals Inc., CA, USA) were coated with a
859 0.1% poly-*l*-lysine solution for 1 h at 37 °C. Seven-day cultures of *Leptospira* were
860 washed once in ultrapure water and suspended to 10^8 cells/mL. Aliquots of 10 µL
861 were added to slide chambers and incubated at 37 °C. Then, the slides were blocked
862 with PBS-FBS 10%, washed twice with PBS-T, and coated for 1 h at 37 °C with pAbs

anti-rErpY-like protein at 1:100 dilution in PBS. The slides were washed again twice with PBS-T and a 1:100 dilution of goat anti-mouse FITC (Fluorescein isothiocyanate) conjugate (Invitrogen, USA) was added, and the slides were incubated at 37 °C in a dark humid chamber for 1 h. After washing with PBS-T, a drop of mounting medium was added and labeling was visualized by fluorescence microscopy (Olympus BX 51). The controls used were the same as those described for ELISA. DNA staining with Hoechst 33258 achieved confirmation of bacteria in the microscopic field.

Statistical analysis

Data are expressed as mean ±SEM (standard error of the mean) and significant differences between groups were determined using analysis of variance and Tukey's post-test. $P < 0.05$ was considered statistically significant. Analyses were performed using Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

Results

In silico analysis and structural modeling

According to cellular localization predictors (PSORT, CELLO, and GNeg-mPLoc), ErpY-like protein is extracellular. The SignalP, SignalCF, and PrediSI programs indicated that the protein has no signal peptide (SP). The analysis in Smart and Pfam web servers revealed an unknown function of ErpY-like protein. Predictors of TMHs (MEMSAT3, TMHMM, HMMTOP, and Phobius) determined that ErpY-like

886 protein does not contain TMH. Finally, the protein was not identified as a lipoprotein
887 by LipoP and SpLip.

888 NetNGlyc analysis revealed the presence of N-glycosylation site at position
889 5. After quality assessment, the RaptorX structure was chosen as the representative
890 protein. The representation of the protein by the Raptor X web server was chosen for
891 presenting a QMean value closer to 0 when compared with the representation of the
892 protein by the I-Tasser web server. Other values, such as Ramachandran Plot,
893 SolvX, and DFire, showed better results on the RaptorX web server. The structure
894 was then aligned with ErpY-like protein, as shown in Figure 1. Quality assessment
895 results are shown in Table 1.

896

897 Presence of *erpY-like* gene in *Leptospira* spp.

898 PCR analysis showed that the *erpY-like* gene is present in all pathogenic
899 serovars tested (*L. interrogans* serovars Pomona, Canicola, and Copenhageni, *L.*
900 *borgpetersenii* serovars Javanica and Sejroe, and *L. kirschneri* serovars
901 Grippotyphosa and Cynopteri) and absent in saprophytic *L. biflexa* serovar Ranarum
902 (Figure 2). *In silico* sequence analysis showed that the predicted *erpY-like* sequence
903 in *L. interrogans* serovar Copenhageni was 99% identical to *erpY-like* sequence in *L.*
904 *interrogans* serovar Pyrogenes, 98% to *L. interrogans* serovar Grippotyphosa, *L.*
905 *interrogans* serovar Pomona, and *L. interrogans* serovar Canicola, 90% to *L.*
906 *noguchii*, 87% to *L. kirschneri*, 85% to *L. weilli*, 82% to *L. santarosai*, and 69% to *L.*
907 *mayottensis*, all of them with a query coverage of 100%. The identity was 73% to *L.*
908 *alstonii* (98% coverage).

909

910 Production of rErpY-like protein and evaluation of antigenicity

911 rErpY-like protein was expressed in *E. coli* in an insoluble form, with the
912 expected size of 13 kDa. After dialysis against PBS, the protocol for solubilization in
913 urea and purification of recombinant protein resulted in a yield of 25 mg/L. rErpY-like
914 protein expression was confirmed by WB assay using an anti–6× His-tag monoclonal
915 antibody (Sigma-Aldrich).

916 Antigenicity of rErpY-like protein was confirmed in an indirect ELISA.
917 Antigenicity of the purified protein was evaluated with sera from naturally infected
918 human, swine, and canine and positive to leptospirosis and with rabbit
919 anti-*Leptospira* hyperimmune sera. rErpY-like protein produced in *E. coli* was
920 recognized by all sera samples evaluated, as shown in Figure 3. Normal mouse
921 serum used as a negative control did not demonstrate a detectable reaction.

922

923 Production of pAb anti-rErpY-like protein and isotyping

924 The pAb against rErpY-like protein was successfully produced, purified, and
925 titrated, reacting with the rErpY-like protein until the dilution of 1:12,800. In the WB
926 assay, the pAb recognized the recombinant protein in the expected size of 13 kDa.
927 As shown in Figure 4, pAb isotyping revealed the presence of IgG1, IgG2a, IgG2b,
928 and IgG3 subclasses. However, IgG1 and IgG2a showed a significantly higher
929 response ($P<0.05$) compared with other subclasses. At OD_{492nm}, IgG1 and IgG2a
930 isotypes obtained mean absorbances close to 3.000, while IgG2b obtained at
931 approximately 2.500 and IgG3 at approximately 1.200. Thus, the isotype profile of
932 pAb against rErpY-like protein observed was IgG1=IgG2a>IgG2b>IgG3, as
933 demonstrated by the mean absorbances obtained in the indirect ELISA, for each
934 subclass of IgG.

935

936 Detection of native ErpY-like protein in *Leptospira* cells using the anti-rErpY-like
937 protein pAb

938 The pAb-produced anti-rErpY-like protein recognized epitopes in native
939 protein either on whole and lysed cells of *L. interrogans*, both in indirect ELISA and
940 IIF. In indirect ELISA, results showed a significant reaction ($P < 0.05$) of pAb with
941 whole and lysed cells of *L. interrogans*, in comparison with normal mouse serum
942 (Figure 5). IIF showed that pAb anti-rErpY-like protein recognized the native protein
943 in whole bacterial cells (Figure 6A). Staining of the bacterial DNA confirmed the
944 presence of leptospires (Figure 6B). The positive control is shown in Figure 6C and
945 6D. FITC-labeled cells were not observed in the negative control, despite the
946 presence of leptospires in lamina (data not shown).

947

948 **Discussion**

949 In this study, we reported the production of ErpY-like protein from *L.*
950 *interrogans*, in a recombinant form using a synthetic gene, and its characterization
951 regarding the structure, cellular localization, conservation, antigenicity, and
952 immunogenicity by *in silico*, *in vitro*, and *in vivo* analyses. ErpY-like protein is
953 annotated as a hypothetical protein and expressed during *in vivo* infection (Eshghi et
954 al. 2009). Our research group demonstrated that ErpY-like protein interacts with the
955 ECM and protects hamsters against mortality from infection with highly virulent
956 *Leptospira* strains (Oliveira et al. 2018). Additionally, in an ELISA from diagnosis of
957 swine leptospirosis, the rErpY-like protein presented 96.8% of sensitivity, 100% of
958 specificity and 99% of accuracy values (Padilha et al. 2019). The identification and
959 characterization of novel leptospiral antigens represent an important advance in the

960 search for new methods to leptospirosis control (Hartwig et al. 2011; Hartleben et al.
961 2013; Hartwig et al. 2013; Nagalingam et al. 2015; Conrad et al. 2017).

962 The *in silico* analysis using bioinformatics tools offers the identification of
963 candidate antigens for subsequent evaluation in the laboratory. This *in silico* step
964 may improve and complement the experimental results obtained for the
965 characterization of proteins, as it enables the discovery of promising antigens, e.g.,
966 those are exposed on the surface of the target microorganism, conserved between
967 pathogenic serovars, and those are antigenic and immunogenic (Dellagostin et al.
968 2017). Our results, obtained using *in silico* analysis, demonstrated that the ErpY-like
969 protein is exposed on the surface, indicating that it could mediate *Leptospira*-host
970 interactions. Additional *in silico* analysis demonstrated that the *erpY-like* gene is
971 conserved at the DNA level (80% overall pairwise identity) in *L. interrogans*, *L.*
972 *noguchii*, *L. santarosai*, *L. kirschneri*, and *L. weilli*, and absent in *L. biflexa*. These
973 results confirm that the *erpY-like* gene is conserved in all pathogenic serovars tested
974 in the present study. Moreover, these data are consistent with the results obtained by
975 the PCR assay.

976 Some authors have reported that proteins are potential vaccine targets
977 because of their conservation in different serovars, such as LipL32 (Haake and
978 Matsunaga 2010), LemA (Hartwig et al. 2013), and LigB (McBride et al. 2009;
979 Conrad et al. 2017). Major efforts have been directed to find potent and effective
980 prophylaxis to leptospirosis. However, the available vaccines do not protect against
981 heterologous *Leptospira* serovars (Dellagostin et al. 2011; Picardeau 2017). The
982 presence and conservation of the *erpY-like* gene in different pathogenic serovars, as
983 demonstrated in this study, is an important feature.

In order to evaluate the antigenic and immunogenic capacity of the ErpY-like protein, we produced this antigen in a recombinant form. The *E. coli*-based expression system was efficient to produce the rErpY-like protein using a synthetic gene with optimized codons to *E. coli*. This bacterium is routinely used in the production of recombinant proteins for various purposes ranging from structural studies to vaccine and diagnostic test development (Dellagostin et al. 2011; Gopal and Kumar 2013; Fernandes et al. 2017). The rErpY-like protein produced was used in the WB assay to evaluate its antigenicity using the sera of naturally infected humans and animals. Zuerner (2015) suggested that the protective immune response against leptospirosis is antibody-based. Therefore, screening to determine the ability of antigens to induce specific antibody responses *in vivo* is an important indicator of the promising potential for a vaccine candidate and to be used in diagnostic assay development.

The most prevalent serovar in human infection is Copenhageni (Faine et al. 1999), while leptospires belonging to the serovar Canicola frequently infect canines (Ye et al. 2014) and leptospires belonging to the serovar Pomona infect swine (Figueredo et al. 2013). Since the rErpY-like protein was recognized by antibodies present in the sera from naturally infected human, canine, and swine, this protein may have a role in the *Leptospira* infection process. This result corresponds with previous reports where RlpA (Gamberini et al. 2005), LemA, LIC10009, LIC11567, LIC13305, LIC13059, and LIC20172 (Hartwig et al., 2011), OmpL36 (Oliveira et al. 2015), and LigB (Conrad et al. 2017) were recognized by antibodies present in sera from naturally and experimentally infected animals.

Antibody-based reagents have provided the basis for a large number of highly specific and reproducible immunoassays (Berry 2005; Monte et al. 2011). Owing to

1009 the high cost for production of monoclonal antibodies (mAbs), the pAb anti-rErpY-like
1010 protein emerges as an important alternative to antigen detection, besides contributing
1011 to protein characterization. The pAb was satisfactorily produced, resulting in high
1012 titers of antibodies against the rErpY-like protein (1:12,800). In isotyping, we
1013 identified the IgG profile of pAb and estimated the specific humoral response
1014 generated by rErpY-like protein in mice, and contributed to the knowledge about
1015 characteristics of the produced pAb.

1016 Monte et al. (2011) used IIF to characterize the mAbs against rLigA and rLigB
1017 proteins and to demonstrate reaction with native proteins in *Leptospira*. The indirect
1018 ELISA and IIF assays demonstrated the reaction of the pAb anti-rErpY-like protein
1019 with epitopes exposed in native proteins either in whole or lysed bacterial cells.
1020 Indirect ELISA showed a reaction of pAb with whole and lysed cells of *Leptospira*. IIF
1021 was performed to study the interaction pAb with native ErpY-like protein in *L.*
1022 *interrogans* strain Fiocruz L1-130. ErpY-like protein was recognized in *Leptospira* by
1023 pAbs produced against the recombinant protein, which indicates its expression with
1024 the same epitopes as those of the native protein. ErpY-like protein was detected both
1025 in whole and lysed cells of *Leptospira*; however, new tests are needed to confirm the
1026 localization of this protein in the bacterial cell.

1027 Generally, diagnostic and vaccine markers induce antibodies against surface
1028 structures (Ko et al. 2009; Seenichamy et al. 2014). Our group demonstrated that
1029 rErpY-like protein, when prepared with an AddaVax™ adjuvant, provided a survival
1030 rate of 62.5% in a hamster animal model. Furthermore, rErpY-like protein has the
1031 ability to bind to fibrinogen, and this may interfere with blood coagulation and in
1032 contribution to hemorrhage and vascular lesion, probably affecting platelet
1033 aggregation and fibrin clot formation (Oliveira et al. 2018). In this study, ErpY-like

protein vaccine preparation induced a high level of IgG antibodies, with a predominance of IgG1/2a response. The presence of high levels of IgG1 indicates a Th2-like response, primarily required against extracellular bacteria such as *Leptospira* spp. However, rErpY-like protein also stimulated the production of IgG2 subclasses in mice, suggesting a mixed Th1/Th2 response, desirable in leptospiral vaccine formulations. This response is attributed to the ErpY-like protein antigen as well as to the adjuvant used in this formulation; Freund's adjuvant is known as an enhancer of the cellular immune response. When rEpY-like protein was administered in hamsters with AddaVax™, a predominant IgG2/3 response was observed (Oliveira et al. 2018). Other studies have reported the induction of a mixed cellular/humoral immunity by recombinant vaccines against leptospirosis with partial protection rates (Conrad et al. 2017; Oliveira et al. 2018). Although these results were obtained in mice, the IgG isotypes are related in hamsters, the animal model recommended for leptospirosis immunoprotection assays. The search, identification, and characterization of new antigens are important steps for the development of effective vaccines and diagnostic tests against leptospirosis. Here, we characterized the ErpY-like protein, which proved to be an important target with potential for use as a vaccine antigen and in diagnostic tests.

Ethical Statement

Animal procedures were performed at the animal facility of the Federal University of Pelotas (UFPel) and approved by the Ethics Committee in Animal Experimentation (CEEA) of UFPel, under protocol number 5429. The CEEA at UFPel is accredited by the Brazilian National Council for Animal Experimentation Control

(CONCEA). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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Conflicts of Interest

The authors BCRP, HQS, TFC, SBF, TLO, RAS, RSW and DDH declares that they no conflict of interest.

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1279 **Figure Legends**

1280

1281 Figure 1 – **3D structure of ErpY-like protein.** The protein structure was predicted by
1282 Raptor X web server.

1283

1284 Figure 2 **– Distribution of *erpY-like* gene among serovars of *Leptospira* spp.** The
1285 genomic DNA of different serovars of *L. biflexa*, *L. interrogans*, *L. borgpetersenii,* and
1286 *L. kirschneri* were submitted to PCR with specific oligonucleotides and visualized on
1287 1% agarose gel.

1288

1289 Figure 3 – **Antigenicity of rErpY-like protein.** rErpY-like protein was used as
1290 antigen in ELISA with pooled sera from human, swine, and canine positives to
1291 leptospirosis. A rabbit anti-*Leptospira* hyperimmune serum was used as positive
1292 control. No detectable reaction was observed in normal mouse serum (negative
1293 control). The results represent mean absorbance ± mean standard error calculated
1294 from the serum samples assayed in duplicate. OD_{492} = optical density at 492 nm. The
1295 significance was determined by the analysis of variance (Tukey's multiple
1296 comparison) and the different letters indicate a significant difference ($P < 0.05$)
1297 between samples.

1298

1299 Figure 4 – **Isotyping of anti-rErpY-like IgG subclasses.** BALB/c mice were
1300 intraperitoneally immunized with rErpY-like (100 µg) in combination with Freund's
1301 complete adjuvant (Sigma Aldrich, USA) in the first dose and Freund's incomplete
1302 adjuvant (Sigma Aldrich, USA) in the subsequent ones (days 14, 21, and 28). Blood
1303 was collected on day 35 post-immunization. The responses of the IgG isotypes

(IgG1, IgG2a, IgG2b, and IgG3) were determined by ELISA with 100 ng/mL of antigen rErpY-like and 1:100 mouse serum dilution. The results represent the mean absorbance ± SEM, calculated from pAb samples assayed in duplicate. The significance was determined by analysis of variance (Tukey's multiple comparison) and the different letters indicate a significant difference ($P < 0.05$) between the samples.

