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Tese

Avaliação da resposta imune induzida pelo antígeno recombinante P42 de *Mycoplasma hyopenumoniae* em suínos a campo

Sérgio Jorge

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RESUMO

JORGE, Sérgio. **Avaliação da resposta imune em suínos imunizados com o antígeno recombinante P42 visando a indução da proteção contra Pneumonia Enzoótica Suína.** 2014. 81 f. Tese (Doutorado) - Programa de Pós - Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

Mycoplasma hyopneumoniae é o agente etiológico da Pneumonia Enzoótica Suína (PES). A bactéria coloniza e paralisa as células epiteliais do trato respiratório, predispondo o hospedeiro a infecções secundárias, causando significativas perdas econômicas. As vacinas atualmente disponíveis são compostas por células bacterianas inteiras inativadas (bacterinas), as quais proporcionam apenas uma proteção parcial aos suínos. Elas são capazes de reduzir as lesões pulmonares e os sinais clínicos, mas não impedem a colonização pelo agente. Com a finalidade de desenvolver uma vacina mais eficiente contra a PES, o uso da tecnologia do DNA recombinante representa uma estratégia promissora. Após o sequenciamento e análise proteômica de quatro cepas de *M. hyopneumoniae*, nosso grupo de pesquisa produziu e avaliou a imunogenicidade e antigenicidade de 35 proteínas recombinantes secretadas e 6 proteínas transmembrana. Algumas destas proteínas apresentaram potencial para serem usadas como antígenos vacinais, onde se destacou a chaperona molecular DnaK P42 (proteína de choque térmico). O objetivo deste estudo foi avaliar a proteina recombinante P42 em formulações vacinais contra a PES. Para tanto, foram realizados ensaios utilizando suínos mantidos em condições de campo numa granja com *status* de positiva para *M. hyopneumonia.* Foram avaliadas as respostas humoral e celular dos grupos de animais imunizados com a rP42 em tampão fosfato salina, com a rP42 emulsificada em adjuvante oleoso e com a rP42 associada à bacterina comercial. Os resultados mostraram que a imunização com rP42 emulsificada em adjuvante oleoso é capaz de induzir anticorpos contra *M. hyopneumoniae* além da expressão da citocina anti-inflamatória IL-10. Estas propriedades imunogênicas tornam o antigeno rP42 um candidato para o desenvolvimento de uma vacina de subunidade recombinante contra a PES.

Palavras-chave: Pneumonia Enzoótica Suína, proteína de choque térmico, vacina recombinante.

ABSTRACT

SÉRGIO, Jorge. **Evaluation of protection in pigs immunized with the recombinant P42 antigen for development of swine enzootic pneumonia vaccine.** 2014. 81 f. Tese (Doutorado) - Programa de Pós - Graduação em Veterinária. Universidade Federal de Pelotas, Pelotas.

Mycoplasma hyopneumoniae is the etiological agent of enzootic pneumonia (EP), a contagious respiratory disease that affects swine production worldwide. *M. hyopneumoniae* colonizes the ciliated epithelial cells of the respiratory tract, damaging the cells and predisposing the infected animals to secondary infections, causing significant economic losses. The commonly used vaccines to control this disease consist of inactivated whole cells (bacterins). These bacterins provide only partial protection and do not prevent the colonization of *M. hyopneumoniae* on the epithelial cells. Efforts to develop a more effective vaccine against mycoplasmas have been proposed and vaccines developed using recombinant DNA technology represents a promissing alternative. Although the genomes of four strains of. *M hyopneumoniae* have been sequenced, few recombinant antigens have been evaluated as candidate vaccines. Our research group produced and evaluated the immunogenicity and antigenicity of 35 secreted recombinant proteins and 6 transmembrane recombinant proteins. Some of these proteins were identified as having the potential to be used as vaccine antigens, including the molecular chaperone DnaK (P42 heat shock protein). The aim of this study was to assess the potential of recombinant P42 in vaccine preparations against EP, using swine animal model housed under field condictions in a *M. hyopneumoniae*-positive farm. Both, humoral and cellular immune responses were elicited when rP42 was delivered in Phosphate buffer saline, when combined to an oil based adjuvant, and when added to a whole cell vaccine preparation. The results indicate that immunization with rP42 emulsified in oil based adjuvant is able to induce antibodies against *M. hyopneumoniae* as well as the expression and the anti-inflammatory cytokine IL-10 in pigs. These immunogenic properties make recombinant antigen P42 a promising candidate for a recombinant subunit vaccine against EP.

Key words: *Mycoplasma hyopneumoniae*, high shock protein, recombinant vaccine

SUMÁRIO

1 INTRODUÇÃO GERAL

As doenças respiratórias estão entre os problemas de saúde mais importantes associados à produção de suínos no mundo todo. *Mycoplasma hyopneumoniae* é o agente etiológico da pneumonia enzoo ótica suína (PES), uma doença respiratória crônica que manifesta-se por tosse seca, atraso no ganho de peso, alta morbidade e baixa mortalidade (MAES et al., 2008; SOBESTIANSKY et al., 1999;). A PES é altamente prevalente, podendo chegar à 100% em granjas comerciais de produção intensiva (MAES et al., 1996).

Mycoplasma hyopenumoniae coloniza as células ciliadas do trato respiratório superior, causando perda da função do epitélio mucociliar, predispondo os suínos à infecções secundárias (THACKER; MINION, 2010). Os prejuízos econômicos decorrentes desta queda de produtividade podem chagar a 20% nas taxas de conversão alimentar e até 30% no ganho de peso (SOBESTIANSKY et al., 1999). Suínos de todas as idades são susceptíveis, sendo mais prevalente nas fases de crescimento e terminação. No entanto, nos rebanhos sem imunidade para a doença, suínos de todas as faixas etárias, incluindo leitões e matrizes, podem ser acometidos (SIBILA et al., 2009).

Atualmente o controle da PES se baseia na otimização daspráticas de manejo, na melhoria das condições santárias das granjas, no uso de antimicrobianos e na vacinação (MAES et al., 1996, 2008; SIBILA et al, 2009; SIMIONAOTTO et al., 2013, VICCA et al.,2004). As vacinas comercais, compostas de células inteiras inativadas (bacterinas), estão disponíveis comercialmente e são mundialmente utilizadas. Estas vacinas reduzem as lesões pulmonares e os sinais clínicos, mas não a colonização do agente e não impedem o estado de portador (MAES et al., 1996; SIBILA et al., 2009; MARCHIORO et al., 2012; SIMIONATTO et al., 2013).

M. hyopneumoniae cresce lentamente em meio de cultivo Friis enriquecido com soro animal e seu isolamento é dificultoso devido à frequente contaminação de outros mycoplasmas como o *M. hyorhinis* e *M. flocculare*. Estas características encarecem a produção das vacinas atualmente disponíveis (ROSS, 1999), além de oferecerem apenas uma proteção parcial aos suínos vacinados (HAESEBROUCK et al., 2004). Diante desta problemática, o uso de vacinologia reversa visando o desenvolvimento de novas vacinas mais eficientes para melhorar o controle e prevenção da PES é estratégico, visto que pode contribuir para a dimunuição da

infecção por *M. hyopnemoniae* e, consequentemente, diminuir a infecção dos agentes secundários, levando a um aumento na oferta de carne suína além de melhorar a saúde e o bem estar dos animais durante o processo produtivo.

Após o sequenciamento e análise proteômica de duas cepas de *Mycoplasma hyopneumoniae* (VASCONCELOS et al., 2005), as vacinas atualmente em fase de pesquisa são baseadas em antígenos recombinantes da bactéria (CHEN et al., 2003; LIN et al., 2003; OKAMBA et al., 2007). Alguns destes antígenos apresentaram resultados promissores quando utilizados como alvos vacinais em ensaios experimentais (FAGAN et al., 1996; CHEN et al., 2003; SHIMOJI et al., 2003; CHEN et al., 2008). Nosso grupo de pesquisas produziu e avaliou a imunogenicidade e antigenicidade de 35 proteínas recombinantes secretadas (SIMIONATTO et al., 2009; SIMIONATTO et al., 2010; SIMIONATTO et al., 2012) e 6 proteínas recombinantes transmembranas (MARCHIORO et al., 2012). Algumas destas proteínas apresentaram potencial para serem usadas como antígenos vacinais, destacando-se a chaperona molecular DnaK P42 (*heat skock protein* - hsp), que monstrou-se altamente imunogênica e antigênica em camundongos (GALLI et al., 2012; SIMIONATTO et al., 2012), além de ser reconhecida por soros de suínos convalescentes (SIMIONATTO et al., 2012). Em ensaios experimentais, os anticorpos anti-P42 foram capazes de se ligar a este antigeno específico, inibindo o crescimento *in vitro* de *M. hyopneumoniae* (CHEN et al., 2003). Apesar da P42 ser um importante antigeno a ser explorado como alvo vacinal no cotrole da PES, todos os trabalhos de pesquisa realizados até o momento avaliaram a P42 utilizando camundongos como modelo animal, não existindo relatados na literatura de avaliação do potencical imunogênico e antigênico da P42 em suínos.

O objetivo principal desse trabalho foi avaliar a resposta imune humoral e celular de suínos vacinados com a proteína recombinante P42, visando contribuir para o desenvolvimento de uma vacina mais eficiente no controle e prevenção da PES. Empregando técnicas de vacinologia reversa e biologia molecular, um fragmento do gene MHP0067 de *M. hyopneumoniae* foi clonado em um vetor de expressão e a proteina correspondente foi expressa em sistema heterólogo utilizando *Escherichia coli.* A rP42 foi utilizada em três diferentes formulações vacinais, as quais foram avaliadas em suínos mantidos em condições de campo. Inicialmente, essa tese apresenta uma revisão bibliográfica apontando características gerais da PES, incluindo aspectos relacionados às particularidades

dos micoplasmas, à sua transmissão, aspectos clínicos, imunologia, métodos diagnósticos e de controle, bem como o desenvolvimento de vacinas.

Os dados gerados nesta tese estão apresentados na forma de artigo científico que trata da clonagem e expressão do antígeno rP42 bem como sua utilização em ensaios vacinais utilizando suínos como modelo animal. O perfil imunológico dos animais foi avaliado considerando a resposta imune celular e humoral; a expressão das citocinas IL-10 e IFN-γ foi quantificada no sobrenadante do cultivo de células mononucleares. Este trabalho foi submetido ao periódico **Comparative Immunology, Microbiology and Infectious Diseases**.