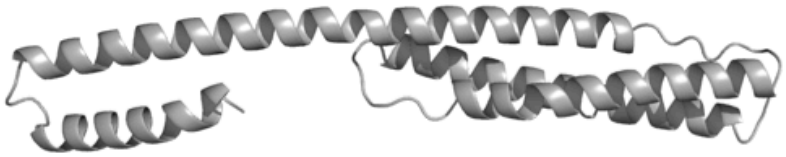
Figure 5 – **pAb anti-rErpY-like protein recognizes the native protein in *Leptospira* cells.** The pAb produced against rErpY-like protein was used to evaluate its reaction with recombinant and native forms of ErpY-like protein by indirect ELISA, in whole and lysed cells of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130. The results are represented as the mean absorbance ± SEM obtained from reactions of pAb anti-rErpY-like or normal mouse serum (negative control) with recombinant or native ErpY-like protein (whole and lysed cells), assayed in duplicate. The significance was determined by the analysis of variance (Tukey's multiple comparison) and the different letters indicate a significant difference ($P < 0.05$) between the samples.

Figure 6 – **Indirect Immunofluorescence (IIF) assay using *L. interrogans* serovar Copenhageni strain Fiocruz L1–130.** The pAb anti-ErpY-like protein was used in the IIF assay to detect the ErpY-like native protein in whole *Leptospira* cells. Panels A and B: *L. interrogans* incubated with pAb against rErpY-like protein. Panels C and D*: L. interrogans* incubated with mAb against rLipL32 protein. The left panels represent reactions with FITC fluorochrome while the right panels represent fluorescence of Hoechst 33258. Visualization was performed on an Olympus BX51

1329 fluorescence microscope and photographed with a digital camera Olympus BP72.
1330 Bars represent 10 μm.
1331

Figures

Fig 1:



1337 Fig 2:
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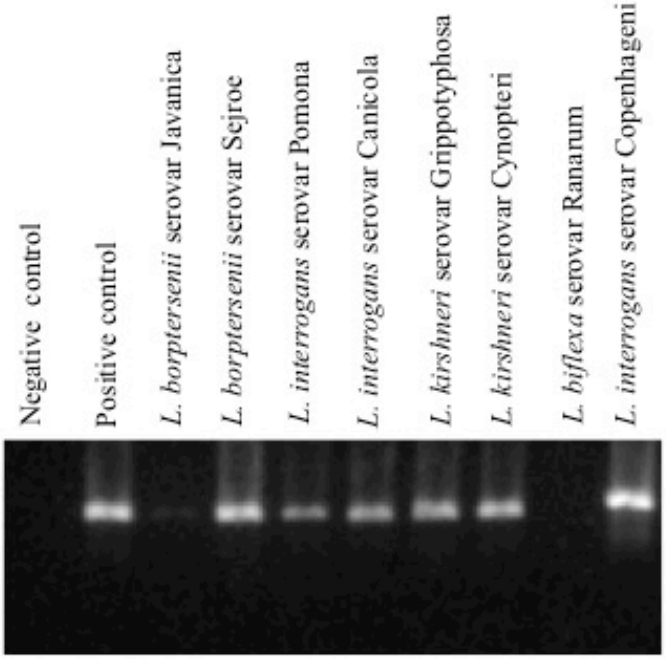

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1340 Fig 3:
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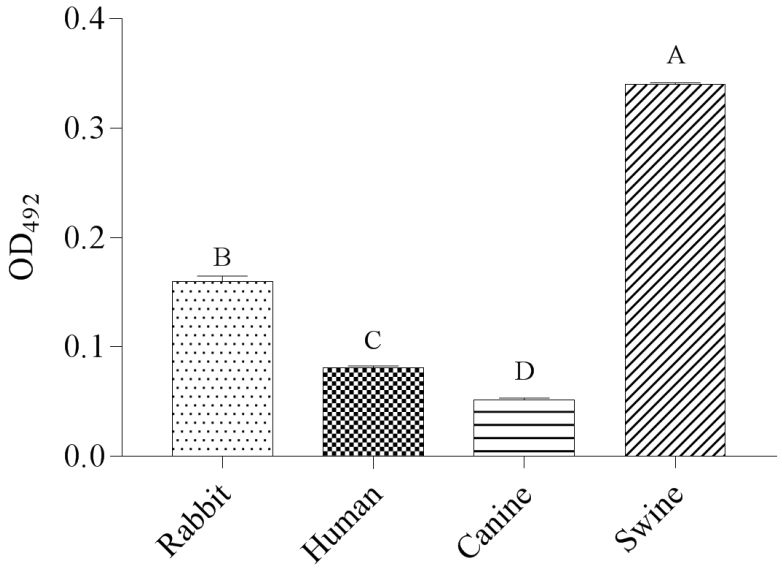

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1344 Fig 4:
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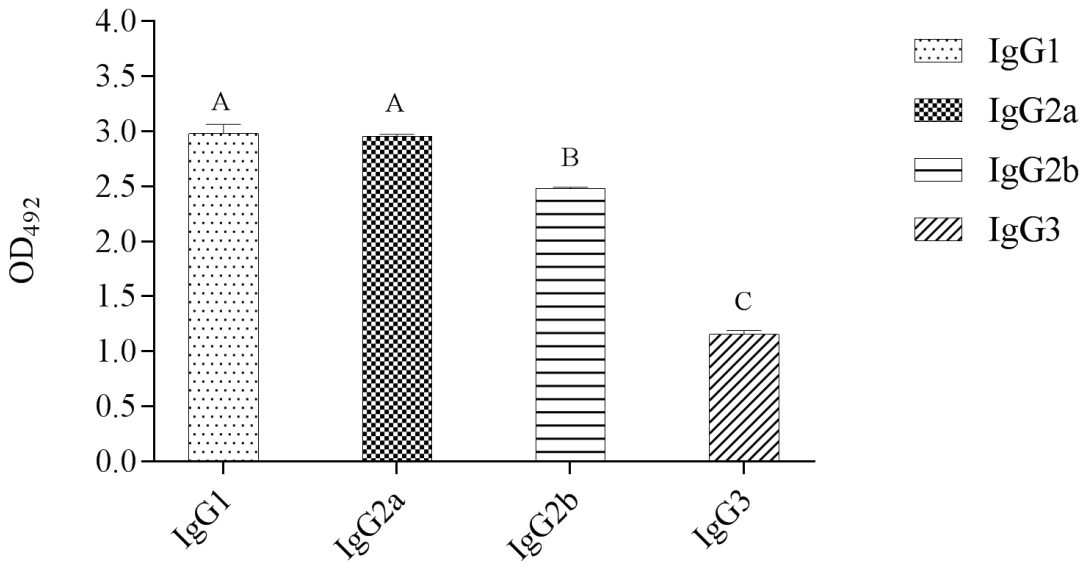

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1348 Fig 5:
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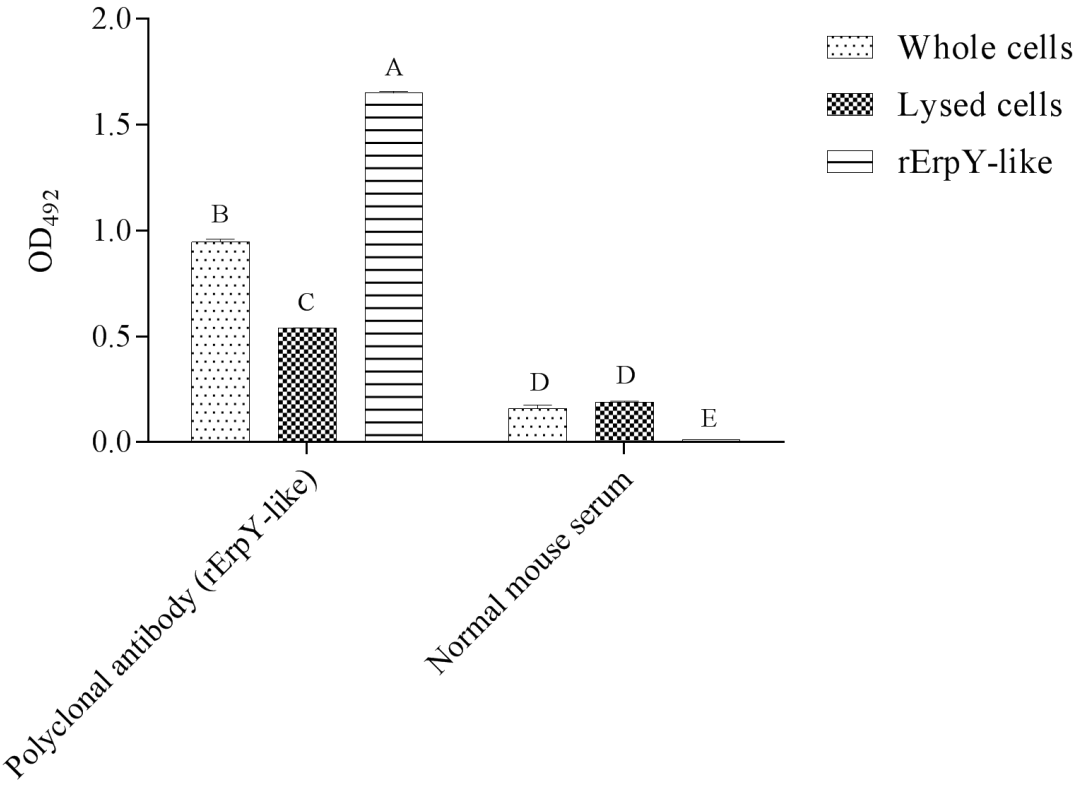

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Fig 6:

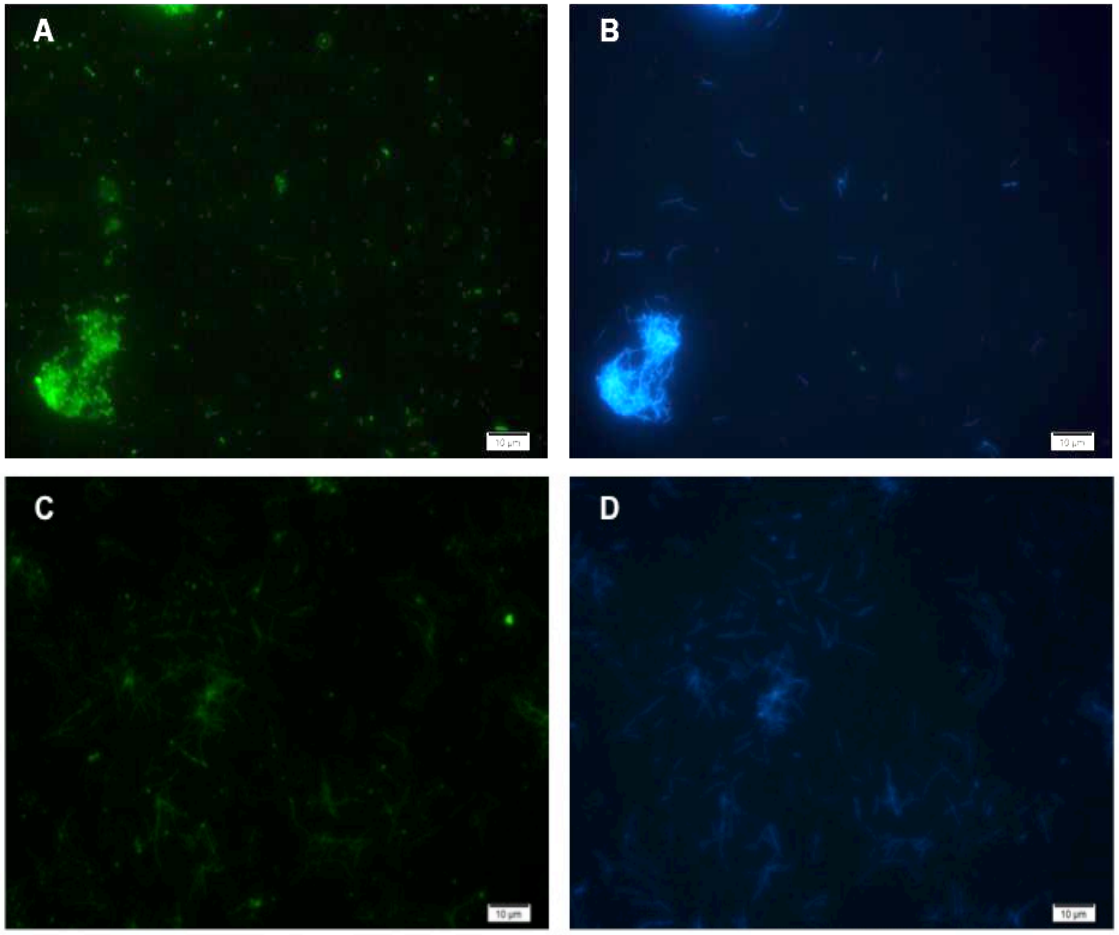

Table 1 – Comparison of the structural metric obtained for the ErpY-like protein from the different web servers, Raptor X, and I-Tasser.

	SolvX	Ramachandran Plot*			Dfire	QMean
		Favored	Allowed	Outlier		
Raptor X	-50,5	155 (98,7%)	1(0,6%)	1(0,6%)	-208,25	-1,52
I-Tasser	10,9	115 (73,2%)	27(17,2%)	15(9,6%)	-287,23	-2,58

* Ramachandram analysis includes the number of amino-acid residues in favored, allowed and outlier positions.

3.3 Manuscrito 3 – The use of ErpY-like recombinant protein from *Leptospira interrogans* in the development of an immunodiagnostic test for swine leptospirosis

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The use of ErpY-like recombinant protein from *Leptospira interrogans* in the development of an immunodiagnostic test for swine leptospirosis.

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Abstract

Swine leptospirosis poses a major problem in the agricultural sector. The gold standard for serodiagnosis of leptospirosis is Microscopic Agglutination Test (MAT). However, the limitations of this technique make the search for alternative diagnostic methods inevitable. In the present study, ErpY-like recombinant protein (rErpY-like), produced in *Escherichia coli* and used as antigen in indirect enzyme-linked immunosorbent assay (ELISA), was evaluated for its efficacy as a novel diagnostic tool for swine leptospirosis. For the study, 72 samples of swine sera characterized by microscopic agglutination test (MAT), were evaluated by indirect ELISA. The sensitivity, specificity and accuracy values obtained from the analysis were 96.8%, 100%, and 99%, respectively, thereby suggesting that rErpY-like ELISA being a sensitive and specific method for antibodies detection in swine populations could be used as an alternative for diagnosis of swine leptospirosis.

Keywords: rErpY-like, diagnosis, indirect ELISA, swine leptospirosis.

1. Introduction

Leptospirosis is a zoonotic disease transmitted to humans and animals by spirochete bacterium of the genus *Leptospira* (Faine et al., 1999). Out of 300 serovars of *Leptospira* known across 30 species, nearly 270 are pathogenic (Picardeau, 2017; Thibeaux et al., 2018). Animals and humans can get infected directly through urine of infected carriers or indirectly through water or soil contaminated with urine from infected carriers (Faine et al., 1999; Jorge et al., 2018). Swine are reservoirs of leptospires, and the *Leptospira* serovars most commonly found in swine are Pomona, Icterohaemorrhagiae, Canicola, Grippotyphosa (Soto et al., 2007; Santos et al., 2011).

Leptospirosis is a major public health concern, besides causing economic losses to agricultural sector (Faine et al., 1999; Levett, 2001; Rocha et al., 2017). Usually, this disease manifests in the form of fever, renal and hepatic insufficiency, pulmonary problems, and reproductive failure (Adler, de la Pena, 2010). In domestic animals, leptospirosis leads to poor reproductive performance, abortions, premature births, and stillbirths resulting in worldwide economic losses to the pork industry (Levett, 2001).

Swine leptospirosis imposes a major economic impact as pigs are important sources of animal protein and by-products. Young pigs suffering from acute leptospirosis rapidly become weak, lose appetite, and may have red eyes, jaundice, and convulsions, resulting in reduced production and quality (Faine et al., 1999; Hartleben et al., 2013). In Brazil, leptospirosis has been reported as one of the major causes of reproductive failure in pigs in several states, mainly the south and southeast regions of the country (Soto et al., 2007). Therefore, this disease has been

included in the National Swine Health Program (PNSS) of Ministry of Agriculture, Livestock and Supply (MAPA). As per the MAPA regulations, all certified herd farms must control leptospirosis and the serological monitoring is obligatory after every six months (MAPA, 2002; Figueredo et al., 2013).

The reference standard technique for leptospirosis diagnosis is the Microscopic Agglutination Test (MAT). This test is performed by incubating the patient serum with various serovars of live leptospires and assessing the degree of agglutination. The results are then read under the dark field microscope. The MAT requires maintaining a collection of living strains, which are used as antigens. MAT is a time consuming and labor intensive test to perform. Besides, it requires periodic verification of each serovar in culture and hence is considered hazardous for laboratory workers (Hartleben et al., 2013; Monte et al., 2011; Picardeau, 2011). Therefore, it is necessary to search for novel diagnostic methods to circumvent the limitations of the standard test. To this intent, sensitive techniques such as enzyme-linked immunosorbent assay (ELISA) using recombinant leptospiral antigens have been developed as alternative diagnostic methods for leptospiral infection (Hartleben et al., 2013; Bomfim et al., 2011; Lizer et al., 2017; Mariya et al., 2006).

In 2009, Eshghi et al. reported the lipoprotein ErpY-like, as a novel potential virulence factor from *L. interrogans*, exhibiting close sequence similarity to the outer membrane lipoprotein ErpY, from *Borrelia burgdorferi*. Furthermore, the upregulation of protein expression during the *in vivo* infection (Eshghi et al., 2009) suggested that ErpY-like protein plays an important role in the infection process and thus could potentially serve as a diagnostic factor and a vaccine antigen. The characterization studies carried out on this protein by our research group have proved it to be an important target in the monitoring of leptospirosis. Also, recent trials conducted in our

laboratory to assess the protective efficacy of ErpY-like recombinant protein (rErpY-like) as a vaccine against *L. interrogans* yielded significant results (Oliveira et al., 2018). In the present study, recombinant ErpY-like (rErpY-like) antigen-based indirect ELISA was developed for the diagnosis of swine leptospirosis.

2. Material and methods

2.1 Bacterial strains and serum samples

E. coli BL21 (DE3) Star host strain (Invitrogen, São Paulo, Brazil) was grown at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37 °C with the addition of 100 μg mL^{-1} ampicillin.

Swine serum samples were obtained from the serum bank of the Laboratory of Molecular Biology of Microorganisms, Federal University of Pelotas, and subsequently evaluated by MAT. A total of 72 samples were collected from animals at a swine farm with reproductive disorders such as abortions, premature births, and stillbirths.

The MAT was carried out according to OIE (2013), with the following reference serovars and strains of leptospires: Australis Ballico, Autumnalis Akiyami A, Bratislava Jez Bratislava, Castellonis Castellon 3, Bataviae Swart, Canicola Hond Utrecht IV, Cynopteri 3522 C, Grippotyphosa Moskva V, Hebdomadis Hebdomadis, Icterohaemorrhagiae RGA, Copenhageni M20, Javanica Veldrat, Batavia 46, Panama CZ 214, Pomona Pomona, Pyrogenes Salinem, Hardjo Hardjoprajitno, Sejroe M 84, Wolffi 3705, Tarassovi Perepeletsin, Patoc Patoc 1. Reciprocal agglutination titers of greater than or equal to 100 were considered positive.

106

107 2.2 Production of rErpY-like protein

108 The ErpY-like gene (≈390 bp), encoding for 125 amino acid protein, was
109 synthesized (GenOne, Rio de Janeiro, Brazil) by using the genomic sequence of *L.*
110 *interrogans* serovar Copenhageni strain Fiocruz L1–130 available in Genbank
111 (Accession Number: AE016823). The e*rpY-like* gene was cloned into a pAE vector,
112 carrying an N-terminal 6×His-tag. The *pAE/erpY-like* plasmid was used to transform
113 *Escherichia coli* BL21 (DE3) Star for rErpY-like production. The protein purification
114 was accomplished by affinity chromatography with Ni^{2+} Sepharose column using the
115 automated liquid chromatography system AKTAprime (GE Healthcare, Chicago, IL),
116 following the manufacturer's instructions. The purified protein was dialyzed in
117 phosphate-buffered saline (PBS) and glycine 0.1%, pH 8.0, decreasing the
118 concentration of urea (starting 5M), in 16 steps over five days, at 4 °C. Western
119 Blotting (WB) with His-tag monoclonal antibody (Mab) (Sigma-Aldrich, St. Louis, MO)
120 was employed to evaluate the rErpY-like purified protein, which was then quantified
121 by the BCA method (BCA Protein Assay Kit - Thermo Scientific, Waltham, MA) and
122 stored at –20 ^{o}C.

123

124 2.3 rErpY-like protein antigenicity

125 The antigenicity of rErpY-like protein was evaluated by Dot Blot (DB) assay
126 using leptospirosis positive sera pool from naturally infected swine (reciprocal MAT
127 titer >1:25,000). A 5µL aliquot of rErpY-like or rLipL32 (used as positive control) was
128 added to a nitrocellulose membrane and dried at 37ºC for 15 min. The membrane
129 was then blocked with PBS-T/casein [0.05% (v/v) Tween 20 and 0.5% (w/v) casein

(PBS-T) in PBS] and incubated with swine sera pool (1:50). The bound antibodies were detected with peroxidase-conjugated goat anti-pig IgG antibody (Sigma-Aldrich, St. Louis, MO), diluted 1:5,000. The membranes were developed with chromogenic substrate solution (6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl, pH 8.0, and 0.03% hydrogen peroxide) for visualization of protein.

2.4 rErpY-like indirect ELISA

Polystyrene ELISA microtiter plates (Nunc, Polysorp - Thermo Scientific, Waltham, MA) were coated with 50 ng of rErpY-like protein per well (determined by previously checkerboard analysis), diluted in carbonate-bicarbonate buffer pH 9.6, for 16 h at 4 ^{o}C. The plates were blocked with a solution of PBS-T/casein [0.05% (v/v) Tween 20 and 0.5% (w/v) casein in PBS], followed by addition of both positive and negative leptospirosis swine sera (diluted 1:50 in PBS-T/casein). Then, goat anti-pig IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO), diluted 1:6,000 in PBS-T/casein was added for sera antibodies detection. The presence of the antigen-antibody complex was revealed by adding a substrate solution containing *o*-phenylenediamine (OPD) (0.4 mg/mL in 0.1 M citrate buffer, pH 5.0) and 0.03% hydrogen peroxide. The reaction was stopped by addition of 0.1 M sulfuric acid and the absorbance was read at 492 nm using VICTOR^{TM} X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). All assay steps were carried out in a total volume of 100 µL/well at 37 °C for 1 h and the plates were washed thrice with PBS-T/casein. The swine sera that tested positive/negative in the MAT for leptospirosis were used as controls.

2.5 Statistical analysis

The results pertaining to specificity, sensitivity, accuracy and a cut-off value of the ELISA test, obtained from 72 serum samples, were evaluated using ROC (*Receiver Operating Characteristic*) curves. The ROC curve analysis was performed with the MedCalc statistical software (version 10.3.0). Test results so obtained were then compared with the standard MAT test.

3. Results

3.1 Production of rErpY-like protein and antigenicity testing

The insoluble rErpY-like protein, nearly 13 kDa in size, was successfully expressed in *E. coli* cells. The purification process yielded approximately 25 mg/L of rErpY-like protein. Western Blot analysis using His-tag mAb (Sigma-Aldrich, St. Louis, MO) (Fig. 1 A) confirmed the protein expression. The capacity of the rErpY-like protein to be recognized by the antibodies, present in leptospirosis positive sera pool obtained from naturally infected swine, was evaluated by Dot Blot assay. The results were positive and are shown in Fig. 1 B.

3.2 rErpY-like ELISA

The 72 swine sera (31 positive and 41 negative), previously characterized by MAT, were evaluated by indirect ELISA. The rErpY-like ELISA was characterized, and the results were correlated with MAT using ROC analysis. All negative sera had the same result as MAT standard, concording with the established cut-off values (Fig. 2).

177

178 3.3 Specificity, sensitivity, accuracy and cut-off

179 The rErpY-like ELISA showed a cut-off value of >0.031 with 96.8% sensitivity
180 and 100% specificity (Fig. 2). The area under the ROC curve indicating the accuracy
181 of immunoassay was found to be 0.990 (99%) (Fig. 3).

182

183 **4. Discussion**

184 Leptospirosis is one of the most important bacterial diseases of worldwide
185 occurrence with a great economic and public health importance owing to its zoonotic
186 nature (Faine et al., 1999; Figueredo et al., 2013). Swine farming plays an enormous
187 role in the national economic scenario. Careful management of farm activities and
188 high level of hygiene are immensely important for the maintenance of healthy
189 animals (Santos et al., 2011; Figueredo et al., 2013).

190 The standard diagnostic for leptospirosis is MAT. However, it is considered
191 unfavorable, mainly because of its time consuming and labor intensive technique as
192 well as the subjectivity of results (Picardeau, 2013). Keeping in view the drawbacks
193 of the standard technique, sensitive methods such as ELISA, using recombinant
194 proteins, have been developed for the diagnosis of leptospirosis in different animal
195 species. ELISA has the advantages of analyzing several samples simultaneously,
196 making the diagnosis faster, besides using recombinant proteins as an antigen which
197 is easy to produce and maintain (Hartleben et al., 2013). rLipL32 (Bomfim et al.,
198 2005), rLipL41 (Mariya et al., 2006), and LigB (Deneke et al., 2014; Nagalingam et
199 al., 2015) are the antigens that are used for serodiagnosis of bovine leptospirosis;
200 LigA for equine leptospirosis (Yan et al., 2013); and a *Leptospira* OMP (Outer

membrane protein) antigen, rLipL21, rLoa22 and rLipL32 for canine leptospirosis (Sathiyamoorthy et al., 2017; Ye et al., 2014). Till now, only two studies have evaluated the efficacy of rLipL32 antigen in indirect ELISA for diagnosis of swine leptospirosis (Hartleben et al., 2013; Monte et al., 2014).

The rErpY-like protein expression was found to be efficient in an *E. coli* based expression system. *E. coli* is routinely used for the production of recombinant proteins for a variety of purposes ranging from structural studies to the development of vaccines (Dellagostin et al., 2011; Fernandes et al., 2017; Gopal, Kumar, 2013).

In silico analysis of the rErpY-like protein carried out by our group evidently illustrated its presence in the serovars most commonly found in swine, viz., Pomona, Icterohaemorrhagiae, Canicola and Grippotyphosa, besides being present in other pathogenic serovars. The observation was consistent with the results of PCR performed on the DNA from these serovars (data not published).

Considering the diagnostic difficulties and the paucity of studies aiming to improve the diagnosis of swine leptospirosis, in the present study, a serological test was developed for the detection of anti-rErpY-like antibodies present in swine serum. ELISA test, although it is an indirect diagnosis test and it is not capable of determining the infective serogroup, as the MAT, offers numerous advantages over the standard test, as it is a quick and easy test which facilitates the analysis of a large number of samples simultaneously. Hartleben et al. (2013) and Monte et al. (2014) reported the development of indirect ELISA for the diagnosis of swine leptospirosis, using rLipL32 antigen. The sensitivity of 100% was reported in both the studies, whereas the specificity of 85.1% and 91.48%, and accuracy of 91.86% and 95.34%, respectively were recorded. The results obtained in the present study

project a higher accuracy and specificity to rErpY-like ELISA, i.e., 99% and 100%, respectively, as compared to that obtained for the same species. The sensitivity values were however slightly lower, i.e., 96.8%. Sensitivity is the ability to identify the truly positives, while specificity indicates truly negatives. For an optimal diagnostic test, high sensitivity and specificity values are expected. In this way, the rErpY-like ELISA can also be used as a screening test to select the samples quickly that will be used for MAT.

The results of indirect ELISA with rErpY-like protein, described in the present study, could aid the evaluation of the disease on a large scale in pigs. Furthermore, the high sensitivity and specificity values obtained suggest that this technique could be effectively used in seroepidemiological investigations in swine populations. However, further studies should be promoted with a larger number of samples and with sera from other animal species to assess the full-scale benefits of this diagnostic technique.

5. Conclusion

The indirect ELISA with rErpY-like was found to be desirably sensitive and specific. Therefore, it could be used as an alternative for the diagnosis of swine leptospirosis, besides being used as a screening test before performing MAT. Thus, it could serve as a reliable tool for antibody detection in swine populations.

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250

251 **Conflict of Interest:** The authors declare that they have no conflict of interest.

252

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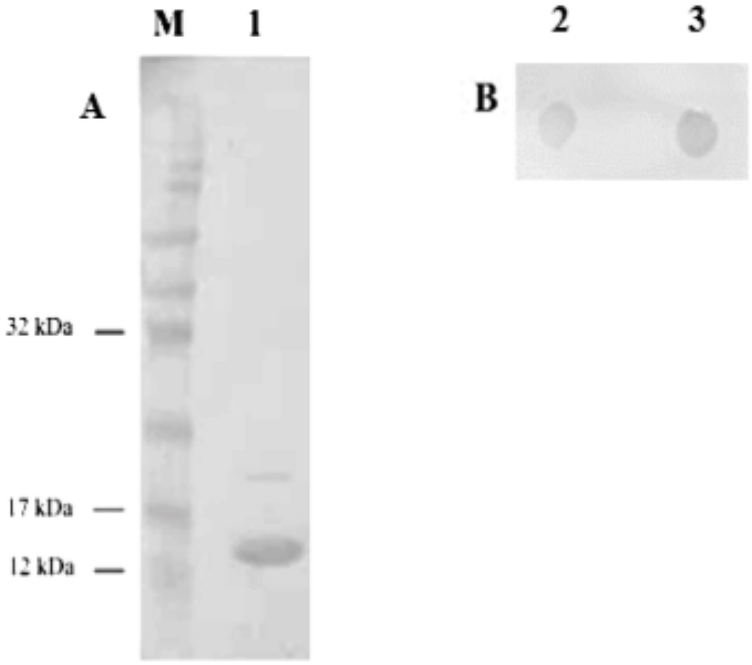
Figure Legends

Figure 1 – **Western Blotting (WB) and Dot Blotting (DB) of rErpY-like protein. (A)** Characterization of purified rErpY-like protein with Mab anti–6×His. M, protein molecular mass markers Rainbow™ (GE Healthcare, USA) (kDa); lane 1, purified rErpY-like protein. **(B)** Antigenicity of rErpY-like using a pool of swine sera positive for leptospirosis. Lane 2, rErpY-like protein and lane 3, rLipL32 protein (positive control).

Figure 2 – **Indirect ELISA performed with rErpY-like protein as antigen.** The protein was tested with 72 swine sera, 31 positive and 41 negative to leptospirosis, as decided by the *Receiver Operating Characteristic* (ROC) curve.

Figure 3 – ***Receiver operating characteristics*** **(ROC) curve for rErpY-like ELISA with the swine sera.** The area under the curve is 0.990 (99% with 95% confidence interval).