2 REVISÃO BIBLIOGRÁFICA

2.1 O gênero Mycoplasma

A ausência de parede celular é um fator de diferenciação fenotípica com outras bactérias, que leva à classificação dos micoplasmas em uma classe denominada de *Mollicutes* (do latino *mollis,* delicado e *cutis,* parede). Esta classe compreende quatro ordens, diversas famílias e aproximadamente 160 espécies (NICOLEI, J., 1996). Apesar de diferenças particulares dos micoplasmas e outras bactérias, inclusive seu tamanho diminuto (RAZIN et al., 1998), alguns aspectos da biologia molecular são similares com as bactérias Gram-positivas (TRACKER; MINION, 2010). Os micoplasmas são organismos difundidos na natureza e acometem o homem, mamíferos, répteis, peixes, artrópodes e plantas. São primariamente encontrados em mucosas do trato respiratório e urogenital, nos olhos, no tubo digestivo, na glândula mamária e nas articulações (NICOLEI, J., 1996; RAZIN et al., 1998), porém, muitas espécies podem se disseminar para outros sítios, levando ao desenvolvimento de uma doença localizada ou sistêmica (RAZIN et al., 1998). Utilizam-se de hospedeiros e tecidos específicos, provavelmente em decorrência da sua exigência nutricional e obrigação do modo de vida parasitária, mas podem eventualmente colonizar outro hospedeiro, muitas vezes sem expressar sua patogenicidade (NICOLEI, J., 1996; RAZIN et al., 1998). Em animais domésticos, integrantes do gênero *Mycoplasma* causam diversas doenças, como a pleuropneumonia contagiosa caprina e bovina (*M. capricolum* subesp. capripneumoniae *e M. mycoides* subsp. mycoides, respectivamente), a agalaxia contagiosa dos ovinos e caprinos (*M. agalactiae*), ceratoconjutivite infecciosa dos ovinos (*M. conjunctivae),* micoplasmose aviária (*M. gallisepticum* e *M*. *synoviae)*, micoplasmose hemotrófica felina (*M. haemofelis*), pneumonia enzoótica suína (*M. hyopneumoniae*), dentre outras (RAZIN et al., 1998; MINION, 2002). No homem, diversas espécies de micoplasma são reconhecidas como patógenos do trato geniturinário (*M. hominis*) (LIU et al, 2014), do trato respiratório (*M. pneumoniae*) (IZUMIKAWA et al, 2013) e mais recentemente *M. genitalium* também tem sido indicado como um dos agentes etiológicos da uretrite não-gonocócica (PLECKO et al, 2013).

2.1.1 *Mycoplasma hyopneumoniae*

M. hyopneumoniae é um patógeno espécie-específico que acomente suínos. Este microorganimo possue um pequeno genoma, com aproximadamente 70% de adenina-timina (A-T). As cepas J, 232, 7448 e 168 de *M. hyopneumoniae* já tiveram seus genomas sequenciados (LIU et al., 2011; MINION et al., 2004; VASCONCELOS et al., 2005). Estudos prévios demonstraram uma ampla diversidade genômica e proteômica (STRAIT et al., 2008; CALUS et al., 2007) e portanto uma variação da virulência entre as cepas (VICCA et al., 2003). Atualmente, os isolados de *M. hyopneumonae* são classificados como de baixa, moderada e alta virulência (VILLARREAL et al., 2011). Devido ao seu reduzido genoma, possuem um metabolismo limitado e possuem poucas vias biossintéticas (RAZIN et al., 1998); a falta de algumas vias implica que precisam obter do ambiente externo, como meio de cultivo, aminoácios, purinas, pirimidinas e componentes da membrana celular (THACKER; MINION, 2010). Devido a este fato, o cultivo *in vitro* é fastidioso e difícil de ser realizado (RAZIN et al., 1998). *M. hyopneumoniae* pode ser cultivado *in vitro*, porém quando utilizado o meio Friis, o enriquecimento com soro suíno ou equino é necessário (FRIIS, 1975). Apesar do “padrão ouro” para o diagnóstico da PES ser o isolamento do *M. hyopneumoniae,* este método é inviável no diagnóstico de rotina (SIBILA et al., 2009). Além das dificuldades do isolamento do agente, a contaminação com outros mycoplasmas como o *M. hyorhinis* (presente no trato respiratórios dos suínos) e *M. flocculare* (espécie não patogênica, também presente no trato respiratório dos suínos, que apresenta similaridades morfológicas, antigênicas e de crescimento com o *M. hyopneumoniae)* é frequente (THACKER; MINION, 2010).

M. hyopneumoniae é um dos agentes que participam intimamente da patogênese do complexo respiratório suíno (*porcine respiratory disease complex* - PRDC). Este complexo envolve tando agentes bacterianos como virais (vírus da síndrome respiratória e reprodutiva suína, circovírus suíno tipo 2, vírus da doença de Aujeszky, vírus da influenza suína e coronavírus suíno). O complexo respiratório suíno afeta animais entre 14 e 20 semanas de idade e é clinicamente caracterizado por diminição nas taxas de crescimento, diminição das taxas de conversão alimentar, anorexia, febre, tosse e dispnéia (DEE, 1996).

2.2 Pneumonia Enzoótica Suína

As doenças respiratórias são principais problemas relacionados à produção de suínos no mundo. *M. hyopneumoniae* é o agente responsável pela Pneumonia Enzoótica Suína (PES), doença respiratória infecciosa caracterizada por alta morbidade e baixa mortalidade. Suínos de todas as idades são susceptíveis a infecção, porém prevalência da doença é particularmente maior em animais em fase de crescimento e terminação nas granjas de criações intensivas. A severidade dos sinais clínicos dependem da cepa envolvida, a presença de infecções secundárias e das condições ambientais (SIBILA et al, 2009). *M. hyopneumoniae* coloniza e paralisa as células epitliais do trato respiratório predispondo, desta forma, à infecções secundárias (TRACKER; MINION, 2010). O dano causado nas células epiteliais são causados pelos sub-produtos do metabolismo da bactéria, como peroxido de hidrogênio (RAZIN et al, 1998). As infecções secundárias ou oportunistas estão frequentemente presentes e relacionadas aos seguintes agentes: *Pasteurella multocida, Actinobacillus pleuroneumoniae, Mycoplasma hyorhinis, Streptococcus suis, Haemophilus parasuis, Bordetella bronchiseptica e Arcanobacterium pyogenes* (THACKER, 2006). Quando a infecção por *M. hyopneumoniae* não é acompanhada por infecções secundárias, a doença apresenta como sinal clínico uma tosse crônica e não produtiva e redução nas taxas de ganho de peso diário e na conversão alimentar. Já quando há envolvimento de infecções secundárias, os sinais clínicos podem incluir dispnéia, hipertermia e morte (MAES et al., 1996)

Os prejuízos para suinocultura são decorrentes de quedas na produtividade que podem chegar a 20% sobre a conversão alimentar e até 30% sobre o ganho de peso (SOBESTIANSKY et al., 1999). Um estudo realizado no Estado de Santa Catarina mostrou que, para cada 100 animais abatidos, há uma perda equivalente de 2,4 suínos devido às pneumonias (SOBESTIANSKY et al., 1990). Na Região Sul do Brasil, que representa cerca de 80% do abate nacional, constatou-se que 55% dos suínos de abate apresentavam lesões sugestivas de PES e 100% dos rebanhos examinados estavam afetados (SOBESTIANSKY et al., 1999).

2.3 Transmissão

Em rebanhos livres de *M. hyopneumoniae*, o agente pode ser introduzido por transmissão direta (BATISTA et al., 2004). Uma vez presente no rebanho, *M. hyopneumoniae* é transmitido entre os animais por meio de gotículas de aerosol geradas pela tosse dos animais infectados e se espalha no rebanho horizontalmente entre os suínos e verticalmente entre as matrizes e seus leitões (MAES et al, 1996). De modo geral, a transmissão horizontal ocorre de animais mais velhos para os mais jovens, podendo ocorrer também entre animais de estágios de produção diferentes dentro de uma mesma granja ou até mesmo entre granjas diferentes. Uma vez infectado, o *M. hyopneumoniae* pode persistir no trato respiratório de animais adultos por até 185 dias (FANO et al., 2005). Os animais infectados frequentemente apresentam infecção subclínica que em muitos casos não é diagnosticada, porém estes animais são capazes de transmitir o patógeno para os animais susceptíveis (RUIZ et al., 2002; PIJOAN, 2005). Um estudo demonstrou que o risco de infecção entre animais num mesmo rebanho está diretamente relacionando com a densidade de animais na mesma granja e também com a proximidade de outras granjas. Uma distância segura entre granjas para que não ocorra a infecção foi calculada em no minimo 3 km (ZHUANG et al, 2002).

2.4 Resposta imune

Pesquisas têm indicado que uma proteção eficaz contra *M. hyopneumoniae* necessida o envolvimento tanto da resposta imune humoral como da resposta mediada por células (THACKER et al, 2000; HAESEBROUCK et al, 2004). Muitos estudos descrevem a dinâmica da soroconversão resultante da infecção por *M. hyopneumoniae* (CALSALMIGLIA et al., 1999; LEON et al., 2001; VICCA et al., 2002; SIBILA et al., 2004; VIGRE et al., 2004). Nos leitões, ocorre uma dimuição progressiva dos anticorpos maternos seguido por um aumento lento de animais soropositivos na fase de terminação (CALSALMIGLIA et al., 1999). Embora o tempo de duração médio dos anticorpos maternais é de 16 dias (MORRIS et al., 1994), eles podem persistir por até 9 semanas quando os títulos iniciais são elevados. A persisêntia de anticorpos maternais contra *M. hyopneumoniae* também é determinada pelo nível de anticorpos presente no soro das matrizes (WALLGREN et

al., 1998). Após este tempo variável de duração dos anticorpos maternais, os animais soroconvertem (iniciam a imunidade ativa) contra *M. hyopneumoniae* (SIBILA et al., 2004; SORENSE et al., 1997; VIGRE et al., 2004). Estudos têm demonstrado que, em condições de campo, o número de animais soropositivos aumenta gradualmente, tanto para rebanhos vacinados como não vacinados, porém somente a soroconversão induzida pela vacinação com bacterina ou pela infecção natural não previne contra uma infecção futura (MAES et al., 1998; MAES et al., 1999).

A compreensão do papel da citocinas na patôgenese da PES tem sido um tema de grande interesse nas pesquisas atuais (VRANCKX et al., 2012). Durante a infecção, ocorre uma interação dos micoplasmas com o sistema imune inato (macrófagos e células polimorfonucleares – PMN) (CHOI et al., 2006), logo após a invasão do agente no hospedeiro, ocorre a estimulação de citocinas pró-inflamatórias, que são responsáveis pelo surgimento das lesões pulmonares (RODRIGUES et al, 2004), sugerindo o envolvimento da resposa imune no desenvolvimento destas lesões (SIMIONATTO et al., 2013). A presença de citocinas indica que os fagócitos mononucleares, possilvelmente os macrófagos alveolares, tem uma importante ação na resposta inflamatória (CHOI et al., 2006), mas, apesar dos macrófagos serem a primeira linha de defesa na resposta imune inata, eles são os menos efetivos durante a infecção. No controle da infecção por *M. hyopneumoniae* as citocinas apresenam um papel chave tanto na resposta humoral como celular, exercendo suas funções biológicas quando se ligam aos seus receptors específicos nas membranas celulares (TRACKER; MINION, 2010). Basedo em sua atividade, as citocinas são classificadas em: citocinas inflamatórias (Interleucina-1 (IL-1), IL-6, interferon-gamma (IFN-γ) e fator de necrose tumoral alfa (TNF-α) ou citocinas anti-inflamatórias (IL-10 e fator de transformação do crescimento beta (TGF-β). A produção de citocinas pró-inflamatórias tem sido associadas ao desenvolviemnto da lesões características PES. Aumento nos níveis de IL-1, IL-2, IL-6, IL-8, IL-12, IFN-γ e TNF-α tem sido relatado em suínos infectados por *M. hyopneumoniae* (CHOI et al., 2006; MUNETA et al., 2006; REDONDO et al., 2009). Evidencias atuais indicam que a produção de citocinas anti-inflamatórias, especialmente IL-10, pode minimizar a lesão pulmonar em decorrência da resposta inflamatória (HAKIMI et., 2014). Num estudo prévio contatou-se que a indução da IL-10 diminui o influxo de macrófagos para o tecido broncoalveolar, sendo portanto,

que esta citocina exerce um papel importante na modulação do processo inflamatório (KINZENBAW et al., 2013).

2.5 Diagnóstico

A técnica “padrão-ouro” para o diagnóstico da infecção por *M. hyopneumoniae* é o isolamento do agente, porém este método é laborioso e lento devido à caraterística fatidiosa do agente (isolamento de amostras de campo necessitam de 4-8 semanas), além disso, na maioria das vezes, o meio de cultura pode ser contaminado com *M. hyorhinis* ou *M. flocculare* (MAES et al., 1996), o que torna o isolamento inviável no diagnóstico de rotina.

Os métodos de diagnóstico por ELISA de captura (sandwich), PCR e inspeção *post-morten* são as técnicas frequentemente usadas para investigar o envolvimento de *M. hyopneumoniae* na doença respiratória suína (SIBILA et al., 2009). Os sinais clínicos podem oferecer um diagnóstico presuntivo, que são baseados em tosse crônica não produtiva, principalmente nos suínos em fase de terminação, nos casos de infecção secundária os sinais evoluem para febre, anorexia e dispnéia. Para avaliar o sinal clínico de tosse não produtiva, os animais precisam ser obervados e devem ser estimulados a se mover, desta forma, quantifica-se o número de tosses em um determinado período de tempo (MEYNS et al., 2006; MAROIS et al., 2007), porém o diagnóstico definitivo não pode ser estabelecido desta forma (SIBILA et al., 2009).

Em abatedouros, o exame *post mortem* do pulmão pode ser realizado objetivando realizar *“score”* das lesões e a prevalênia da infecção nos rebanhos suínos. Recomenda-se que no mínimo os pulmões de 30 animais devem ser analisados para oferecer uma media confiável (DAVIES et al., 1995). Porém, estes dados são limitados, pois oferece informações a respeito da infecção somente na idade de abate (NOYES et al., 1990).

Os testes sorológicos são os mais frequentemente utilizados para monitorar a presença/prevalência da infecção nos rebanhos suínos. O perfil sorológico por meio do ELISA de um rebanho deve ser realizado testando simultaneamente grupos de animais de diferentes idades (estudo transversal) ou um grupo no mesmo ciclo produtivo (estudo longitudinal). Existem disponíveis no mercado kits ELISA utilizados para detectar anticorpos contra *M. hyopneumoniae*: ELISA de bloqueio (IDEI,

Mycoplasma hyopneumoniae EIA kit, Oxoid) e dois testes ELISA indiretos (HerdCheck, IDEXX and Tween 20-ELISA). Diversos estudos comparativos foram realizados e mostraram diferenças na sensibilidade e especificidade destes kits, que podem variar de 98-100% e 93-100%, respectivamente (STRAIT et al., 2004; AMERI-MAHABADI et al., 2005; ERLANDSON, 2005). Caso o diagnóstico por ELISA não seja conclusivo, o imunoensaio *Western blot,* utilizando diversos antígenos, pode ser realizado como teste confirmatório (AMERI et al., 2006).

Embora os testes de imunohistoquímica (IHC) e imunofluorescência (IFA) detectam o *M. hyopneumoniae* em tecidos pulmonares, estas técnicas têm a desvantagem de poderem ser aplicadas somente no *post-mortem*. Outra desvantagem é que, uma pequena amostra do pulmão é utilizada para a realização das técnicas, aumentando o risco de um resultado falso-negativo (CAI et al., 2007), além disso, a qualidade dos anticorpos utilizados nestas técnicas também pode limitar a deteção específica de *M. hyopneumoniae,* uma vez que anticorpos policlonais podem se ligar com outras espécies relacionadas de *Mycoplasma* (CHEIKH SAAD BOUH et al., 2003).

A técnica de hibridização *in situ* é também é outra opção para diagnóstico, e pode ser utilizada para detectar, em um determinado local, DNA de *M. hyopneumoniae* (KWON e CHAE, 1999). Sondas marcadas do gene 16S ribossomal podem ser utilizadas para a identificação espécie-específica de *M. hyopneumoniae*, *M. hyosynoviae* e *M. hyorhinis* (BOYE et al., 2001). O PCR é outra técnica utlizada para a detecção de DNA de *M. hyopneumoniae* a partir de diferentes amostras; é uma técnica mais rápida que o isolamento e deve-se considerar que o PCR amplifica tanto DNA do micro-organismo vivo ou morto, o que pode não ser conclusivo se a infecção está ativa ou inativa. Considerando que o *M. hyopneumoniae* se adere ao epitélio muco-ciliar do trato respiratório, swabs nasais e traqueo-bronqueal e lavado bronqueo-alveolar (BALF), são materiais preferenciais para a detecção do DNA do agente, tanto de animais vivos como mortos (KURTH et al., 2002). O uso de *nested* PCR (nPCR) para diagnósitco de PES a partir de swabs nasais tem sido amplamente utilizado e tem sido considerado um método diagnóstico eficaz para o monitoramento da infecção de um rebanho ou em nível individual (OTAGIRI et al., 2005). Além disso, detecção de *M. hyopneumoniae* por nPCR é um método sensível para diagnosticar a infecção em animais nos quais ainda não tenha ocorrido a soroconversão (ANDREASEN et al., 2000; LEON et al., 2001).

2.6 Vacinas atuais e perpectivas

Todas as vacinas utilizadas atualmente são compostas por células inteiras inativadas (bacterinas) de *M. hyopneumoniae* (cepa J) em adjuvantes (HAESEBROUCK et al., 2004). Contudo, as vacinas atuais não são capazes de garantir a ausência da infecção por *M. hyopneumoniae* nos rebanhos (SIMIONATTO et al, 2012), pois não impedem a colonização do agente no trato respiratório (SIBILA et al*.,* 2008). Ensaios para avaliar a eficácia da vacinação têm sido realizados em diferentes países e os resultados obtidos têm comprovado que a vacinação traz uma proteção parcial e variável entre os rebanhos. Esta variabilidade, que ocorre tanto na proteção como no desenvolvimento clínico da PES, pode variar entre 8% a 52% no que se refere a sroconversão, é atribuída a diferentes fatores como o número de células infectantes, a idade de exposição dos animais, a variabilidade de virulência entre as cepas e a eficácia das vacinas (CALUS et al., 2007; VILLARREAL et al., 2011; VICCA et al., 2002). Porém, apesar da proteção parcial proporcionada por estas vacinas, esta estratégia ainda é considerada a prática mais efetiva no controle da infecção (MATEUSEN et al., 2002), pois oferece vantagens do ponto de vista econômico e de sanidade (HAESEBROUCK et al., 2004). As principais vantagens incluem aumento de 2-8% no ganho de peso diário e 2-5% nas taxas de conversão alimentar, diminuição no tempo para atingir o peso de abate, redução dos sinais clínicos e das lesões pulmonares (MAES et al., 1999; HAESEBROUCK et al., 2004).

Diante deste quadro, esforços têm sido feitos por diversos grupos de pesquisa com a finalidade de desenvolver uma vacina mais eficiente afim de previnir e controlar a PES. O genoma de tamanho reduzido dos micoplasmas, bem como seu número limitado de proteínas secretadas ou de superfície, têm favorecido o uso da tecnologia do DNA recombinante na busca de novos alvos vacinais mais eficazes contra a PES. Na busca de novos alvos, deve-se considerar que, em micoplasmas, o códon de terminação (*stop codon*) TGA, codifica para o aminoácido triptofano (RAZIN et al., 1998), o que dificulta a expressão de genes contendo o códon TGA em *E. coli*. Porém, mutações sítio-dirigidas capazes de substituir os códons TGA por TGG já foram eficientemente realizadas (SIMIONATTO et al., 2009). A identificação e caracterização de proteínas recombinantes antigênicas e imunogênicas é um passo importante para seleção de novos alvos vacinais a serem explorados (SIMIONATTO et al*.,* 2009). Vários estudos tem demonstrado o potencial de

diversas proteínas recombinantes, que foram avaliadas em várias formulações e formas de administração, como alvos vacinais, das quais alguns foram testadas individualmente (GALLI et al., 2012; SIMIONATTO et al., 2012), outros foram associados a bactérias atenuadas ou vetores virais (CHEN et al., 2006a,b; FAGAN et al., 2001; OKAMBA et al., 2010; OKAMBA et al., 2007; SHIMOJI et al., 2003; ZOU et al., 2011), fusionados à adjuvantes de mucosa (CONCEIÇÃO et al., 2006) e ainda alguns ensaios experimentais foram realizados utilizando *cocktail* de antígenos (CHEN et al., 2008).