364 **Figure 1**



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367 **Figure 2**

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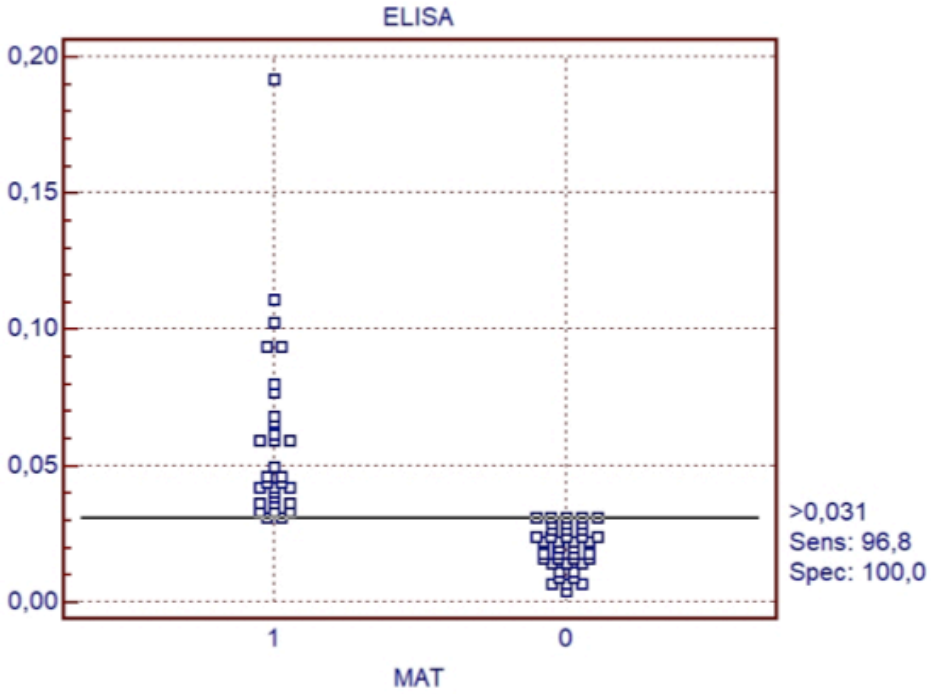

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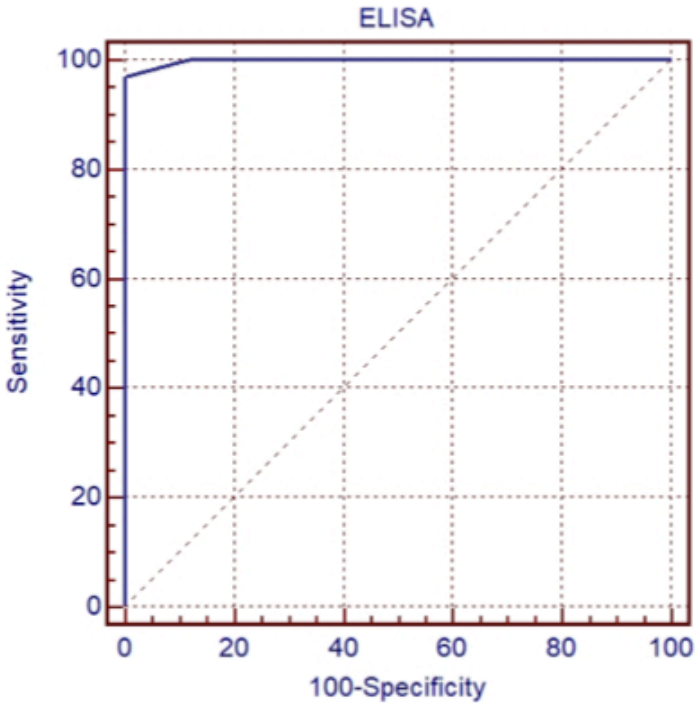
372 **Figure 3**



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3.3 Patente – Utilização de ErpY-like como imunobiológico no controle da leptospirose

Este documento foi enviado para a Coordenação de Inovação Tecnológica (CIT) da UFPel, para solicitação de depósito de patente junto ao Instituto Nacional da Propriedade Industrial (INPI) – Número do processo: BR 10 2016 018203 4

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RESUMO

UTILIZAÇÃO DE ErpY-like COMO IMUNOBIOLÓGICO NO CONTROLE DA LEPTOSPIROSE

A patente refere-se ao isolamento de uma molécula de DNA, originalmente derivada de *L. interrogans*. Sua sequência codifica para a proteína ErpY-like (LIC11966), reportada como uma proteína que possui sequência muito similar a fatores de virulência encontrados em outros patógenos. Além disso, foi demostrada a expressão desta proteína durante a infecção *in vivo*, o que a torna alvo promissor na terapêutica contra a infecção, quer seja de forma preventiva como uma vacina ou componente vacinal, na formulação de kits de diagnóstico da leptospirose, ou ainda na produção de ferramentas como anticorpos monoclonais, a serem utilizados no tratamento da infecção. De acordo com a presente invenção a proteína ErpY-like, bem como a sequência que a codifica, poderão ser usadas no diagnóstico, tratamento e prevenção da infecção por cepas patogênicas de *Leptospira* spp., agente etiológico da leptospirose – doença de importância global afetando a saúde humana e animal.

RELATÓRIO

UTILIZAÇÃO DE ErpY-like COMO IMUNOBIOLÓGICO NO CONTROLE DA LEPTOSPIROSE

FUNDAMENTOS DA INVENÇÃO

CAMPO DA INVENÇÃO

[001] A presente invenção, enquadrada internacionalmente nas classificações de patentes A61K 39/02, C12N 15/09, C12N 15/31 e G01N 33/53, refere-se a uma sequência de DNA (ácido desoxirribonucléico) presente no genoma da bactéria *Leptospira* spp. que codifica a proteína ErpY-like. As invenções referem-se à utilização desta proteína como imunobiológico, seja como produto, utilizando a proteína ErpY-like em formulações vacinais, insumo para produção de anticorpos monoclonais e policlonais, desenvolvimento de testes diagnósticos ou novas alternativas de tratamento para leptospirose, visando, dessa forma, a prevenção e/ou terapia da infecção por *Leptospira* spp. em humanos e animais.

DESCRIÇÃO DO ESTADO DA TÉCNICA

[002] A leptospirose é uma enfermidade classificada como uma doença tropical negligenciada - que são doenças causadas por agentes infecciosos ou parasitas, consideradas endêmicas em populações de baixa renda, apresentam indicadores inaceitáveis e investimentos reduzidos em pesquisas, produção de medicamentos e em seu controle. Esta doença pode acometer uma ampla variedade de animais domésticos, de companhia e de interesse econômico, incluindo cães, bovinos e suínos, além de animais silvestres e o homem **(BHARTI, A. R., NALLY, J. E., RICALDI, J. N., et al. Leptospirosis: a zoonotic disease of**

global importance. Lancet Infectious Diseases, v.3, n.12, p.757-771, 2003; FAINE, S., ADLER, B., BOLIN, C. et al. *Leptospira* and Leptospirosis. MedSci, Melbourne, 2nd ed, p.272, 1999; MWACHUI, M.A., CRUMP, L., HARTSKEERL, R. et al. Environmental and Behavioural Determinants of Leptospirosis Transmission: A Systematic Review. *PLOS Neglected Tropical Diseases*, v.9, n.9, 2015; VINETZ, J. M. Leptospirosis. Current Opinion in Infectious Diseases, v.14, n.5, p.527-538, 2001).

[003] É uma enfermidade de origem bacteriana, causada por *Leptospira* spp. patogênicas, relacionada principalmente à condições precárias de saneamento, e assim, é mais frequente em países subdesenvolvidos **(BHARADWAJ, R. Leptospirosis - a reemerging disease? Indian Journal of Medical Research. v.120, n.3, p.136-138, 2004).** Possui ampla distribuição mundial e maior ocorrência em regiões tropicais e subtropicais, podendo também estar associada ainda a atividades recreacionais, esportivas ou a desastres naturais **(DESAI, S., VAN TREECK, U., LIERZ, M., et al. Resurgence of field fever in a temperate country: an epidemic of leptospirosis among seasonal strawberry harvesters in Germany in 2007. Clinical Infectious Diseases, v.48, n.6, p.691-697, 2009).**

[004] Os roedores são os principais hospedeiros de leptospiras, sendo os pertencentes ao gênero *Rattus* as fontes mais importantes de infecção humana no ambiente urbano. A espécie *Rattus norvegicus* considerada o principal reservatório de *Leptospira*, especialmente do sorogrupo Icterohaemorrhagiae, que é o responsável pelo maior número de casos de leptospirose humana no Brasil e na América Latina **(ATHANAZIO, D. A., SILVA, E. F., SANTOS, C. S., et al. *Rattus norvegicus* as a model for persistent renal colonization by pathogenic *Leptospira interrogans*. Acta Tropica, v.105, n.2, p.176-180, 2008;**

[005] Em humanos, mais de um milhão de casos de leptospirose são relatados a cada ano, e estima-se que cerca de 10.000 casos da forma severa precisem de hospitalização

anualmente em todo o mundo **(RAJAPAKSE, S., RODRIGO, C., HANDUNNETTI, S. M., et al. Current immunological and molecular tools for leptospirosis: diagnostics, vaccine design, and biomarkers for predicting severity. Annals of Clinical Microbiology and Antimicrobials. V.14, n.2, p.2-8, 2015).** Em sua forma aguda, a leptospirose pode desencadear uma série de sinais clínicos e afetar múltiplos órgãos, incluindo o fígado (icterícia), rins (nefrite), pulmões (hemorragia pulmonar) e cérebro (meningite), com taxas de mortalidade em humanos de 10-15%, associadas à doença de *Weil*, chegando a 70%, nos casos de síndrome hemorrágica pulmonar grave **(GOUVEIA, E. L, METCALFE, J., DE CARVALHO, A. L., et al. Leptospirosis-associated Severe Pulmonary Hemorrhagic Syndrome, Salvador, Brazil. Emerging Infectious Diseases, v.14, n.3, p.505-508, 2008; SEGURA, E. R., GANOZA, C. A., CAMPOS, K., et al. Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. Clinical Infectious Diseases, v.40, n.3, p.343-51, 2005).** Nestes casos graves, mesmo com estratégias de intervenção agressivas, as taxas de mortalidade permanecem altas.

[006] O diagnóstico da leptospirose é realizado através da análise da sintomatologia clínica e de diferentes testes estudados, que são divididos, basicamente, em: métodos de detecção do microrganismo em culturas ou amostras biológicas; métodos imunológicos (sorológicos); e, métodos genômicos (**HARTLEBEN, C.P., LEAL, F.M., MONTE, L.G., et al. Serological analysis by enzyme-linked immunosorbent assay using recombinant antigen LipL32 for the diagnosis of swine leptospirosis. Current Microbiology. v.66, n.2, p.106-109, 2013; JORGE, S., MONTE, L. G., DE OLIVEIRA, N.R., et al. Phenotypic and molecular characterization of *Leptospira interrogans* isolated from canis familiaris in southern brazil. Current Microbiology 4:496-500, 2015**).

[007] Dentre os métodos sorológicos utilizados, a soroaglutinação microscópica (MAT), que utiliza cepas de leptospiras vivas para a reação com soros de humanos e animais, é considerada o "padrão ouro" para o diagnóstico da doença (**OIE. Leptospirosis. In: Manual of diagnostic tests and vaccines for terrestrial animals. World Organization for Animal Health, Paris, 2014**). Entretanto, a MAT apresenta baixa sensibilidade na fase aguda da doença, quando os anticorpos são difíceis de detectar e há maior quantidade de IgM, o qual não é diferenciado do IgG (mais presente nas demais fases da doença) por este método (**MCBRIDE, A. J. A., ATHANAZIO, D. A., REIS, M. G., et al. Leptospirosis. Current Opinion in Infectious Diseases, v.18, n.5, p.376-386, 2005**). Outra falha apresentada pela MAT é o alto grau de reações cruzadas entre diferentes sorovares.

[008] Devido às dificuldades encontradas para o controle da leptospirose, a busca por métodos alternativos de diagnóstico torna-se cada vez mais necessária. Nos últimos anos tem havido um grande esforço no desenvolvimento de testes diagnósticos mais sensíveis, como o Ensaio imunoadsorvente ligado à enzima (*Enzyme-linked immunosorbent assay* – ELISA) e outros ensaios imunoquímicos utilizando antígenos recombinantes de *Leptospira,* que veem sendo desenvolvidos como métodos alternativos para o diagnóstico da leptospirose (**BOMFIM, M. R., KO, A., & KOURY, M. C. Evaluation of the recombinant LipL32 in enzyme-linked immunosorbent assay for the serodiagnosis of bovine leptospirosis. Veterinary Microbiology, v.1, n.2, p.89-94, 2005; MARIYA, R., CHAUDHARY, P., KUMAR, A. A., et al. Evaluation of a recombinant LipL41 antigen of *Leptospira interrogans* serovar canicola in ELISA for serodiagnosis of bovine leptospirosis. Comparative Immunology, Microbiology & Infectious Diseases, v.5, n.6, p.269-277, 2006**).

[009] Como em outras espiroquetas, o genoma da *Leptospira* spp. codifica para mais lipoproteínas que outras bactérias, contendo aproximadamente 145 genes que codificam para prováveis lipoproteínas e OMPs (**YANG, H. L., ZHU, Y. Z., QIN, J. H., et al. In silico and microarray-based genomic approaches to identifying potential vaccine candidates against *Leptospira interrogans*. *BMC Genomics*, v.16, n.7, p.293, 2006**). Existem diversas proteínas, presentes na superfície das leptospiras, que tiveram sua localização comprovada. Dentre essas, se destacam: as Ligs, Loa22, LipL32, OmpL1, LenA, LenD, e as OMPs, OmpL36, OmpL37, OmpL47 e OmpL54 (**KO, A. I., GOARANT, C., PICARDEAU, M. *Leptospira*: the dawn of the molecular genetics era for an emerging zoonotic pathogen. Nature Reviews Microbiology, v.7, n.10, p.736-747, 2009).**

[010] Proteínas são os principais alvos da atual pesquisa para desenvolvimento de vacinas contra a leptospirose. Os esforços para desenvolver vacinas recombinantes contra esta doença se concentraram em proteínas conservadas de membrana externa (*Outer Membrane Proteins* - OMPs), fatores de mobilidade bacteriana, LPS e fatores de virulência que representam alvos potenciais para os mecanismos de defesa imunológicos (**DELLAGOSTIN, O. A, GRASSMANN, A. A., HARTWIG, D. D., et al. Recombinant vaccines against Leptospirosis. Human Vaccines, v.7, n.11, p.1215-24, 2011; OLIVEIRA, T. L., GRASSMANN, A. A., SCHUCH, R. A., et al. Evaluation of the *Leptospira interrogans* outer membrane protein OmpL37 as a vaccine candidate. PLoS One, v.10, n.11, 2015**). Várias lipoproteínas de leptospiras, como LipL41, LipL32, LIC10325, LIC13059 e LIC11058, mostraram estimular uma resposta imune protetora contra a leptospirose, indicando o seu potencial como candidatos a vacinas (**HAAKE, D. A., MAZEL, M. K., MCCOY, A. M., et al. Leptospiral outer membrane proteins OmpL1 and LipL41 exhibit synergistic immunoprotection. Infection and Immunity, v.67, n.12, p.6572-6582, 1999; HARTWIG, D. D, FORSTER, K. M, OLIVEIRA, T. L., et**

al. A prime-boost strategy using the novel vaccine candidate, LemA, protects hamsters against leptospirosis. Clinical and Vaccine Immunology, v.20, n.5, p.747-52, 2013.

[011] As primeiras vacinas utilizadas para o controle da leptospirose humana e animal foram baseadas em células inteiras de *Leptospira* inativadas com formalina (bacterinas). Vários estudos relatam que estas vacinas têm eficácia por um curto período, requerendo reforços periódicos, além de serem sorovar-específicas (sua proteção é dependente dos sorovares isolados localmente) **(DELLAGOSTIN, O. A, GRASSMANN, A. A., HARTWIG, D. D., et al. Recombinant vaccines against Leptospirosis. Human Vaccines, v.7, n.11, p.1215-24, 2011; OLIVEIRA, T. L., GRASSMANN, A. A., SCHUCH, R. A., et al. Evaluation of the *Leptospira interrogans* outer membrane protein OmpL37 as a vaccine candidate. PLoS One, v.10, n.11, 2015; RAJAPAKSE, S., RODRIGO, C., HANDUNNETTI, S. M., et al. Current immunological and molecular tools for leptospirosis: diagnostics, vaccine design, and biomarkers for predicting severity. Annals of Clinical Microbiology and Antimicrobials. V.14, n.2, p.2-8, 2015).** Portanto, há a necessidade de avaliar novos alvos, bem como diferentes estratégias vacinais, adjuvantes e/ou veículos para estas vacinas.

[012] Dentre eles, tem sido avaliado o potencial de uma série de LICs, presentes em leptospiras patogênicas (**HARTWIG, D. D., SEIXAS, F. K., CERQUEIRA, G.M., et al. Characterization of the immunogenic and antigenic potential of putative lipoproteins from *Leptospira interrogans*. Current Microbiology, v.62, n.4, p.1337-1341, 2011; HARTWIG, D. D., FORSTER, K. M., OLIVEIRA, T. L., et al. A prime-boost strategy using the novel vaccine candidate, LemA, protects hamsters against leptospirosis. Clinical and Vaccine Immunology, v.20, n.5, p.747-52, 2013**), como a lipoproteína ErpY-like, ainda não estudada.