Dentre estes alvos, um fragmento da uma proteína chaperona molecular (MHP0067), a P42 têm se mostrado promissora como antígeno vacinal, tanto como vacina de subunidade como vacina de DNA. Em um estudo prévio, a proteína rP42 foi capaz de induzir soroconversão em camundongos imunizados (SIMIONATTO et al., 2012). Quando testada como vacina de DNA, a P42 induziu tanto resposta humoral como celular em camundongos, especialmente na expressão das citocinas IL-4, IL-10 e INF-γ, além disso os anticorpos anti-P42 induzidos foram capazes de inibir o crescimento i*n vitro* de *M. hyopneumoniae* (CHEN et al., 2003). Poucos antígenos recombinantes foram avaliados quanto ao seu potencial vacinal utilizando suínos como modelo animal, dentre os que foram avaliados, alguns se mostraram promissores e se mostraram capazes de induzir proteção parcial (FAGAN et al*.,* 2001; OKAMBA et al*.,* 2010; SHIMOJI et al., 2003), no entanto, a avaliação da imunogenicidade e antigenicidade do fragmento de 42 Kda da chaperona molecular MHP0067 utilizando suínos como modelo animal nunca foram relatados na literatura.

Desde 1987, o termo “chaperona molecular” tem sido utilizado para decrever uma família de proteínas não relacionadas entre si que tem a capacidade de participar no processo de *folding* de outras proteínas. Desde então, mecanismos estruturais e funcionais destas chaperonas, denominadas como Hsp (*Heat shock protein*) 60 e Hsp 70, têm sido estudados (ELLIS, 1987, DE MAIO, 1999, MAYER; BUKAU, 2005). As Hsp são expressas quando as células são submetidas a perturbações fisiológicas e outros fatores de estresse, como por exemplo, mudanças bruscas de temperatura (DE MAIO, 1999). Esta classe de proteínas age como chaperonas moleculares, atuando no processo de enovelamento da proteina, sem fazer parte da estrutura final da proteina (ELLIS, 1987). As Hsps70 são o grupo de proteínas dominantes dentre as proteínas de choque térmico, sendo que podem ser encontradas no citossol e em outras regiões da célula. Trabalhos de pesquisa já

demonstraram que as Hsp possuem função imunológica, sendo relacionadas como carreadoras de antígenos além de induzirem uma resposta imune antígeno-específica e atuarem como adjuvante (RICO et al., 1999; ROMAN; MORENO, 1996; SUZUE; YOUNG, 1996). As Hsps são altamente conservadas entre organismos procarióticos e são reconhecidas como antígenos imunodominates em muitas bactérias, desta forma, a importância destas proteínas como antígenos vacinais em várias infecções parasitárias e bacterianas são objetos de pesquisas (KARLIN; BROCHIERI, 1998; ZHANG et al., 2013).

3 OBJETIVOS

3.1 Objetivo geral

Avaliar a resposta imune celular e humoral em suínos vacinados com o antígeno recombinante P42 de *Mycoplasma hyopneumoniae*, visando conhecer o seu potencial para o desenvolvimento de uma vacina mais eficiente contra a Pneumonia Enzoótica Suína.

3.2 Objetivos específicos

- Amplificar e clonar o fragmento gênico correspondente ao antígeno P42 (MHP0067) em vetor de expressão (pAE) em *E. coli*
- Expressar em *E. coli* e purificar o antígeno rP42;
- Vacinar suínos aos 21 dias de idade com formulações vacinais que incluam o antígeno rP42 em granja com *status* de positiva para a PES;
- Avaliar a resposta imune humoral e quantificar as citocinas Interleucina-10 e Interferon-γ no sobrenadante do cultivo *in vitro* de células mononucleares dos suínos mantidos em condições de campo.

4 ARTIGO

THE *Mycoplasma hyopneumoniae* RECOMBINANT HEAT SHOCK PROTEIN P42 INDUCES AN IMMUNE RESPONSE IN PIGS UNDER FIELD CONDITIONS

(Artigo submetido ao periódico *Comparative Immunology, Microbiology and Infectious Diseases*)

THE *Mycoplasma hyopneumoniae* RECOMBINANT HEAT SHOCK PROTEIN P42 INDUCES AN IMMUNE RESPONSE IN PIGS UNDER FIELD CONDITIONS

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ABSTRACT

Enzootic pneumonia (EP), resulting from *Mycoplasma hyopneumoniae* infection is one of the most prevalent diseases in pigs and is a major cause of economic losses to the swine industry worldwide. EP is often controlled by vaccination with inactivated, adjuvanted whole-cell bacterin. However, these bacterins provide only partial protection and do not prevent *M. hyopneumoniae* colonization. Attempts to develop vaccines that are more efficient have made use of the recombinant DNA technology. The objective of this study was to assess the potential of recombinant *M. hyopneumoniae* heat shock protein P42 in vaccine preparations against EP, using piglets housed under field conditions in a *M. hyopneumoniae*-positive farm. The cellular and humoral immune responses were elicited after a single intramuscular inoculation of rP42 in an oil-based adjuvant, or in conjunction with whole-cell vaccine preparation. The expression of INF-γ and IL-10 cytokines was quantified in the supernatant of the cultured mononuclear cells. The rP42 emulsified in oil-based adjuvant was able to trigger a strong humoral immune response. Further, it induced a cellular immune response, accompanied by the production of antibodies that reacted with the native *M. hyopneumoniae* protein. The rP42-mediated induction of cellular and humoral immune response in the host suggests that rP42 emulsified in an oil-based adjuvant holds promise as an effective recombinant subunit vaccine against EP.

Key Words: Enzootic pneumonia, subunit vaccine, heat shock protein.

1. INTRODUCTION

Mycoplasma hyopneumoniae is the causative agent of enzootic pneumonia (EP), a highly prevalent respiratory disease, responsible for significant economic losses to the swine industry. This disease is characterized by chronic non-productive coughing, poor growth rate, low feed conversion, and delayed weight gain, with high morbidity and low mortality. In addition, EP increases the host's susceptibility to other respiratory pathogens and secondary infections [1;2].

Vaccination with inactivated, adjuvanted whole-cells is frequently used worldwide to control *M. hyopneumoniae* infections [3], but their efficiency is questionable. The production costs of these vaccines are very high because of the difficulties associated with the *in vitro* cultivation of *M. hyopneumoniae* [4]. Furthermore, they provide only partial protection [4;5] and are unable to prevent the transmission or establishment of the microorganism in the lungs [6;7]. Thus, there is a need to develop more effective and less expensive vaccines against EP. Recombinant DNA technology can be useful in overcoming the common problems encountered with conventional vaccines, and the small genome of this pathogen, as well as the limited number of secreted or surface proteins, favors the use of reverse vaccinology approach [8].

The genomes of four strains of *M. hyopneumoniae* have been sequenced [9-11] and proteomic analysis has been performed for two *M. hyopneumoniae* strains [11]. Our research group produced 35 secreted [12;13] and six transmembrane recombinant proteins [14] expressed in *Escherichia coli*, and evaluated their immunogenic and antigenic properties. A few of these proteins were identified as potential vaccine antigens, including well-known immunodominant antigens found in several bacterial species, such as the Heat shock protein (Hsp) family members that

are highly conserved in prokaryotes [15;16]. One such Hsp family member is the *M. hyopneumoniae* molecular chaperone DnaK (P42 heat shock protein) that has been shown to induce strong immune responses in mice [12;17] and was recognized by sera from convalescent pigs [12].

In this work, the cellular and humoral immune responses stimulated by recombinant Heat shock protein P42 (rP42) subunit vaccine preparations were evaluated in piglets under field conditions in a *M. hyopneumoniae*-positive farm.

2. MATERIAL AND METHODS

2.1. Cloning, expression, and purification of rP42

Genomic DNA extraction of *M. hyopneumoniae* strain 7448 was performed using the illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The *p42* gene was amplified using the primer pair P42_F (5′-taGGATCCATGGCGCTTACAAGAC) and P42_R (5′-cggGGTACCTTAATCCTGCTTG). The PCR reaction mixture consisted of 50 ng of *M. hyopneumoniae* genomic DNA, 0.2 mM dNTP, 2.5 mM $MgCl_2$, 10 pmol of each primer, 2.5 units of Platinum *Pfx* DNA polymerase (Life Technologies, New York, USA), 1x reaction buffer and 1x enhancer buffer in a final volume of 25 µL. DNA amplification was performed using a Gradient Mastercycler (Eppendorf, New York, USA) with the following settings: 7 min at 95 °C, followed by 30 cycles of 60 s at 95 °C, 60 s at 55 °C, and 60 s at 68 °C, and then a final extension of 7 min at 68 °C. The amplicon was cloned into pAE expression vector [18], and the ligation products were transformed *E. coli* TOP10 electro competent cells. Recombinant clones were identified by agarose gel electrophoresis of extracted plasmid DNA and confirmed by restriction enzyme digestion. The integrity of the insert was confirmed by DNA sequencing using the DYEnamic ET Dye Terminator Cycle Sequencing Kit for

MegaBACE DNA Analysis Systems–MegaBACE 500 (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Purification of rP42 was performed by a previously described method [12]. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Thermo Scientific, Rockford, IL, USA).

2.2 Western blot analysis

The purified rP42 was separated on 12% SDS–PAGE, electrotransferred onto a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and blocked with 5% non-fat dry milk in phosphate buffered saline (PBS) at 37 °C for 2 h. Two Western blot analyses, one using an anti-6× His antibody (Sigma Aldrich, Saint Louis, Missouri, USA), and another using a polyclonal anti-rP42 antibody, previously produced in mice [12] were performed to confirm the presence of the purified protein, to determine the apparent molecular mass of the recombinant protein, and to assess the antigenicity. After washing with PBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse IgG monoclonal antibody. Immunoreactive protein bands were detected with 0.005% (w/v) 4-chloro-1-naphthol and 0.015% (v/v) hydrogen peroxidase in PBS.