SUMÁRIO DA INVENÇÃO

[013] Neste âmbito, os eventos descritos a seguir, apresentam a invenção baseada em uma proteína hipotética de *Leptospira* spp., denominada ErpY-like (LIC11966), visando explorar seu potencial terapêutico para uso como vacina, diagnóstico e/ou tratamento da leptospirose humana e animal.

[014] O primeiro objetivo da presente invenção trata de uma sequência de DNA específica, de *Leptospira* spp. e o respectivo polipeptídeo por ela codificado. O outro objetivo refere-se ao uso isolado ou combinado deste polipeptídeo, dos anticorpos produzidos experimentalmente contra este polipeptídeo e da sequência de DNA que codifica para ErpY-like, como insumos para tratar, prevenir e/ou diagnosticar a infecção por *Leptospira* spp.

DESCRIÇÃO DETALHADA DO INVENTO

[015] Por conveniência o significado de alguns termos empregados nas especificações estão descritos abaixo.

[016] ErpY-like – é uma lipoproteína hipotética de *Leptospira interrogans*, conservada, cuja massa molecular é de aproximadamente 13 kDa.

[017] *erpY-like* – refere-se à sequência de nucleotídeos, de 414 pb, que codifica para a proteína rErpY-like.

[018] LIC11966 - nomenclatura genômica de *L. interrogans* sorovar Copenhageni, LIC. LIC11966 refere-se à sequência genômica correspondente ao antígeno de interesse desta invenção (ErpY-like).

[019] Na presente invenção a identificação do antígeno foi baseada na análise *in silico* de antígenos candidatos à vacina recombinante contra a leptospirose. A sequência alvo *ErpY-like* referida nesta invenção pode ser inserida como DNA heterólogo em vetores de expressão para produção do polipeptídeo ErpY-like ou fragmentos do mesmo. A proteína ErpY-like descrita nesta invenção é imunogênica, uma vez que animais experimentalmente imunizados com este polipeptídeo produzem anticorpos específicos contra a mesma, representando potencial alvo vacinal.

[020] A sequência LIC11966 (SEQ ID N°:1) apresenta trezentos e setenta e oito (378) nucleotídeos que codificam para cento e vinte e cinco (125) aminoácidos, os quais originam uma proteína com massa molecular de cerca de 13 kDa, denominada ErpY-like.

[021] Com base na sequência codificadora do antígeno recombinante rErpY-like (SEQ ID N°:2), produzido a partir da clonagem parcial de LIC11966 (SEQ ID N°1), seguiram-se os processos de clonagem, expressão, purificação, avaliação do potencial deste antígeno como imunobiológico e caracterização do anticorpo policlonal produzido contra a proteína ErpY-like, segundo os procedimentos a seguir:

[022] **Passo 1:** DNA genômico de *L. interrogans*, *L. borgpetersenii*, *L. kirshneri, L. santarosai e L. biflexa* foram extraídos usando GFX Genomic DNA purification kit (GE Healthcare), segundo instruções do fabricante para bactérias Gram-negativas. O DNA extraído foi analisado através de eletroforese em tampão 1x tris-borato-EDTA (TBE) (89 mM de Tris, 89 mM de ácido bórico e 2 mM de EDTA), utilizando gel de agarose 1% (6 g de agarose, 60 mL de 1x TBE), a uma voltagem de 100 V, e este corado com GelRed™ (Invitrogen), com intuito de avaliar sua qualidade e integridade, sendo posteriormente

estocado à -20°C. Os fragmentos de DNA foram amplificados utilizando oligonucleotídeos iniciadores desenhados de acordo com o genoma de *L. interrogans* sorovar Copenhageni (GenBank: AE016823), originalmente isolada de um paciente com leptospirose severa: e*rpY-like* F (SEQ ID N°4): 5′-ACTCTCGAGGAAATCATGAACGCT-3′ e e*rpY-like* R (SEQ ID N°5): 5′-ACGGAATTCTTGAGAAGCGTATTC-3′. As reações de PCR foram realizadas de acordo com as seguintes condições: 94 °C por 5 min, seguido de 35 ciclos de 94 °C por 1 min, 50 °C por 1 min e 72 °C por 1 min, com uma extensão final de 72 °C por 7 min. As reações foram realizadas em um volume final de 25 μl utilizando a enzima *Taq* DNA Polimerase (*kit* GoTaq Coloress Master Mix - ProMega) em termociclador Mastercycler (Eppendorf). O produto das amplificações foi visualizado através de eletroforese em gel de agarose 1%, conforme descrito acima. Estes resultados podem ser observados na Figura 1.

[023] **Passo 2:** Os *primers* para a amplificação do gene *erpY-like* (NC_005823.1) foram desenhados a partir da seqüência genômica de *L. interrogans* sorovar Copenhageni L1-130 (FIOCRUZ, BA_GeneBank: AE016823), com o auxílio do *software* VectorNTI 11 (Invitrogen). Para clonagem do gene *erpY-like* no vetor de expressão pAE, foram utilizados os mesmos oligonucleotídeos descritos acima, no passo 1, denominados *erpY-like* F (SEQ ID N°4) e e*rpY-like* R (SEQ ID N°5). Os sítios das enzimas de restrição *Xho*I e *Eco*RI estão destacados. A reação em cadeia da polimerase (PCR) foi empregada para amplificação do gene alvo (*erpY-like*) sob as mesmas condições descritas acima, no Passo 1. O produto da amplificação foi visualizado através de eletroforese em gel de agarose 1% com tampão 1x TBE, a uma voltagem de 100 V utilizando GelRed™ (Invitrogen) como corante.

[024] **Passo 3:** A clonagem de *erpY-like* foi realizada conforme descrito por Sambrook e Russell **(SAMBROOK, J., RUSSELL, D. W. Molecular Cloning: A Laboratory Manual. 3rd edn,**

2001), com algumas modificações. O fragmento amplificado por PCR foi clonado no vetor pAE, que possui as seguintes características: origem de replicação em *Escherichia coli*, sítio de múltipla clonagem, gene de resistência ao antibiótico ampicilina e promotor do fago T7, que é reconhecido pela RNA polimerase de *E. coli*. Além disso, esse vetor permite a expressão das proteínas recombinantes fusionadas a uma cauda de seis resíduos de histidinas em sua porção amino-terminal. Uma representação gráfica deste vetor pode ser observada na Figura 2. O gene amplificado por PCR foi checado em gel de agarose 1% e purificado utilizando o GFX™ PCR DNA and Gel Band Purification Kit (GE Heathcare), seguindo orientações do fabricante. As moléculas (produto da PCR e plasmídeo pAE) foram digeridas com suas respectivas enzimas de restrição (*Eco*RI e *Xho*I) e re-purificados com o mesmo kit. Para a reação de ligação foi utilizada a enzima T4 DNA ligase (Invitrogen) e concentrações eqüimolares de inserto e vetor. A reação de ligação foi mantida à 4 °C por 16-20 h. O produto da ligação foi utilizado para transformar células competentes de *E. coli* TOP10 (capacitância de 25 µF, resistência de 200 Ω e voltagem de 2 kV). As células transformadas foram plaqueadas em ágar Luria-Bertani (LB) (1% de triptona, 0,5% de extrato de levedura, 0,5% de NaCl) acrescido de 100 $\mu g.mL^{-1}$ de ampicilina e incubadas a 37 °C, por 12–20 h. As colônias que cresceram nas placas, oriundas do processo de transformação, foram submetidas a uma triagem rápida com fenol-clorofórmio. Os clones caracterizados como recombinantes nesta triagem, ou seja, que apresentavam o plasmídeo pAE mais o inserto *ErpY-like*, foram selecionados e cultivados em 5 mL de meio LB líquido acrescido de 100 $\mu g.mL^{-1}$ de ampicilina, a 200 rpm de agitação, 37 °C, de 12–20 h. Deste cultivo utilizou-se 3 mL para extração de DNA plasmidial com GFX™ Micro Plasmid Prep Kit (GE Heathcare), e o DNA resultante desta extração foi submetido à PCR (mesmas condições já descritas) e digestão com

as enzimas de restrição utilizadas na clonagem, para checar a presença do inserto. O vetor construído foi chamado de pAE/*erpY-like*.

[025] **Passo 4:** Para expressão da proteína rErpY-like (SEQ ID N°3) fusionada a uma cauda de seis histidinas, o vetor recombinante pAE/*erpY-like* foi utilizado para transformar *E. coli* BL21 Star (DE3) por choque térmico, utilizando 100 µL de $CaCl_2$, uma colônia isolada de *E. coli* BL21 Star DE3 e 2 µL de pAE/*erpY-like* (incubação por 5 min à 0 °C, seguido de 1 min à 42 °C e 1 min à 0 °C). Um clone recombinante foi utilizado para inocular 10 mL de LB contendo 100 $\mu g.mL^{-1}$ de ampicilina e cultivado sob agitação de 200 rpm, por 12-20 h à 37 °C. Esta cultura foi utilizada para inocular 500 mL de LB com 100 $\mu g.mL^{-1}$ de ampicilina, que foi incubado à 37 °C e agitação de 200 rpm até a fase exponencial de crescimento (DO_{600} entre 0,6 e 0,8), quando então a expressão de rErpY-like (ErpY-like recombinante) foi induzida com 1 mM de IPTG (isopropiltio-β-D-galactosideo), durante 3 h. Após este período, a cultura foi fracionada em tubos de 250 mL, centrifugada a 6.000 × *g* por 15 min à 4 °C. O pellet foi suspendido em um tampão de solubilização (8 M de uréia, 0,1 M de Tris-base, 0,3 M de NaCl e 20 mM de imidazole, pH 8,0). As células foram então sonicadas (3 ciclos de 30 s, 20 kHz), centrifugadas (10.000 x *g* por 30 min a 4 °C) e o sobrenadante coletado e filtrado em membrana de 0,8 µM (Millipore). A proteína rErpY-like foi purificada através de cromatografia de afinidade utilizando colunas Ni^2 Sepharose HisTrap, sendo a proteína eluída da coluna com 250 mM de imidazole. A fração contendo a proteína eluída foi identificada através de SDS-PAGE 15%. A Figura 3 representa a purificação da proteína rErpY-like. As alíquotas eluídas contendo a proteína recombinante foram dialisadas de forma lenta, utilizando-se membrana de celulose para diálise (25 mm x 16 mm) (Sigma). A diálise foi realizada à 4 °C contra tampão contendo 100 mM de Tris-base e 300 mM de Nacl em

concentrações decrescentes de uréia. Por fim, a proteína foi dialisada contra tampão fosfato-salino (PBS) (pH 7,2) por 16-18 h à 4 °C e armazenada à –20 °C. A concentração da proteína purificada foi determinada usando BCA Protein Assay Kit (Pierce, USA), com uma curva padrão de albumina sérica bovina (BSA). O rendimento da expressão da proteína rErpY-like foi de 25,5 $mg.L^{-1}$.

[026] **Passo 5:** A proteína recombinante purificada e dialisada foi submetida a um Western Blot**(SAMBROOK, J., RUSSELL, D. W. Molecular Cloning: A Laboratory Manual. 3rd edn, 2001**) com anticorpo monoclonal contra cauda de seis histidinas (MAb anti-6xHis). A proteína rErpY-like purificada foi submetida a um SDS-PAGE 15% e eletrotransferida para membrana de nitroceluse Hybond™ ECL™ (Amersham Biosciences). A membrana foi bloqueada com PBST acrescido de 5% de leite em pó desnatado, à 4 °C, 12–20 h e, após este período, lavadas 3 vezes com PBST. Posteriormente, a membrana foi incubada durante 1 h à temperatura ambiente com MAb anti-6xHis numa diluição de 1:2000. Após 3 lavagens com PBST, foi adicionado o anticorpo secundário anti-IgG de camundongo conjugado com peroxidase (Sigma) na diluição de 1:6000 (contra MAb anti-6xHis). Após 3 lavagens com PBST, a reação foi revelada com diaminobenzidina (DAB) e H_2O_2. Este resultado pode ser observado na Figura 4.

[027] **Passo 6:** Dois camundongos isogênicos BALB/c fêmeas (Biotério Central, Universidade Federal de Pelotas, RS, Brasil) foram inoculados intraperitonealmente com a proteína rErpY-like produzida em *E. coli*. A dose administrada foi de 100 µg de proteína recombinante, acrescido do volume correspondente de adjuvante de Freund completo (dia 0) ou incompleto (dias 14, 21 e 28). O intervalo entre as inoculações foi de 14 dias para a 2ª dose, e de 7 dias para as doses seguintes. Amostras de sangue foram coletadas através de punção do plexo venoso retro-ocular 7 dias após cada

inoculação, centrifugadas à 4.000 *x g* por 10 min, e o soro foi armazenado à –20 °C. Após a última dose, amostras de sangue foram coletadas, centrifugadas e purificadas por cromatografia de afinidade usando colunas CL-4B de sefarose carregadas com proteína A (GE Healthcare), de acordo com as instruções do fabricante. O soro foi titulado através de ELISA indireto utilizando a proteína rErpY-like (200 ng/cavidade) diluída em tampão carbonato bicarbonato, pH 9,8, como antígeno, em placas de poliestireno de 96 cavidades (Polysorp Surface, Nunc), diluições seriadas do anticorpo policlonal (1:100–1:512.000) e diluição 1:6000 do conjugado anti-IgG de camundongo (Sigma). Este soro também foi caracterizado através de Western Blot quanto à sua capacidade de reconhecimento de rErpY-like. Esta técnica foi conduzida com anticorpo policlonal anti-rErpY-like (1:100) produzido em camundongos e conjugado anti-IgG de camundongo (Sigma) (1:6000), e revelado com diaminobenzidina (DAB) e H_2O_2, conforme descrito acima, no passo 5. Este resultado pode ser observado na Figura 5.

[028] **Passo 7:** Placas de poliestireno de 96 cavidades (Polysorp Surface, Nunc) foram sensibilizadas com uma solução de poli-L-lisina 0,1% por 1 h à 37 °C contendo leptospiras inteiras ou lisadas pelo calor. Para tanto, cultivos de sete dias de *Leptospira interrogans* foram lavados uma vez com PBS e suspendidos para uma densidade de 10^8 células/mL. As placas foram bloqueadas com 1% de soro fetal bovino. Posteriormente, o anticorpo policlonal anti- rErpY-like foi adicionado na diluição de 1:100 em PBS por um período de 2 h à temperatura ambiente sob agitação (50 rpm). Após lavagem com PBS-T, foi adicionado o anticorpo anti-camundongo conjugado com peroxidase na diluição de 1:6000 (Sigma Aldrich, USA). Como controles positivo e negativo, foram utilizados anticorpo monoclonal (mAb) anti-rLipL32 e soro normal de camundongo, respectivamente. Da mesma forma, lâminas de microscópio foram cobertas por 1 h à 37 °C com poli-L-lisina diluída o,1%.

Cultivos de sete dias de *Leptospira interrogans* foram lavados com água ultrapura e suspendidos para 10^8 células/mL. Alíquotas de 10 µL desta suspensão foram adicionadas às laminas e incubadas por 1 h à 37 °C. As lâminas foram bloqueadas com 10% de soro fetal bovino, lavadas duas vezes com PBS-T e então, o anticorpo policlonal anti-ErpY-like foi adicionado em uma diluição de 1:100. As laminas foram novamente lavadas com PBS-T e o anticorpo anti-camundongo conjugado a FITC (Invitrogen, USA) foi adicionado em uma diluição de 1:100 e incubado em câmara úmida à 37 °C por 1 h. Após lavagem, foi adicionado o meio de montagem e a reação foi visualizada em microscópio de fluorescência (Olympus BX 51). Como controles positivo e negativo, foram utilizados o anticorpo monoclonal anti-rLipL32 e soro normal de camundongo, respectivamente. O DNA foi corado com Hoechst 33258 a fim de confirmar a presença da bactéria. Os resultados destes ensaios podem ser visualizados nas figuras 6 e 7, respectivamente.