2.3. Experimental design

The study was performed after obtaining the approval of the Ethical Committee for Animal Experiments of the Universidade Federal de Pelotas (approval number 5614). All the animals belonged to a commercial farm in Southern Brazil and were maintained under field conditions. The herd was screened using a commercial indirect ELISA kit (IDEXX *M*. *hyo*. antibody ELISA, Westbrook, Maine, USA) and nested PCR. Forty piglets at 21 days of age (8 animals per group) were randomly allocated into five treatment groups and immunized intramuscularly (IM) with a single dose of the following: group 1, oil-adjuvant only (Marcol-Montanide®, SEPPIC, Paris,

France); group 2, commercial *M. hyopneumoniae* inactivated whole-cell vaccine (RespiSureOne®, Pfizer Animal Health, Florham Park, New Jersey, USA); group 3, RespiSureOne® + rP42; group 4, Marcol-Montanide® + rP42 (used at a ratio of 1:1); and group 5, PBS + rP42 (Table 1). Blood samples were collected from the jugular vein at 1, 21, 42, 63, and 84 days post inoculation (d.p.i.). The herd used was not vaccinated for EP before the study began, and antibiotics effective against *M. hyopneumoniae* were not used.

2.4. Nested PCR for detection of *M. hyopneumoniae* DNA

Nasal swabs were collected at 1 day from each piglet used in this study were placed in a tube containing 1 mL of PBS and tested for *M. hyopneumoniae* by *nested* PCR (nPCR). The tubes were centrifuged at 14,000 *g* for 30 min, and the pellet resuspended in 250 µL of PBS. Bacterial suspensions were boiled for 5 min, DNA was extracted with phenol:chloroform, and precipitated with ethanol and sodium acetate (pH 5.2, final concentration of 0.5 M). The DNA was resuspended in 40 µL of double-distilled water, and nPCR was performed as previously described [19].

2.5. Evaluation of the humoral immune response with ELISA using rP42 protein

Dilutions (1:50) of specific antibodies against recombinant rP42 in sera from piglets from control groups, or from groups inoculated with the experimental vaccine formulations, were assayed using the indirect ELISA method. Microtiter plates were coated with 50 ng/well of purified recombinant protein in 50 mM carbonate-bicarbonate buffer (pH 9.8), at 4 °C overnight. The plates were washed with PBS (pH 7.4) containing 0.05% (v/v) Tween 20 (PBS-T) and incubated with 5% blocking buffer (non-fat dry milk) at 37 °C for 2 h. After washing with PBS-T, the wells were incubated with swine serum diluted 1:50 in blocking buffer, at 37 °C for 2 h. After washing with PBS-T, the wells were incubated with rabbit anti-pig IgG peroxidase

conjugate (Sigma) diluted 1:6000, at 37 °C for 1 h. The reactions were developed using o-phenylenediamine dihydrochloride (Sigma) and hydrogen peroxide, after PBS-T washes. The color reaction was allowed to develop for 15 min and stopped with 50 µL of 2 M H_2SO_4. Absorbance was determined at 492 nm with a microplate reader. The mean and standard deviation (S.D.) values for triplicate samples were calculated.

2.6. ELISA with extract of *M. hyopneumoniae* 7448 strain as antigen

ELISA with *M. hyopneumoniae* extract was performed with sera from piglets inoculated with vaccine formulations to verify whether the antibodies induced by this antigen were able to recognize native proteins of *M. hyopneumoniae*. For this experiment, the cells of *M. hyopneumoniae* strain 7448 were centrifuged and lysed using detergent buffer (Triton X-100). The crude extract (1 µg), dissolved in 0.1 M sodium carbonate buffer (pH 9.6), was added to each well of the microtiter plate. The plates were incubated overnight at 4 °C, then incubated at −70 °C for 2 h and thawed at room temperature for 30 min. The following steps were performed as previously described in section 2.5.

2.7. Peripheral Blood Mononuclear Cells culture

Blood samples were collected in sterile Vacutainer tubes (Becton Dickinson, Grenoble, France) containing 100 U/mL of heparin (Choay, Paris, France). After lysis of erythrocytes using ammonium chloride (74.7%) and subsequent centrifugation, the pelleted cells were washed, resuspended in PBS with 1 mM of EDTA, and cultivated in Gibco Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, New York, USA) at a concentration of 10^6 cells/mL. Lymphocytes from the animals of the each group were pooled, seeded in 24-well microtitre plates (TPP), and incubated at 37°C in 5% CO_2 atmosphere. Monomorphonuclear cells in Gibco DMEM Medium alone (no

stimulation), concanavalin A (7 µg/well) or with rP42 antigen (15 µg/well) were added to the wells. After an incubation period of 24 h, the contents of the wells were harvested and stored frozen at -20 °C until use.

2.8 Cytokine quantification in the supernatant of mononuclear cell culture

Cell culture supernatants were harvested and analyzed for cytokine production by ELISA using Invitrogen Swine Interleukin-10 and Swine INF-γ kits, according to the manufacturer's instructions. The optical density (OD) at 450 nm was measured and the cytokine levels were quantified using a standard curve.

2.9 Statistical analysis

Descriptive statistics were performed to check the normality of the variables (cytokines and serum immunoglobulins). The Bonferroni ANOVA was used. Results were considered significantly different at a *P* value of <0.05 (two-sided test). All the analyses were performed with GraphPad Prism® Version 5.01 for Windows (SPSS inc. Illinois, USA).

3. RESULTS

3.1 Cloning, expression, and purification of rP42

PCR amplified coding sequence for P42 was purified and cloned into pAE expression vector. After confirmation of the insert by restriction enzymes digestion and DNA sequencing, the recombinant protein was expressed in *E. coli* BL21 (DE3) Star and purified by affinity chromatography. The results of SDS-PAGE showed that the expressed recombinant protein was insoluble (data not shown), and N-Lauroylsarcosine (Sigma-Aldrich) was used for solubilization.

3.2 Antigenicity of the recombinant protein

Western blot anlaysis revealed that different antibodies recognized rP42 (Figure 1). Accordingly, the recombinant protein was recognized by polyclonal anti-

rP42 (Figure 1A) and anti-histidine monoclonal antibody (Figure 1B), confirming its antigenicity.

3.3 Detection of *M. hyopneumoniae* DNA from nasal swab

Nested PCR of the 16S ribosomal DNA using two species-specific primer sets was performed to confirm the status of the herd. *M. hyopneumoniae* DNA was detected in 85% of the nasal swabs from piglets at 21 days of age used in this study (Figure 2).

3.4 Humoral immune response against rP42

The serological profile of the animals is shown in Figure 3. Twenty-one days post inoculation (d.p.i.), significant seroconversion ($P < 0.05$) was detected in piglets vaccinated with Respisure-One®+rP42 (Group 3) and Montanide™+rP42 compared with those in the negative and positive control groups (Montanide™ and Respisure-One®, respectively). The levels of specific antibodies against rP42 remained high for over 84 d.p.i. in animal groups 3 and 4. The negative control group inoculated with oil-based adjuvant, revealed seroconversion ($P < 0.05$) at twenty-one d.p.i., compared with that on the pre-immune day.

3.5 Detection of *M. hyopneumoniae*-specific serum antibodies

ELISA was performed to quantify the humoral antibody response against *M. hyopneumoniae* whole cell extract (Figure 4). Significant levels of circulating antibodies were detected ($P < 0.05$) in piglets vaccinated with rP42 + Montanide™ preparation (Group 4) at 21, 42, and at 63 d.p.i., compared with all the other experimental groups. At 84 d.p.i., no statistical difference was detected in piglets vaccinated with Respisure-One® + rP42 and rP42 + Montanide™ when compared.

3.6 IFN-γ and IL-10 secretion by peripheral blood mononuclear cells

IL-10 and IFN-γ concentrations in the supernatant of mononuclear cells isolated from peripheral blood are shown in figure 5 and figure 6, respectively. Significantly higher amounts of IL-10 were obtained from the group vaccinated with rP42 + Montanide™ ($P < 0.05$), compared to those from all the other groups, when stimulated with rP42 antigen. No significant differences in IFN-γ were observed between groups, when the cells were stimulated with rP42 antigen at the time points evaluated.

4. DISCUSSION

Enzootic pneumonia is a chronic respiratory disease occurring worldwide, and currently available vaccines are costly and fail to offer complete protection. To this end, efforts to develop more effective vaccines against EP are in progress, and vaccines developed using recombinant DNA technology present a viable alternative [20]. In this study, we evaluated the immune responses induced by *M. hyopneumoniae* recombinant heat shock protein (Hsp) P42, an antigen that is expressed in large amounts, exposed on the surface of the pathogen, and is possibly involved in the pathogenesis of EP [12;13;17]. Both, humoral and cellular immune responses were induced when rP42 was delivered in an oil-based adjuvant (Montanide™) or in conjunction with the whole-cell vaccine preparation. The results indicate that immunization with rP42 + Montanide™ is a promising approach for inducing antibodies against *M. hyopneumoniae* and induction of IL-10 in pigs under field conditions.

Hsp family members are highly conserved in prokaryotic organisms and are known to be immunodominant antigens in many bacteria. Hsp are a class of functionally related proteins, whose expression is increased when cells are exposed to physiological perturbations or other stress factors [21;22]. Microbial Hsp70s have

acquired special significance in immunity, since they have been shown to be potent activators of the innate immune system and generate specific immune responses against infectious agents [16]. Moreover, the Hsp proteins have demonstrated immunomodulatory properties, which can be exploited in the development of potent vaccine adjuvants for stimulating cytokine-mediated immune responses [15]. Previous studies had already reported that a recombinant Hsp from *M. hyopneumoniae*, the heat shock protein P42, was able to induce seroconversion in a mice model [12;17] and anti-P42 antibodies have been shown to bind specifically to the antigenic heat shock protein of *M. hyopneumoniae* and inhibit cell growth [23]. Moreover, Western blot analysis revealed that rP42 was recognized by sera from convalescent pigs in the antigenicity assay [12]. However, experiments to evaluate the immunogenicity of rP42 subunit vaccine preparations had never been performed in swine model. In this study was to assess the potential of recombinant P42 as a subunit vaccine and/or as adjuvant, when used in combination with a conventional whole-cell vaccine for inducing humoral and cellular immune responses against EP, using piglets under field conditions in a *M. hyopneumoniae*-positive farm.

Recombinant P42 expressed in *E. coli* and emulsified in oil-based adjuvant (Montanide®), significantly increased the humoral and cellular responses, but had no significant effect when administered as a recombinant subunit vaccine in PBS in the absence of an adjuvant. The oil-based adjuvant therefore, plays an important role in the enhancement of the immunogenicity of rP42 vaccine preparation. Oil in water emulsions are well tolerated, induce strong immune responses, and can be safely used for fattening pigs in order to enhance antibody responses against bacterial infections. In addition, the manufacturing process is very convenient, and oil based adjuvants are used in commercially available *M. hyopneumoniae* vaccines [24-27].

Interestingly, the piglets immunized with rP42 + Montanide™ showed significantly higher specific IgG level against *M. hyopneumoniae* extract at 21, 42, and 63 d.p.i. when compared to the levels of IgG in the all other groups inoculated with the experimental vaccine formulations. Moreover, seroconversion was higher in groups immunized with rP42 + Montanide™ and with rP42 + commercial vaccine (RespiSureOne®), than that in the other groups, from 21 d.p.i. As expected, the negative control group inoculated with oil-based adjuvant revealed significant seroconversion ($P < 0.05$) at 21 d.p.i. compared with that on the pre-immune day, due to *M. hyopneumoniae* infection in piglets under field conditions. The significant seroconversion ($P < 0.05$) was detected when negative control group was compared with all other groups at time points.

Previous reports have suggested that protection against *M. hypneumoniae* requires humoral and cell-mediated immune responses [3;6]. The role of cytokines in the pathogenesis of EP has been a topic of considerable interest; they exert biological effects by binding to specific receptors on target cell membranes [28;29]. In the present study, pigs immunized with rP42 + Montanide™ responded with higher levels of anti-inflammatory IL-10 cytokine when compared with levels detected in the all other experimental group, with no significant expression of pro-inflammatory IFN-γ cytokine. Current evidence suggests that anti-inflammatory cytokine production may minimize disease severity by reducing the development of lesions in the lung. IL-10 plays a major role in the inhibition of apoptosis [30;31]. Indeed, in a previously reported study, induction of IL-10 secretion resulted in a lower influx of macrophages in the bronchoalveolar lymphoid tissue [28], an important factor that regulates the inflammatory process [30;31].

Although immunity is known to control and prevent infections, immune responses can also promote the formation of inflammatory lesions associated with mycoplasmal disease [28;29;32]. The presence of inflammatory cytokines, the products of activated macrophages, suggests that mononuclear phagocytes, possibly alveolar macrophages, have an important role in the initiation of the inflammatory response [33]. The increased levels of IFN-γ and other pro-inflammatory cytokines, has been shown to be associated with the development of *M. hyopneumoniae*-induced pneumonia [34;35]. In this study, we observed no difference in IFN-γ levels across vaccinated groups, compared to the control group. Even in the group that received adjuvant only, IFN-γ expression was observed after stimulating the cells with rP42, probably due to the fact that the animals were naturally infected with *M. hyopneumoniae*.

Current vaccines against EP cannot prevent colonization of *M. hyopneumoniae* in the respiratory tract, nor significantly reduce the transmission of the pathogen [20;29;36;36]. Identification and characterization of immunogenic recombinant proteins is an important approach towards the development of improved vaccines [13;17]. This strategy may potentially yield a new vaccine efficient enough to control *M. hyopneumoniae* infection. However, only three recombinant antigens have been tested in pigs until now, and all of them were able to provide only partial protection in vaccinated animals [37-39]. In the present work, piglets immunized with rP42 in oil-based adjuvant were able to produce a humoral immune response that recognized the native antigen present in *M. hyopneumoniae*. In addition, rP42 stimulated a significant expression of anti-inflammatory IL-10 cytokine.

In conclusion, the present study showed that the *M. hyopneumoniae* heat shock protein P42 administered in oil-adjuvant induced both humoral and cellular

immune responses in pigs under field conditions in a mycoplasma-positive farm. The data presented here indicate that rP42 is a promising candidate antigen for use in vaccine for the management and successful control of EP.

Conflict of interest

The authors declared no conflicts of interest.

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Figures and tables list

Figure 4. ELISA using *M. hyopneumoniae* extract as an antigen. Detection of the antibodies in groups of piglets immunized with four vaccine formulations and negative control at days 1, 21, 42, 63, and 84. Group 1, oil-adjuvant only (Marcol-Montanide®); group 2, commercial *M. hyopneumoniae*-inactivated whole-cell vaccine (RespiSureOne® vaccine, Pfizer); group 3, RespiSureOne® plus 100 µg rP42; group 4, 100 µg rP42 + Montanide® (used at a ratio of 50/50), and group 5, 100 µg rP42 + PBS. Numbers are the mean values in each group, and error bars demonstrate standard deviations (SD). Statistical significance was defined at *P* values of <0.05. The significant seroconversion was detected when RespiSureOne® plus 100 µg rP42 and rP42 plus Montanide was compared with all other groups at time points.

Figure 5. Expression of IL-10 after stimulation of lymphocytes from peripheral blood mononuclear cells with rP42 in piglets immunized with the distinct vaccine formulations. Values are shown as the mean ± SD of pool of 8 piglets per group, performed in triplicate. Significantly higher amounts of IL-10 were obtained from the group vaccinated with rP42 in oil-based adjuvant ($P < 0.05$) in comparison with those from all the others groups stimulated with rP42 antigen.

Figure 6. Expression of IFN-γ after stimulation of lymphocytes from peripheral blood mononuclear cells with rP42 in piglets immunized with the distinct vaccine formulations. Values are shown as the mean ± SD of pool of 8 piglets per group, performed in triplicate. No significant differences were observed between the groups stimulated with rP42 antigen at the time points evaluated.

Table 1

Group	Immunogen	Dose	Route
Group 1	Oil-adjuvant* (control)	2 mL	i.m.
Group 2	Respisure-One®	2 mL	i.m.
Group 3	Respisure-One® + rP42	2 mL + 100 µg	i.m.
Group 4	Oil-adjuvant* + rP42	2 mL + 100 µg	i.m.
Group 5	PBS + rP42	2 mL + 100 µg	i.m.