[029] **Passo 8:** O potencial imunoprotetor da proteína rErpY-like como vacina de subunidade recombinante foi avaliado em hamsters. Para tanto, machos de 4–6 semanas de idade foram divididos em 2 grupos de 8 animais cada, com alimento e água *ad libitum*. Os animais foram imunizados com a proteína recombinante rErpY-like (80 µg) ou solução salina acrescidos do adjuvante Addavax™ (1:1) através da via intramuscular. Um grupo de 4 animais foi imunizado com 10^9 células de leptospiras inativadas pelo calor, como controle positivo do desafio. As vacinas foram administradas em duas doses com 14 dias de intervalo entre as doses. Amostras de sangue foram coletadas pelo plexo retro-ocular após administração de colírio anestésico antes de cada imunização e também do desafio, sendo o soro coletado como descrito anteriormente e estocado à –20 °C. Vinte e oito dias após a primeira dose todos os animais foram desafiados intraperitonealmente com uma dose de 10^3 leptospiras, que equivale a 5x DL_{50} de *L. interrogans* serovar

Copenhageni. Os hamsters foram monitorados diariamente para sinais clínicos de leptospirose e eutanasiados quando sinais de doença terminal aparecessem. Os animais sobreviventes até o final do experimento foram eutanasiados no 30° dia pós-desafio.

[030] **Passo 9:** Os hamsters que sobreviveram até 30 dias após o desafio (Figura 8) foram eutanasiados para avaliação da capacidade esterilizante das vacinas, através de cultura a partir de tecido renal. Neste caso, os rins foram macerados e transferidos para 5 mL de meio Ellinghausen-McCullough-Johnson-Harris (EMJH) suplementado com *Leptospira* Enrichment EMJH (Difco, USA), sendo as culturas mantidas à 30 °C. Durante oito semanas as culturas foram observadas através de microscopia de campo escuro, para identificar crescimento de leptospiras.

Breve Descrição das figuras

[031] A figura 1 ilustra a distribuição do gene *erpY-like* (~378 pb) entre sorovares de *Leptospira* spp. Os DNAs genômico de diferentes sorovares de *L. biflexa, L. interrogans*, *L. borgpetersenii*, *L. kirshneri* e *L. santarosai* foram submetidos à PCR com oligonucleotídeos específicos, desenhados de acordo com o genoma de *L. interrogans* sorovar Copenhageni;

[032] A Figura 2 é a representação gráfica do vetor de clonagem e expressão, pAE, utilizado para produção da proteína recombinante ErpY-like em *Escherichia coli*;

[033] A Figura 3 demonstra a produção da proteína rErpY-like (~15,0 kDa), expressa em *E. coli* BL21 (DE3) STAR e purificada por cromatografia de afinidade ao níquel, através de eletroforese em gel de poliacrilamida (SDS-PAGE) 15% corado com Comassie Blue. Coluna 1, Marcador de massa molecular *Full-*

Range Rainbow Molecular Weight Marker (GE Healthcare); Coluna 2, rErpY-like purificada.

[034] A Figura 4 representa um *Western blotting* com anticorpo Monoclonal anti-cauda de poli-histidina conjugado com peroxidase. A reação foi revelada com 3,3-diaminobenzidina e peróxido de hidrogênio (DAB/H_2O_2). Coluna 1, Marcador de massa molecular *Full-Range Rainbow Molecular Weight Marker* (GE Healthcare); Coluna 2, rErpY-like; Coluna 3, rLipL32 (32 kDa) usada como controle positivo; Coluna 4, BSA usada como controle negativo.

[035] A Figura 5 demonstra a caracterização do anticorpo policlonal anti-rErpY-like produzido em camundongos através de *Western blot*. Coluna 1, Marcador de massa molecular *Full-Range Rainbow Molecular Weight Marker* (GE Healthcare); Coluna 2, rErpY-like; Coluna 3, rLipl32 usada como controle negativo.

[036] A Figura 6 representa a reação de ErpY-like na forma nativa e na forma recombinante com o anticorpo policlonal anti-rErpY-like através de ELISA indireto. Como controle positivo, foi utilizado anticorpo monoclonal anti-rLipL32 e como controle negativo foi utilizado soro normal de camundongo. As reações do pAb anti-rErpY-like com as células inteiras de *Leptospira* (barras cinza claras), com as células lisadas de *Leptospira* (barras cinza escuras) e com a proteína rErpY-like (barras pretas) são expressas como a absorbância média num comprimento de onda de 450 nm.

[037] A Figura 7 representa o ensaio de imunofluorescência indireta usando *L. interrogans* cepa Fiocruz L1-130. As imagens A e B referem-se à incubação de *L. interrogans* com o pAb anti-rErpY-like, e indicam a fluorescência de FITC e de Hoestch 33258, respectivamente. As imagens C e D referem-se à incubação de *L. interrogans* com mAb anti-rLipL32 e indicam a

fluorescência de FITC e de Hoestch 33258, respectivamente. As reações foram visualizadas em microscópio de fluorescência Olympus BX51 e fotografadas com câmera digital Olympus BP72. As barras representam 10 µm.

[038] A Figura 8 representa a curva de sobrevivência dos animais vacinados com rErpY-like acrescida do adjuvante Addavax e desfiados com cepa virulenta de *L. interrogans* sorovar Copenhageni (10^3 leptospiras, que equivale a 5x DL_{50}). O teste Wilcoxon Log-rank e o teste de Fischer foram usados para determinar diferenças significativas entre os grupos imunizados e os grupos controles (*P<0,05).

REIVINDICAÇÕES

1) Utilização de ErpY-like como imunobiológico no controle da leptospirose, **caracterizada por** utilizar este antígeno como imunobiológico, seja como produto, onde a proteína ErpY-like será utilizada em formulações vacinais, produção de anticorpos monoclonais e policlonais, desenvolvimento de testes diagnósticos ou novas alternativas de tratamento para leptospirose, visando, dessa forma, a prevenção e/ou terapia da infecção por *Leptospira* spp. em humanos e animais.

2) Utilização de ErpY-like como imunobiológico no controle da leptospirose, **caracterizada por** compreender o uso do polinucleotídeo que codifica para a proteína ErpY-like, para obtenção de um produto ou ferramenta molecular;

3) Utilização de ErpY-like como imunobiológico no controle da leptospirose, **caracterizada por** compreender o processo de obtenção do polipeptídeo ErpY-like ou fragmentos do mesmo pela inserção da sequência alvo *erpY-like* como DNA heterólogo em vetores de expressão em *Escherichia coli* e alternativamente outros organismos procariotos ou eucariotos;

4) Utilização de ErpY-like como imunobiológico no controle da leptospirose, **caracterizada por** compreender formulações com potencial vacinal baseadas no uso individual ou combinado do antígeno ErpY-like de *Leptospira* spp. para induzir resposta imune específica contra espiroquetas patogênicas do gênero *Leptospira*, correspondendo a proteína purificada ou sequências funcionalmente equivalentes, contendo Addavax™ ou alternativamente outro composto adjuvante;

5) Utilização de ErpY-like como imunobiológico no controle da leptospirose, caracterizada por compreender formulações com potencial imunoterápico baseadas em anticorpos gerados contra a proteína ErpY-like de *Leptospira* spp. que correspondam a uma quantidade imunologicamente eficaz de anticorpos que reconhecem peptídeos ou sequências funcionalmente equivalentes de ErpY-like em um veículo farmaceuticamente aceitável visando a neutralização da bactéria durante a infecção;

6) Utilização de ErpY-like como imunobiológico no controle da leptospirose, caracterizada por compreender composições para diagnóstico direto ou para detecção *in vitro* da ocorrência de infecção por *Leptospira* spp. em amostras suspeitas, que contenham um reagente que se liga a componentes específicos do patógeno, que podem ser oligonucleotídeos para a identificação do gene *erpY-like*, ou anticorpos contra a proteína ErpY-like ou sequências funcionalmente equivalentes, e reagentes de detecção desta ligação.

7) Utilização de ErpY-like como imunobiológico no controle da leptospirose, caracterizada por compreender kit para diagnóstico *in vitro* de infecção por *Leptospira* spp. visando a detecção de anticorpos que reconhecem ErpY-like ou suas sequências equivalentes em amostras de organismos possivelmente infectados, contendo um ou mais recipientes que acondicionam individual ou sinergeticamente a proteína ErpY-like, para detecção de anticorpos anti-ErpY-like ou funcionalmente equivalentes ao mesmo produzidos durante a infecção por *Leptospira* spp.

FIGURAS

Figura 1

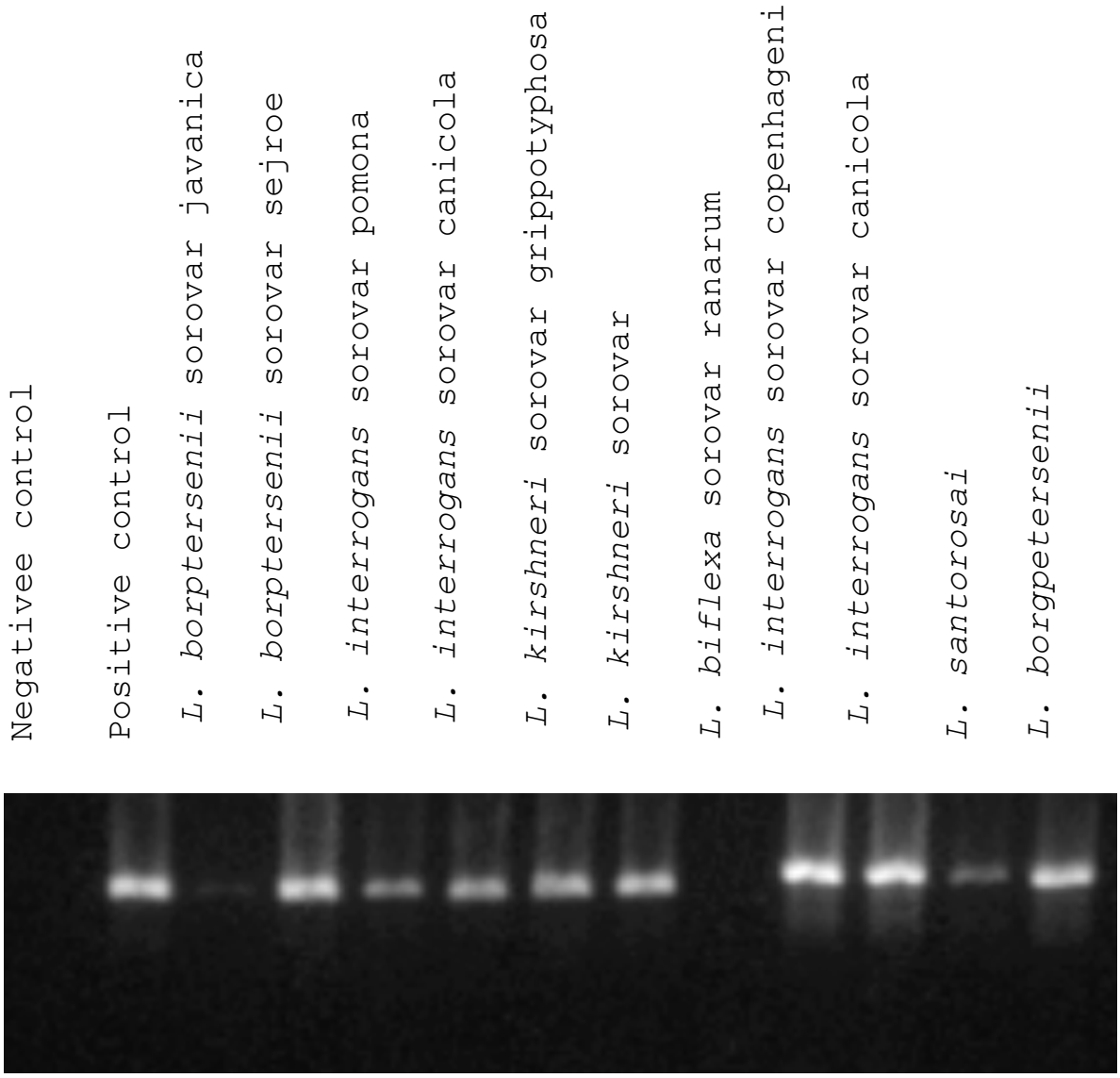
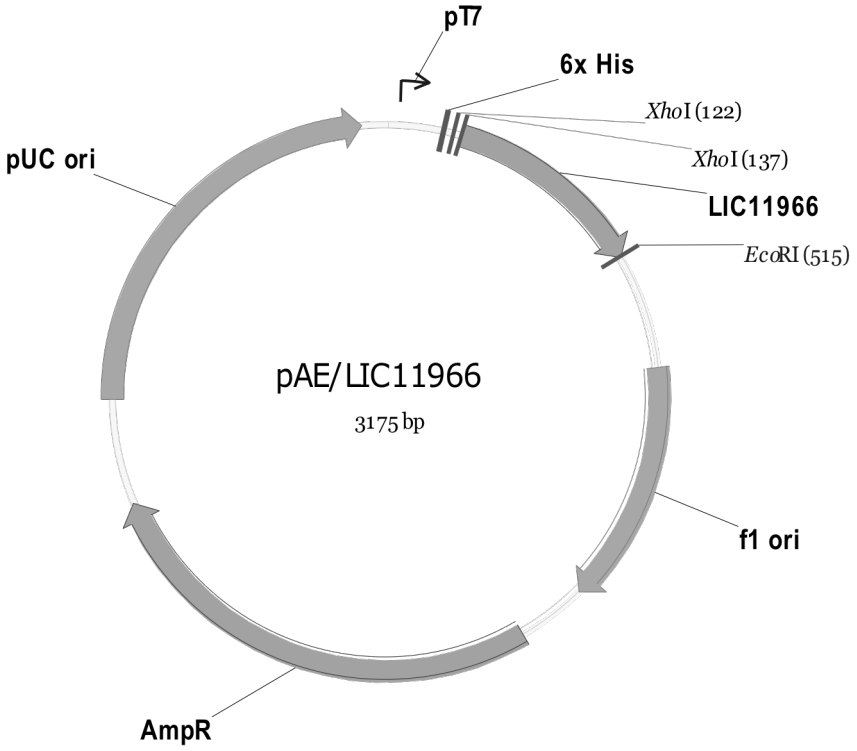


Figura 2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6x His
XhoI (122)
XhoI (137)
LIC11966
EcoRI (515)
pUC ori
pAE/LIC11966
3175 bp
f1 ori
AmpR

Figura 3

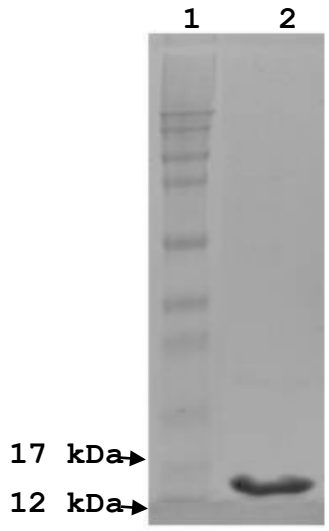


Figura 4

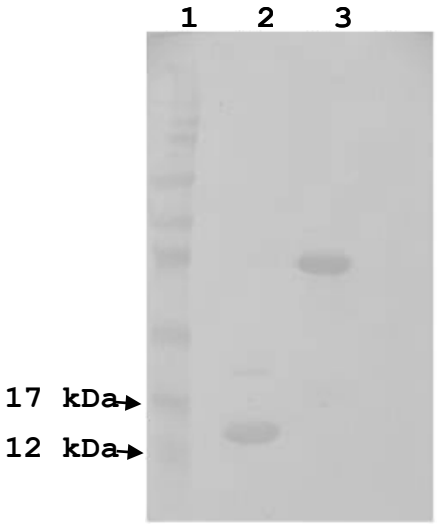


Figura 5

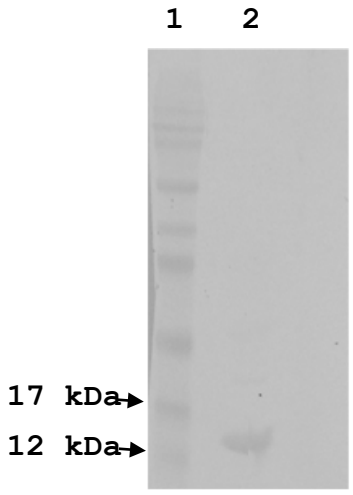


Figura 6

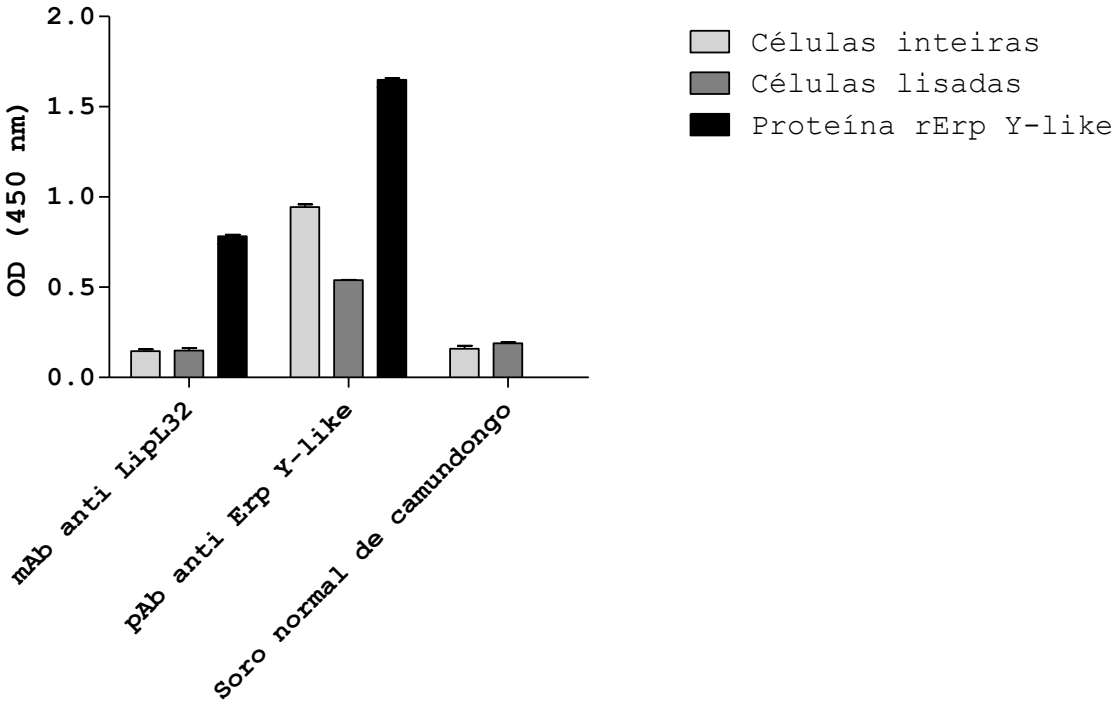
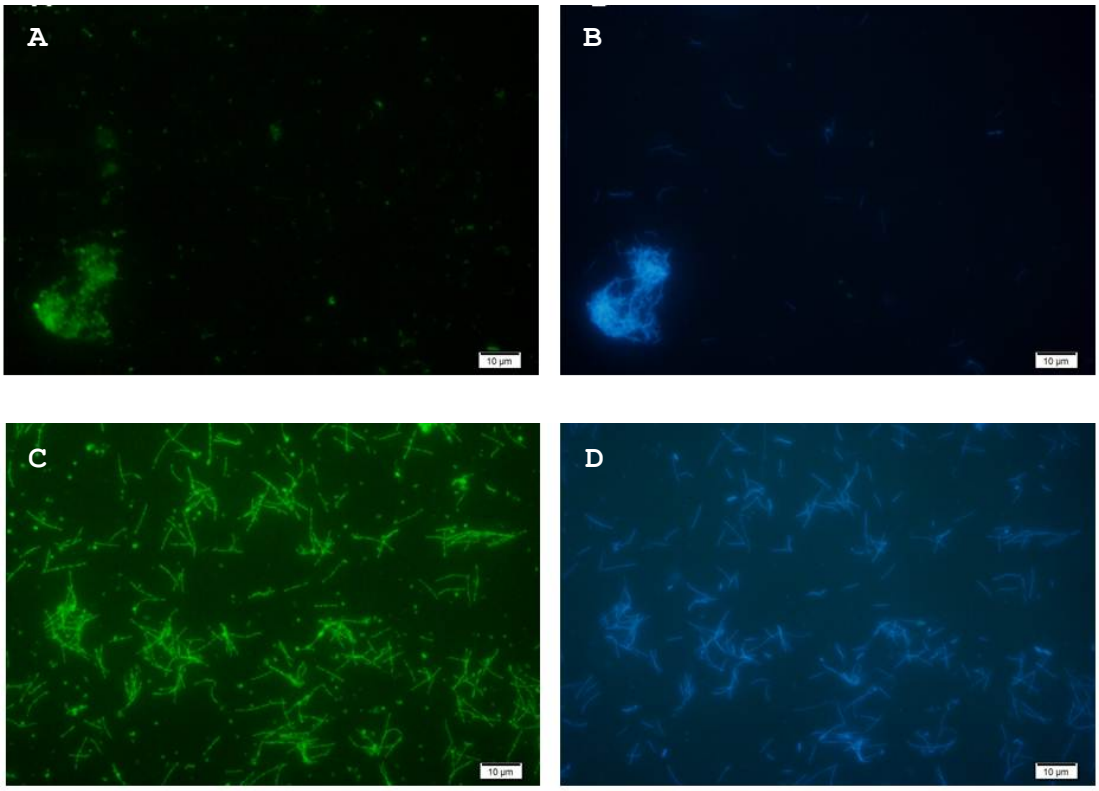
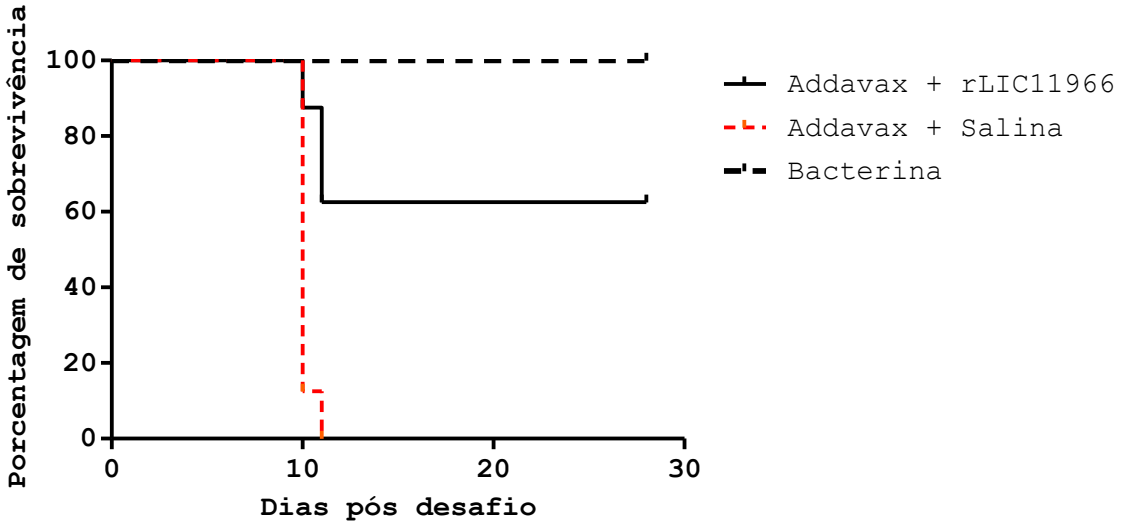

2.0
1.5
1.0
0.5
0.0
OD (450 nm)
mAb anti LipL32
pAb anti Erp Y-like
Soro normal de camundongo
Células inteiras
Células lisadas
Proteína rErp Y-like

Figura 7



1028 **Figura 8**

1029



1030

LISTA DE SEQUÊNCIAS

SEQ ID NO: 1

DNA de *Leptospira interrogans*

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gaagacctta caaacgcaga aaatgttaga aaagcttggg aaacaaagct agtttcttca 120

ctcgataagc ttaaaggaat cagtgatttt aaaggagatt ccagttttaa aaatgcaagc 180

gtccaagctc tcgaaactta tttaaacata gtaagtaaag actacaaacg tttgatcgaa 240

ttacgaggat taggtgacaa agcagactca aatgaaatca accaagttct caatcgtatt 300

aatcaggatt ttgaaaaagc tgtaaatact ctcaatgctg cttctgataa atttgcgaaa 360

gaatacgctt ctcaataa 378

SEQ ID NO: 2

CDS do antígeno recombinante rErpY-like, produzido a partir da clonagem parcial do gene erpY-like de *Leptospira interrogans* no vetor pAE

```
atg cat cac cat cac cat cac ctc gag gga tcc gac ctc gag gaa atc        48
Met His His His His His His Leu Glu Gly Ser Asp Leu Glu Glu Ile
 1               5                  10                  15

atg aac gct tct aca aac gat tta gat gcg tta aac gca gcc atg gaa        96
Met Asn Ala Ser Thr Asn Asp Leu Asp Ala Leu Asn Ala Ala Met Glu
             20                  25                  30

aag gaa gac ctt aca aac gca gaa aat gtt aga aaa gct tgg gaa aca       144
Lys Glu Asp Leu Thr Asn Ala Glu Asn Val Arg Lys Ala Trp Glu Thr
         35                  40                  45

aag cta gtt tct tca ctc gat aag ctt aaa gga atc agt gat ttt aaa       192
Lys Leu Val Ser Ser Leu Asp Lys Leu Lys Gly Ile Ser Asp Phe Lys
     50                  55                  60

gga gat tcc agt ttt aaa aat gca agc gtc caa gct ctc gaa act tat       240
Gly Asp Ser Ser Phe Lys Asn Ala Ser Val Gln Ala Leu Glu Thr Tyr
65                  70                  75                  80

tta aac ata gta agt aaa gac tac aaa cgt ttg atc gaa tta cga gga       288
Leu Asn Ile Val Ser Lys Asp Tyr Lys Arg Leu Ile Glu Leu Arg Gly
                 85                  90                  95

tta ggt gac aaa gca gac tca aat gaa atc aac caa gtt ctc aat cgt       336
Leu Gly Asp Lys Ala Asp Ser Asn Glu Ile Asn Gln Val Leu Asn Arg
            100                 105                 110

att aat cag gat ttt gaa aaa gct gta aat act ctc aat gct gct tct       384
Ile Asn Gln Asp Phe Glu Lys Ala Val Asn Thr Leu Asn Ala Ala Ser
        115                 120                 125

gat aaa ttt gcg aaa gaa tac gct tct caa                              414
Asp Lys Phe Ala Lys Glu Tyr Ala Ser Gln
    130                 135
```

SEQ ID NO: 3

Sequência de aminoácidos do antígeno recombinante rErpY-like, produzido a partir da clonagem parcial do gene erpY-like de *Leptospira interrogans* no vetor pAE

```
Met His His His His His His Leu Glu Gly Ser Asp Leu Glu Glu Ile   16
 1               5                  10                  15

Met Asn Ala Ser Thr Asn Asp Leu Asp Ala Leu Asn Ala Ala Met Glu   32
            20                  25                  30

Lys Glu Asp Leu Thr Asn Ala Glu Asn Val Arg Lys Ala Trp Glu Thr   48
        35                  40                  45

Lys Leu Val Ser Ser Leu Asp Lys Leu Lys Gly Ile Ser Asp Phe Lys   64
    50                  55                  60

Gly Asp Ser Ser Phe Lys Asn Ala Ser Val Gln Ala Leu Glu Thr Tyr   80
65                  70                  75                  80

Leu Asn Ile Val Ser Lys Asp Tyr Lys Arg Leu Ile Glu Leu Arg Gly   96
                85                  90                  95

Leu Gly Asp Lys Ala Asp Ser Asn Glu Ile Asn Gln Val Leu Asn Arg  112
            100                 105                 110

Ile Asn Gln Asp Phe Glu Lys Ala Val Asn Thr Leu Asn Ala Ala Ser  128
        115                 120                 125

Asp Lys Phe Ala Lys Glu Tyr Ala Ser Gln                          138
    130                 135
```

SEQ ID NO: 4

Primer *Forward* para amplificaçã de fragmento de 372 pb contendo parte do gene erpY-like de *Leptospira interrogans* e sítio de restrição para a enzima XhoI entre os nucleotídeos 4..9

actctcgagg aaatcatgaa cgct 23

SEQ ID NO: 5

Primer *Reverse* para amplificação de fragmento de 372 pb contendo parte do gene erpY-like de *Leptospira interrogans* e sítio de restrição para a para a enzima EcoRI entre os nucleotídeos 4..9

acggaattct tgagaagcgt attc 25

4 CONCLUSÃO GERAL

- O diagnóstico da leptospirose através da MAT é eficaz, porém diversos métodos alternativos são descritos para melhorar a acurácia diagnóstica, reduzir o tempo e subjetividade do teste. Muitos estudos têm mostrado resultados promissores, trazendo novas perspectivas para o diagnóstico da leptospirose.
- Com a avaliação da proteína ErpY-like foi possível conhecer características que podem contribuir para escolha deste antígeno como insumo no controle da leptospirose, seja através do uso em preparações vacinais ou no diagnóstico .
- O teste de ELISA utilizando a proteína rErpY-like é sensível e específico, e assim, pode ser usado para o diagnóstico da leptospirose suína, como ferramenta para análise epidemiológica de rebanhos ou como teste de triagem antes da realização do MAT.
- A proteína ErpY-like mostrou-se uma ferramenta importante, que pode ser usada no diagnóstico da leptospirose.

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6 ANEXOS

Anexo A – Artigo publicado no periódico *Acta Tropica*

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The use of ErpY-like recombinant protein from *Leptospira interrogans* in the development of an immunodiagnostic test for swine leptospirosis

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ABSTRACT

Swine leptospirosis poses a major problem in the agricultural sector. The gold standard for serodiagnosis of leptospirosis is Microscopic Agglutination Test (MAT). However, the limitations of this technique make the search for alternative diagnostic methods inevitable. In the present study, ErpY-like recombinant protein (rErpY-like), produced in *Escherichia coli* and used as antigen in indirect enzyme-linked immunosorbent assay (ELISA), was evaluated for its efficacy as a novel diagnostic tool for swine leptospirosis. For the study, 72 samples of swine sera characterized by microscopic agglutination test (MAT), were evaluated by indirect ELISA. The sensitivity, specificity and accuracy values obtained from the analysis were 96.8%, 100%, and 99%, respectively, thereby suggesting that rErpY-like ELISA being a sensitive and specific method for antibodies detection in swine populations could be used as an alternative for diagnosis of swine leptospirosis.

1. Introduction

Leptospirosis is a zoonotic disease transmitted to humans and animals by spirochete bacterium of the genus *Leptospira* (Faine et al., 1999). Out of 300 serovars of *Leptospira* known across 30 species, nearly 270 are pathogenic (Picardeau, 2017; Thibeaux et al., 2018). Animals and humans can get infected directly through urine of infected carriers or indirectly through water or soil contaminated with urine from infected carriers (Faine et al., 1999; Jorge et al., 2018). Swine are reservoirs of leptospires, and the *Leptospira* serovars most commonly found in swine are Pomona, Icterohaemorrhagiae, Canicola, Grippotyphosa (Soto et al., 2007; Santos et al., 2011).

Leptospirosis is a major public health concern, besides causing economic losses to agricultural sector (Faine et al., 1999; Levett, 2001; Rocha et al., 2017). Usually, this disease manifests in the form of fever, renal and hepatic insufficiency, pulmonary problems, and reproductive failure (Adler, de la Pena, 2010). In domestic animals, leptospirosis leads to poor reproductive performance, abortions, premature births, and stillbirths resulting in worldwide economic losses to the pork industry (Levett, 2001).

Swine leptospirosis imposes a major economic impact as pigs are important sources of animal protein and by-products. Young pigs suffering from acute leptospirosis rapidly become weak, lose appetite, and may have red eyes, jaundice, and convulsions, resulting in reduced production and quality (Faine et al., 1999; Hartleben et al., 2013). In Brazil, leptospirosis has been reported as one of the major causes of reproductive failure in pigs in several states, mainly the south and southeast regions of the country (Soto et al., 2007). Therefore, this disease has been included in the National Swine Health Program (PNSS) of Ministry of Agriculture, Livestock and Supply (MAPA). As per the MAPA regulations, all certified herd farms must control leptospirosis and the serological monitoring is obligatory after every six months (MAPA, 2002; Figueredo et al., 2013).