*mineral oil based adjuvant Montanide™; i.m., intramuscular injection

Figure 1

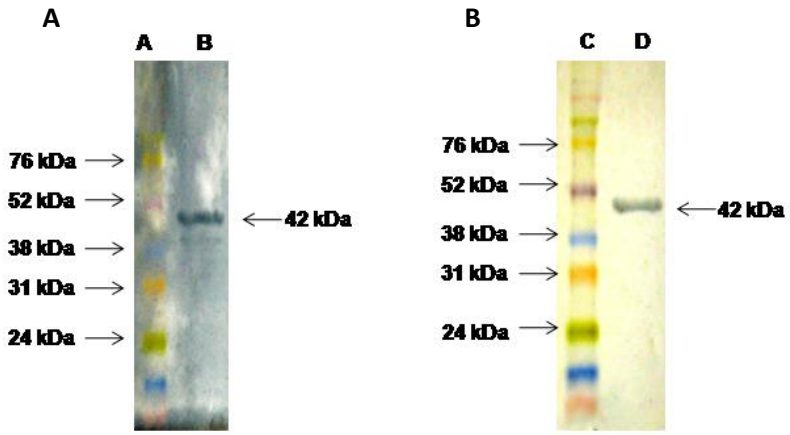


Figure 2

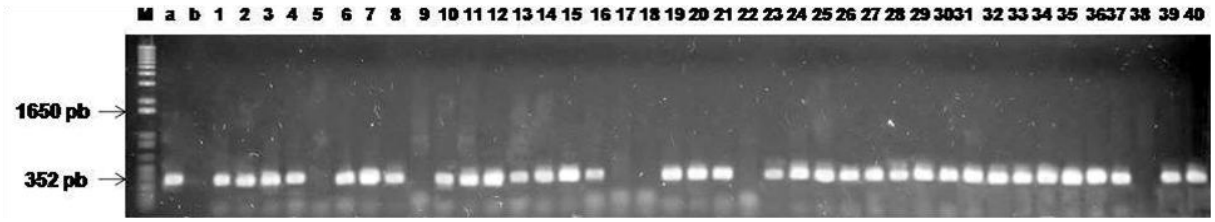


Figure 3

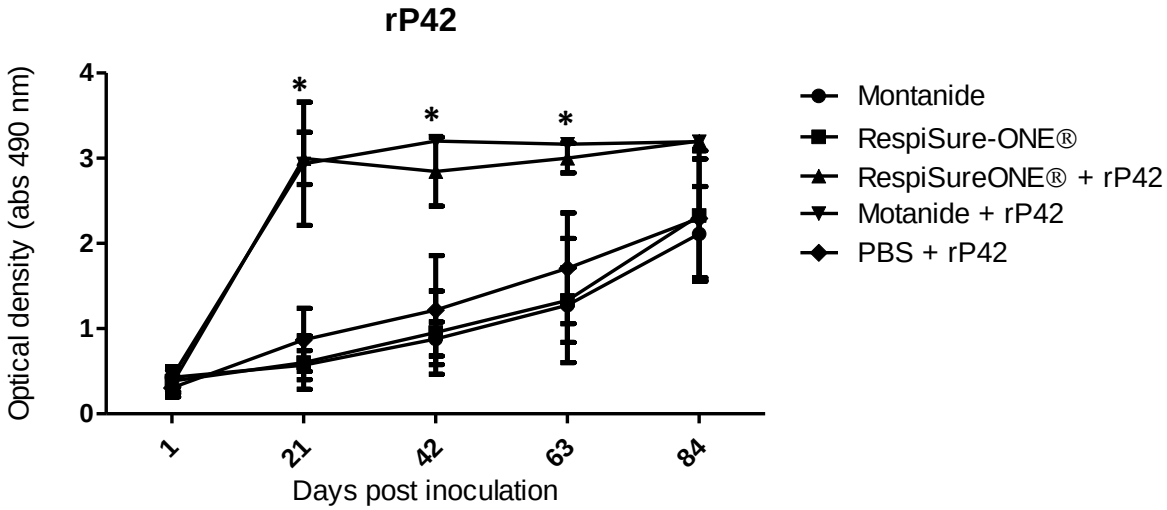


Figure 4

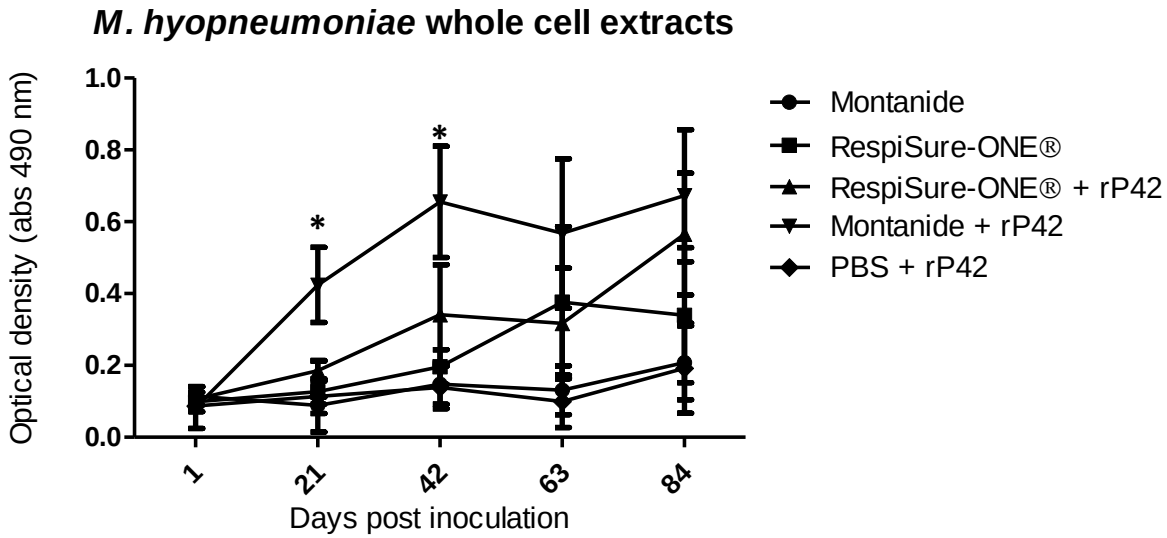


Figure 5

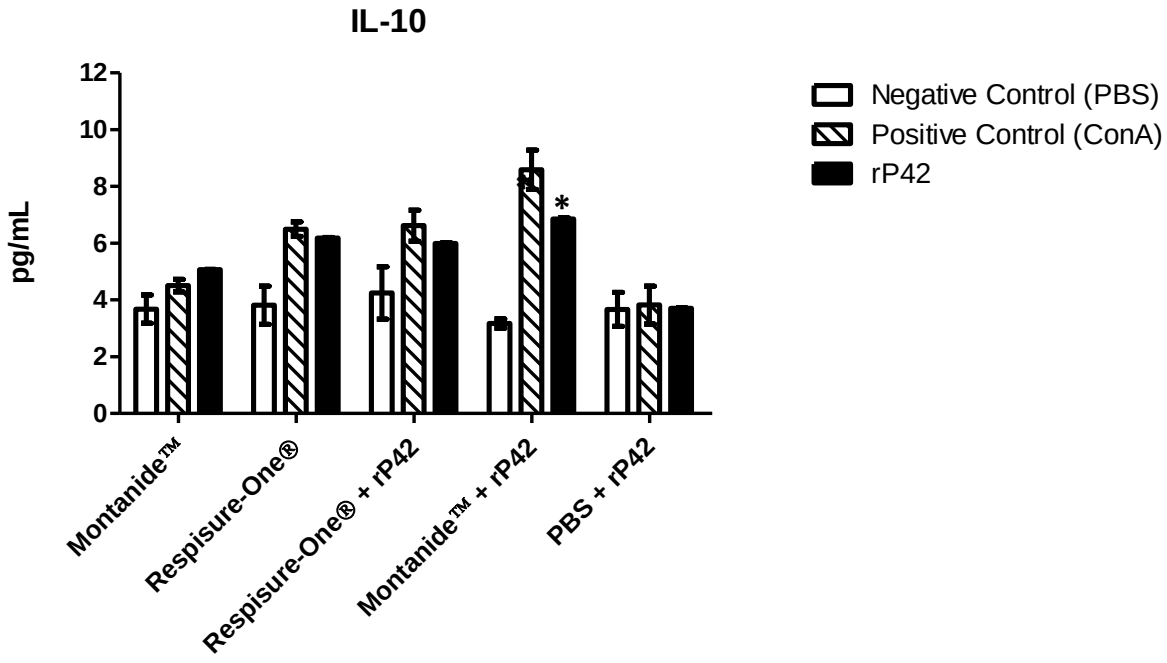
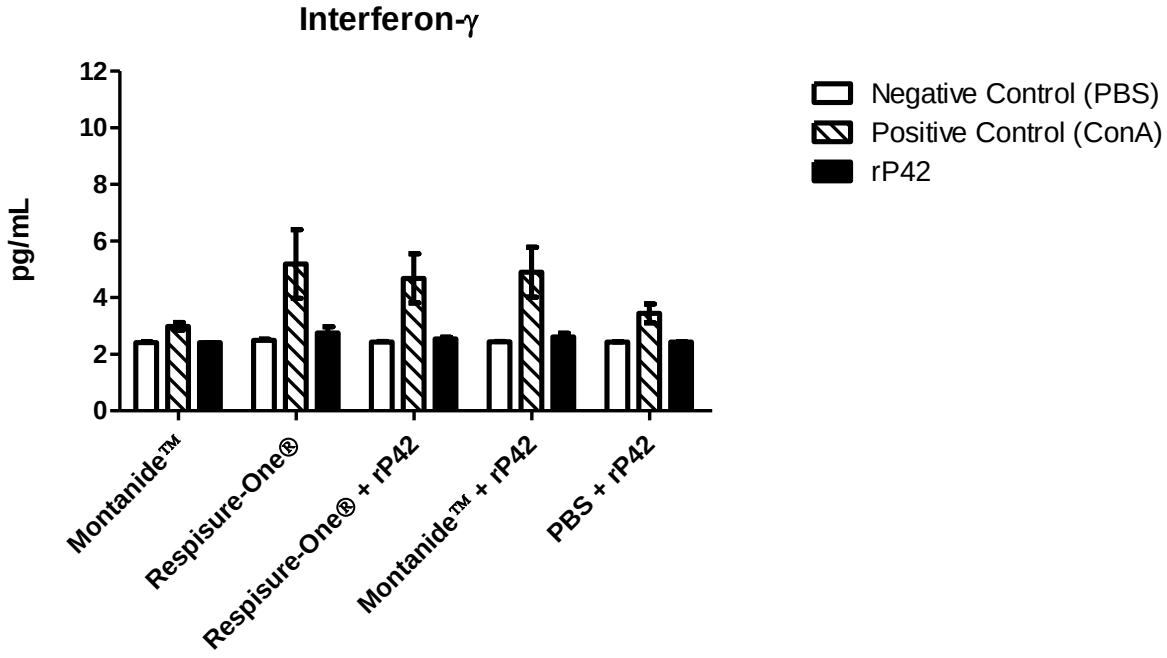


Figure 6



5 CONCLUSÕES

- A proteína recombinante desenvolvida é imunogênica em suínos, sendo que quando emulsificada em adjuvante oleoso é capaz de induzir uma resposta imune específica nos animais mantidos em condições de campo.
- A vacinação intramuscular com rP42 é capaz de estimular a expressão da citocina anti-inflamatória IL-10 em maiores níveis que a vacina comercial em cutivo *in vitro* de células mononucleares.
- Os anticorpos produzidos pela imunização com a rP42 são capazes de reconhecer o *M. hyopenumoniae*.
- rP42 é um antígeno promissor para a composição de uma nova e efetiva vacina recombinante contra pneumonia enzoótica suína.

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ANEXOS

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Leptospira borgpetersenii from free-living white-eared opossum (*Didelphis albiventris*): First isolation in Brazil

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ABSTRACT

Leptospirosis is a zoonotic disease that occurs all over the world, caused by bacteria of the genus *Leptospira*. Marsupial and didelphidae families are considered susceptible to infection caused by a wide range of *Leptospira* serovars for which they serve as reservoirs. Thirty-three free-living white-eared opossums (*Didelphis albiventris*) were captured in Southern Brazil and bodily fluids were collected. From the urine samples it was possible to obtain an isolate identified as *Leptospira borgpetersenii* by *rpoB* gene sequencing and belonging to serovar Castellonis by Multilocus Variable-Number Tandem-Repeat Analysis. This is the first report of the isolation of *Leptospira* spp. from the white-eared opossum in Brazil. In addition, the new strain was also virulent in the hamster model of lethal leptospirosis. The microscopic agglutination test (MAT) was used for detecting the presence of antibodies against *Leptospira* spp. in white-eared opossum, human, cattle and canine sera using a panel of 59 *Leptospira* strains that included the new isolate. The inclusion of the new strain in the MAT battery increased the MAT sensitivity for canine sera. These findings suggest that the white-eared opossum is an important reservoir of pathogenic *Leptospira* spp.

1. Introduction

Leptospirosis is a worldwide zoonosis with high mortality and morbidity rates, usually transmitted to humans through contaminated water or direct exposure to the urine of infected animals (Cerqueira and Picardeau, 2009; Ko et al., 2009; Levett, 2001). Tropical countries offer greater opportunities for exposure of the human population to infected animals. The presence of infected wildlife and domestic animals poses a persistent public health threat. Leptospires usually gain access to new hosts by passage across mucous membranes or through skin abrasions, often from environmental sources such as urine-contaminated water (Faine et al., 1999; Levett, 2001). The disease is seasonal, with peak incidence occurring in the summer or fall in temperate regions and during rainy seasons in warm-climate regions (Levett, 2001).

The causative agent of leptospirosis belongs to the genus *Leptospira*, which contains both saprophytic and pathogenic species (Faine et al., 1999; Levett, 2001). Pathogenic leptospires are classified into at least 12 genomic species, with *Leptospira interrogans* and *Leptospira borgpetersenii* being the main cause of human disease worldwide (Dolhnikoff et al., 2007; Isturiz et al., 2006; Yasuda et al., 1987). Based on serological criteria, strains of *Leptospira* spp. are differentiated into serovars, which represent the basic taxonomy. Antigenically related serovars are grouped into serogroups. Serogroups are of clinical and epidemiological importance and the use molecular techniques provides the prompt identification of *Leptospira* species (Cerqueira and Picardeau, 2009; Ko et al., 2009; Levett, 2001).

Since the discovery of *Leptospira* spp., this spirochete has been isolated from the environment and a wide spectrum of animals. Many domestic and wild mammals have been found to be either natural reservoirs or accidental hosts for various serovars (Faria et al., 2008; Glosser et al., 1974; Liceras de Hidalgo and Sulzer, 1984; Lins and Lopes, 1984; Santa Rosa et al., 1980) and information on clinical leptospirosis in feral animals has been gathered mainly from observations on captured animals (Faine et al., 1999).