The reference standard technique for leptospirosis diagnosis is the Microscopic Agglutination Test (MAT). This test is performed by incubating the patient serum with various serovars of live leptospires and assessing the degree of agglutination. The results are then read under the dark field microscope. The MAT requires maintaining a collection of living strains, which are used as antigens. MAT is a time consuming and labor intensive test to perform. Besides, it requires periodic verification of each serovar in culture and hence is considered hazardous for laboratory workers (Hartleben et al., 2013; Monte et al., 2011; Picardeau, 2011). Therefore, it is necessary to search for novel diagnostic methods to circumvent the limitations of the standard test. To this intent, sensitive techniques such as enzyme-linked immunosorbent assay (ELISA) using recombinant leptospiral antigens have been developed as

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alternative diagnostic methods for leptospiral infection (Hartleben et al., 2013; Bomfim et al., 2011; Lizer et al., 2017; Mariya et al., 2006).

In 2009, Eshghi et al. reported the lipoprotein ErpY-like, as a novel potential virulence factor from *L. interrogans*, exhibiting close sequence similarity to the outer membrane lipoprotein ErpY, from *Borrelia burgdorferi*. Furthermore, the upregulation of protein expression during the *in vivo* infection (Eshghi et al., 2009) suggested that ErpY-like protein plays an important role in the infection process and thus could potentially serve as a diagnostic factor and a vaccine antigen. The characterization studies carried out on this protein by our research group have proved it to be an important target in the monitoring of leptospirosis. Also, recent trials conducted in our laboratory to assess the protective efficacy of ErpY-like recombinant protein (rErpY-like) as a vaccine against *L. interrogans* yielded significant results (Oliveira et al., 2018). In the present study, recombinant ErpY-like (rErpY-like) antigen-based indirect ELISA was developed for the diagnosis of swine leptospirosis.

2. Material and methods

2.1. Bacterial strains and serum samples

E. coli BL21 (DE3) Star host strain (Invitrogen, São Paulo, Brazil) was grown at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, and 2% agar) at 37 °C with the addition of 100 μg mL^{-1} ampicillin.

Swine serum samples were obtained from the serum bank of the Laboratory of Molecular Biology of Microorganisms, Federal University of Pelotas, and subsequently evaluated by MAT. A total of 72 samples were collected from animals at a swine farm with reproductive disorders such as abortions, premature births, and stillbirths.

The MAT was carried out according to OIE (2013), with the following reference serovars and strains of leptospires: Australis Ballico, Autumnalis Akiyami A, Bratislava Jez Bratislava, Castellonis Castellon 3, Bataviae Swart, Canicola Hond Utrecht IV, Cynopteri 3522 C, Grippotyphosa Moskva V, Hebdomadis Hebdomadis, Icterohaemorrhagiae RGA, Copenhageni M20, Javanica Veldrat, Batavia 46, Panama CZ 214, Pomona Pomona, Pyrogenes Salinem, Hardjo Hardjoprajitno, Sejroe M 84, Wolffi 3705, Tarassovi Perepeletsin, Patoc Patoc 1. Reciprocal agglutination titers of greater than or equal to 100 were considered positive.

2.2. Production of rErpY-like protein

The ErpY-like gene (≈390 bp), encoding for 125 amino acid protein, was synthesized (GenOne, Rio de Janeiro, Brazil) by using the genomic sequence of *L. interrogans* serovar Copenhageni strain Fiocruz L1–130 available in Genbank (Accession Number: AE016823). The *erpY-like* gene was cloned into a pAE vector, carrying an N-terminal 6×His-tag. The *pAE/erpY-like* plasmid was used to transform *Escherichia coli* BL21 (DE3) Star for rErpY-like production. The protein purification was accomplished by affinity chromatography with Ni^{2+} Sepharose column using the automated liquid chromatography system AKTAprime (GE Healthcare, Chicago, IL), following the manufacturer's instructions. The purified protein was dialyzed in phosphate-buffered saline (PBS) and glycine 0.1%, pH 8.0, decreasing the concentration of urea (starting 5 M), in 16 steps over five days, at 4 °C. Western Blotting (WB) with His-tag monoclonal antibody (Mab) (Sigma-Aldrich, St. Louis, MO) was employed to evaluate the rErpY-like purified protein, which was then quantified by the BCA method (BCA Protein Assay Kit - Thermo Scientific, Waltham, MA) and stored at −20 °C.

2.3. rErpY-like protein antigenicity

The antigenicity of rErpY-like protein was evaluated by Dot Blot (DB) assay using leptospirosis positive sera pool from naturally infected swine (reciprocal MAT titer > 1:25,000). A 5 μL aliquot of rErpY-like or rLipL32 (used as positive control) was added to a nitrocellulose membrane and dried at 37 °C for 15 min. The membrane was then blocked with PBS-T/casein [0.05% (v/v) Tween 20 and 0.5% (w/v) casein (PBS-T) in PBS] and incubated with swine sera pool (1:50). The bound antibodies were detected with peroxidase-conjugated goat anti-pig IgG antibody (Sigma-Aldrich, St. Louis, MO), diluted 1:5,000. The membranes were developed with chromogenic substrate solution (6 mg diaminobenzidine, 0.03% nickel sulfate, 50 mM Tris-HCl, pH 8.0, and 0.03% hydrogen peroxide) for visualization of protein.

2.4. rErpY-like indirect ELISA

Polystyrene ELISA microtiter plates (Nunc, Polysorp - Thermo Scientific, Waltham, MA) were coated with 50 ng of rErpY-like protein per well (determined by previously checkerboard analysis), diluted in carbonate-bicarbonate buffer pH 9.6, for 16 h at 4 °C. The plates were blocked with a solution of PBS-T/casein [0.05% (v/v) Tween 20 and 0.5% (w/v) casein in PBS], followed by addition of both positive and negative leptospirosis swine sera (diluted 1:50 in PBS-T/casein). Then, goat anti-pig IgG peroxidase conjugate (Sigma-Aldrich, St. Louis, MO), diluted 1:6,000 in PBS-T/casein was added for sera antibodies detection. The presence of the antigen-antibody complex was revealed by adding a substrate solution containing o-phenylenediamine (OPD) (0.4 mg/mL in 0.1 M citrate buffer, pH 5.0) and 0.03% hydrogen peroxide. The reaction was stopped by addition of 0.1 M sulfuric acid and the absorbance was read at 492 nm using VICTOR™ X5 Multilabel Plate Reader (Perkin Elmer, Waltham, MA). All assay steps were carried out in a total volume of 100 μL/well at 37 °C for 1 h and the plates were washed thrice with PBS-T/casein. The swine sera that tested positive/negative in the MAT for leptospirosis were used as controls.

2.5. Statistical analysis

The results pertaining to specificity, sensitivity, accuracy and a cut-off value of the ELISA test, obtained from 72 serum samples, were evaluated using ROC (*Receiver Operating Characteristic*) curves. The ROC curve analysis was performed with the MedCalc statistical software (version 10.3.0). Test results so obtained were then compared with the standard MAT test.

3. Results

3.1. Production of rErpY-like protein and antigenicity testing

The insoluble rErpY-like protein, nearly 13 kDa in size, was successfully expressed in *E. coli* cells. The purification process yielded approximately 25 mg/L of rErpY-like protein. Western Blot analysis using His-tag mAb (Sigma-Aldrich, St. Louis, MO) (Fig. 1 A) confirmed the protein expression. The capacity of the rErpY-like protein to be recognized by the antibodies, present in leptospirosis positive sera pool obtained from naturally infected swine, was evaluated by Dot Blot assay. The results were positive and are shown in Fig. 1 B.

3.2. rErpY-like ELISA

The 72 swine sera (31 positive and 41 negative), previously characterized by MAT, were evaluated by indirect ELISA. The rErpY-like ELISA was characterized, and the results were correlated with MAT using ROC analysis (Fig. 2). The comparison of MAT and ErpY-like ELISA results was also demonstrated in Table 1. All negative sera had the same result as MAT standard, concording with the established cut-off values.

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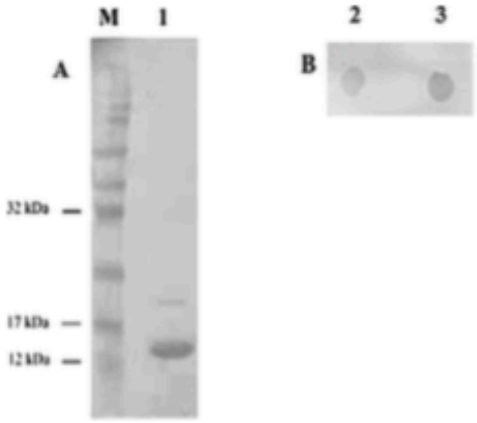

Fig. 1. Western Blotting (WB) and Dot Blotting (DB) of rErpY-like protein. **(A)** Characterization of purified rErpY-like protein with Mab anti-6×His. M, protein molecular mass markers Rainbow™ (GE Healthcare, USA) (kDa); lane 1, purified rErpY-like protein. **(B)** Antigenicity of rErpY-like using a pool of swine sera positive for leptospirosis. Lane 2, rErpY-like protein and lane 3, rLipL32 protein (positive control).

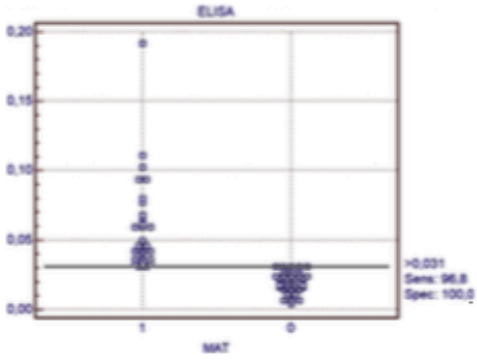

Fig. 2. Indirect ELISA performed with rErpY-like protein as antigen. The protein was tested with 72 swine sera, 31 positive and 41 negative to leptospirosis, as decided by the *Receiver Operating Characteristic* (ROC) curve.

3.3. Specificity, sensitivity, accuracy and cut-off

The rErpY-like ELISA showed a cut-off value of > 0.031 with 96.8% sensitivity and 100% specificity (Fig. 2). The area under the ROC curve indicating the accuracy of immunoassay was found to be 0.990 (99%) (Fig. 3).

4. Discussion

Leptospirosis is one of the most important bacterial diseases of worldwide occurrence with a great economic and public health importance owing to its zoonotic nature (Faine et al., 1999; Figueredo et al., 2013). Swine farming plays an enormous role in the national economic scenario. Careful management of farm activities and high level of hygiene are immensely important for the maintenance of healthy animals (Santos et al., 2011; Figueredo et al., 2013).

Table 1
Results obtained in MAT and rLipL32/ELISA assays for swine leptospirosis diagnosis.

MAT		ELISA positive		ELISA negative	
No. of samples	Titre[a] (≤100)	No. of samples	OD ± SD[b]	No. of samples	OD ± SD[b]
6	100	6	≤0.093(0.056)	–	–
5	200	5	≤0.080 (0.127)	–	–
2	400	2	≤0.040 (0.084)	–	–
5	800	5	≤0.093 (0.267)	–	–
5	1,600	5	≤0.102 (0.295)	–	–
5	3,200	5	≤0.191 (0.425)	–	–
3	6,400	3	≤0.066 (0.167)	–	–
40	–	–	–	40	≤0.031 (0.007)

[a] The antibodies titers for MAT are expressed as the reciprocal of the highest serum dilution that resulted in 50% agglutination of leptospires.
[b] Mean scores of the ODs obtained of three experimental replication and standard deviation (SD).

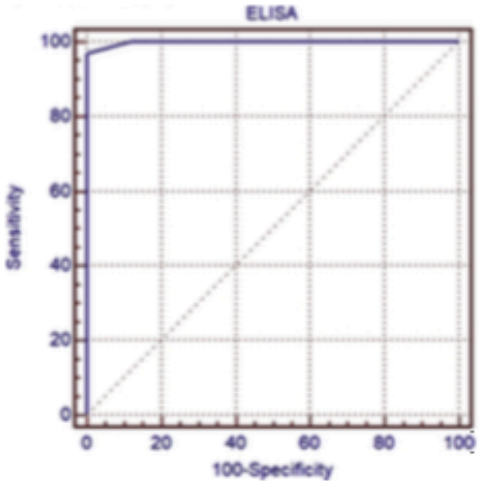

Fig. 3. Receiver operating characteristics (ROC) curve for rErpY-like ELISA with the swine sera. The area under the curve is 0.990 (99% with 95% confidence interval).

The standard diagnostic for leptospirosis is MAT. However, it is considered unfavorable, mainly because of its time consuming and labor intensive technique as well as the subjectivity of results (Picardeau, 2013). Keeping in view the drawbacks of the standard technique, sensitive methods such as ELISA, using recombinant proteins, have been developed for the diagnosis of leptospirosis in different animal species. ELISA has the advantages of analyzing several samples simultaneously, making the diagnosis faster, besides using recombinant proteins as an antigen which is easy to produce and maintain (Hartleben et al., 2013). rLipL32 (Boonfiet et al., 2005), rLipL41 (Mariya et al., 2006), and LigB (Deneke et al., 2014; Nagalingam et al., 2015) have been evaluated as antigens for serodiagnosis of bovine leptospirosis; LigA for equine leptospirosis (Yan et al., 2013); and a *Leptospira* OMP (Outer membrane protein) antigen, rLipL21, rLoa22 and rLipL32 for canine leptospirosis (Sathiyamoorthy et al., 2017; Ye et al., 2014). Till now, only two studies have evaluated the efficacy of rLipL32 antigen in indirect ELISA for diagnosis of swine leptospirosis

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(Hartleben et al., 2013; Monte et al., 2014).

The rErpY-like protein expression was found to be efficient in an *E. coli* based expression system. *E. coli* is routinely used for the production of recombinant proteins for a variety of purposes ranging from structural studies to the development of vaccines (Dellagostin et al., 2011; Fernandes et al., 2017; Gopal, Kumar, 2013).

In silico analysis of the rErpY-like protein carried out by our group evidently illustrated its presence in the serovars most commonly found in swine, viz., Pomona, Icterohaemorrhagiae, Canicola and Grippotyphosa, besides being present in other pathogenic serovars. The observation was consistent with the results of PCR performed using the DNA from these serovars (data not published).

Considering the diagnostic difficulties and the paucity of studies aiming to improve the diagnosis of swine leptospirosis, in the present study, a serological test was developed for the detection of anti-rErpY-like antibodies present in swine serum. The ELISA test, although it is an indirect diagnosis test that is not capable of determining the infective serogroup, offers numerous advantages over the standard test, once it allows to analyze a large number of samples simultaneously, there is no need to paired samples and a battery of live serovars of *Leptospira* spp. Hartleben et al. (2013) and Monte et al. (2014) reported the development of indirect ELISA for the diagnosis of swine leptospirosis, using rLipL32 antigen. The sensitivity of 100% was reported in both the studies, whereas the specificity of 85.1% and 91.48%, and accuracy of 91.86% and 95.34%, respectively were recorded. The results obtained in the present study project a higher accuracy and specificity to rErpY-like ELISA, i.e., 99% and 100%, respectively, as compared to that obtained for the same species. The sensitivity values were however slightly lower, i.e., 96.8%. Sensitivity is the ability to identify the truly positives, while specificity indicates truly negatives. For an optimal diagnostic test, high sensitivity and specificity values are expected. In this way, the rErpY-like ELISA can also be used as a screening test to select the samples quickly that will be used for MAT.

The results of indirect ELISA with rErpY-like protein, described in the present study, could aid the evaluation of the disease on a large scale in pigs. Furthermore, the high sensitivity and specificity values obtained suggest that this technique could be effectively used in seroepidemiological investigations in swine populations. However, further studies should be performed with a larger number of samples and with sera from other animal species to assess the full-scale benefits of this diagnostic technique.

5. Conclusion

The indirect ELISA with rErpY-like was found to be desirably sensitive and specific. Therefore, it could be used as an alternative for the diagnosis of swine leptospirosis, besides being used as a screening test before performing MAT. Thus, it could serve as a reliable tool for antibody detection in swine populations.

Conflict of interest

The authors declare that they have no conflict of interest.

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Anexo B – Pedido de patente

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INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 018203 4

Dados do Depositante (71)

Depositante 1 de 1

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