The same animal species inhabiting distinct ecological niches in different countries may represent different ecosystem types for specific *Leptospira* serovars (Hathaway et al., 1981). The white-eared opossum is a species highly capable of adapting to environmental variations and is widespread in some countries in

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South America: Argentina, Bolivia, Brazil, Paraguay and Uruguay. It is a terrestrial and, sometimes, arboreal animal, living in a wide range of different habitats. It is an omnivorous animal, feeding on invertebrates, small vertebrates, fruits and plants (Eisenberg and Redford, 2000). This animal often comes in direct or indirect contact with man, both in rural and in urban areas. Thus, serological surveys of these animals in different ecosystems are important for an initial assessment of the prevalence of certain serogroups of *Leptospira* spp. within the sampled population to elucidate the importance of this species in leptospirosis transmission. However, the infecting serovar cannot be unequivocally determined by this means. Isolation of the agent is necessary to identify the infecting serovar which should be included in the battery of strains used in the microscopic agglutination test (MAT) (Levett, 2001).

In this study, 33 free-living white-eared opossum were captured in Southern Brazil and bodily fluids were collected. From the urine samples it was possible to obtain an isolate that was characterized by *rpoB* gene sequencing and Multilocus Variable-Number Tandem-Repeat Analysis (MLVA). The new isolate was inoculated in Golden Syrian hamsters for histopathological studies and the MAT was performed in order to verify the presence of antibodies to this isolate among cattle, dogs, humans and captured white-eared opossums in the study area.

2. Materials and methods

2.1. Capture of animals and specimen collection

Thirty-three white-eared opossum were captured in Capão do Leão (31°48′S, 52°24′W) and Pelotas cities (31°46′S, 52°20′W) in the state of Rio Grande do Sul, Brazil. Animals were taken to the *Núcleo de Reabilitação da Fauna Silvestre/Centro de Triagem de Animais Silvestres* of the *Universidade Federal de Pelotas* (NURFS/CETAS-UFPel). The animals were chemically restrained according to protocols previously described (Pachaly and Brito, 2001). A loop diuretic (Furosemide 2 mg/kg) and anesthetic solution containing tiletamine and zolazepam (Zoletil) was used. Urine samples were collected by bladder puncture. Blood was collected by cardiac puncture, centrifuged and serum was stored at −20 °C until use. The procedures used in the present study were approved by the relevant federal authorities (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis – IBAMA), authorization number 13755-1. After the specimen collection procedures, the animals were maintained under observation for 4 days receiving water and food and then they were released at the place of capture.

2.2. Isolation of leptospires

Urine samples were immediately inoculated into culture media, diluted 10^{-1}, 10^{-2} and 10^{-3}. A volume of 500 μL of each dilution was inoculated in tubes containing 4.5 ml of Ellinghausen–McCullough–Johnson–Harris (EMJH) medium supplemented with Leptospira Enrichment EMJH (Difco, USA). The cultures were incubated at 30 °C and examined weekly by dark field microscopy for 2–4 months.

2.3. Genomic DNA extraction

For DNA extraction, a 7-day culture was harvested by centrifugation (15,000 × g, for 30 min) at 4 °C and the pellet was frozen at −20 °C. Genomic DNA was extracted using the Illustra bacteria genomicPrep Mini Spin Kit (GE Healthcare) following the protocol recommended for Gram negative bacteria. The extracted DNA was analyzed by agarose gel electrophoresis, the DNA was quantified and evaluated with regard to its integrity and then stored at −20 °C.

2.4. Polymerase chain reaction and partial rpoB gene sequencing

The partial *rpoB* gene was amplified and sequenced using primers previously described (La Scola et al., 2006). The primers used were Lept 1900f (CCTCATGGGTTCCAACATGCA) and Lept 2500r (CGCATCCTCRAAGTTGTAWCCTT). PCR amplification was carried out with one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 2 min and a final extension at 72 °C for 7 min. Aliquots were evaluated by agarose gel electrophoresis. Before sequencing, PCR products were purified by GFX PCR DNA and Gel Band purification kit according to manufacturer instructions (GE Healthcare). The sequencing was performed in a MegaBACE 500 DNA sequencer (GE Healthcare) by the use of the Dynamic ET-terminator technology. Chromatograms were assembled and analyzed using ContigExpress module of Vector NTI 10.0 suite (Invitrogen). The assembled sequence was submitted to BLAST alignment (http://www.ncbi.nlm.nih.gov/BLAST) against other *rpoB* sequences available in GenBank.

2.5. Multilocus Variable-Number Tandem-Repeat Analysis

The MLVA was performed using two discriminatory pair of primers: Lb4a (AAGAAGATGATGGTAGAGACG), Lb4b (ATTGCGAAACCAGATTTCCAC), Lb5a (AGCGAGTTCGCCTACTTGC) and Lb5b (ATAAGACGATCAAGGAAACG). The DNA was amplified under the following conditions: one cycle at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min. The amplicons were evaluated by agarose gel electrophoresis and the number of repeats was estimated as previously described (Salaun et al., 2006).

2.6. The microscopic agglutination test

The MAT was carried out to detect antibodies in the serum samples against the various *Leptospira* serovars (Faine et al., 1999). The MAT titer was expressed as the reciprocal of the highest serum dilution that resulted in 50% agglutination of leptospires. Cut-off titers were 100 for domestic animals, humans and white-eared opossums. Thirty negative and 30 positive serum samples from humans, cattle and dogs were randomly selected from the sera bank of *Centro de Controle de Zoonoses* (CCZ) of UFPel. In addition, 33 serum samples from the captured white-eared opossums were tested by the MAT using recommended serovars and other local isolates as antigen. The recommended serovars were Australis Ballico, Autumnalis Akiyami A, Castellonis Castellòn 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. The following serovars were also included: Grippotyphosa Duyster, Grippotyphosa Mandemakers, Icterohaemorrhagiae Ictero 1, Icterohaemorrhagiae Kantorovic, Icterohaemorrhagiae Verdum, Copenhageni Winjberg, Hardjo Lely 607, Bratislava Jez Bratislava, Rachmati Rachmati, Butembo Butembo, Ballum Mus 127, Bataviae Van tienen, Whitcombi Whitcomb, Celledoni Celledoni, Cynoptery 3522 C, poi Poi, Proechimys 1161 U, Saxkoebing Mus 24, Shermani 1342 K, Andamana CH11, Andamana Bovedo, Semaranga Veldrat Semarang 173, Sentot Sentot, djasiman Djasiman, Mini Sari, and Rufino; and local *Leptospira* isolates from CCZ/UFPel: Canicola Kito, Canicola Tande, Canicola Mike, Copenhageni Kade, plus the white-eared opossum isolate.

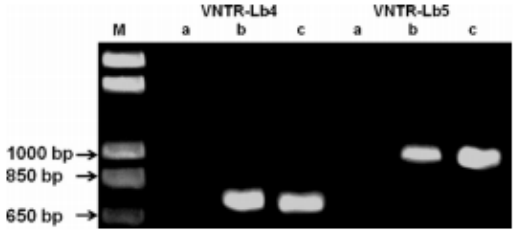

Fig. 1. MLVA products in 2% agarose gel. Molecular markers (1 kb Plus DNA Ladder). (A) Negative control; (B) positive control (*Leptospira borgpetersenii* serovar Castellonis strain Castellon3) and (C) white-eared opossum *Leptospira* isolate.

2.7. Evaluation of virulence of the isolate

The isolate was grown in EMJH medium and 1 ml (10^8 bacterial cells) was inoculated intraperitoneally in four male Golden Syrian hamsters (*Mesocricetus auratus*). The animals were monitored daily for the presence of clinical signs, including evidence of external hemorrhaging, dehydration, ruffled hair coat, decreased activity and survivors were euthanized after 10 days. A hamster injected with PBS served as the negative control. For histopathological studies, lung, liver and kidney tissue samples were fixed in 10% formalin (pH 7.0), and then embedded in paraffin. Six sections of 5–6-μm thickness from each organ were stained with hematoxylin and eosin.

The Golden Syrian hamsters used in this experiment were provided by the central animal house of UFPel. The experimental animals were housed at the animal facility of the *Centro de Desenvolvimento Tecnológico – Núcleo de Biotecnologia* and maintained in accordance with the guidelines of the Ethics Committee in Animal Experimentation of UFPel throughout the experimental period.

2.8. Statistical analysis

The results were analyzed (Pearson's chi-square test) using Sigma Stat for Windows Version 3.0 (SPSS, Inc.). The $P < 0.05$ was considered statistically significant.

3. Results

One *Leptospira* isolate was obtained from 33 urine samples of white-eared opossum following two months of incubation. A PCR product of approximately 600 pb from the *rpoB* gene was amplified and sequenced in order to perform the taxonomic classification of the isolate. A BLAST analysis of the sequence of this PCR product demonstrated 100% identity only to *L. borgpetersenii*. Analysis of the MLVA profile revealed the presence of four tandem repeats at the VNTR-Lb4 locus and six tandem repeats at the VTNR-Lb5 locus. This pattern is identical to that of *L. borgpetersenii* serogroup Ballum serovar Castellonis (Salaun et al., 2006). In order to confirm the identity, PCR amplification was carried out using DNA isolated from the Castellon3 strain and the amplified products were analyzed by electrophoresis, side-by-side with the products amplified from the isolate (Fig. 1).

The MAT using the new isolate as antigen was performed with 60 canine serum samples, 30 positive and 30 negative samples previously characterized using the standard MAT battery. Of the 30 positive samples, a total of 14 (46.7%) also reacted with the new isolate. From the 30 negative samples, four (13.3%) reacted with the new isolate with titers ranging from 100 to 800 (Table 1). However, there was no significant statistical difference observed when the new isolate was included in the MAT characterization of human and cattle serum samples, although two human and one cattle serum

Table 1
MAT titers of thirty canine serum samples collected from dogs with suspected leptospirosis.

Serovars/strains	Canine serum samples[a]																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Bratislava Jez Bratislava							100																							
Autumnalis Akiyami A					400																									
Canicola Hond Utrecht IV	100	3200					200					100	100		200	100	200	100		100		200		100		100	100	100	100	100
Copenhageni M20	100	400	100					200	200	200				200					200									100		
Icterohaemorrhagiae RGA				100																		100	100	200				100		100
Icterohaemorrhagiae Kantarowic								100		100			200	400																
Copenhageni Kade															100		200													
Canicola Kito		1600	200				100	3200		800	100	100			100	100	200			100										
Canicola Mike								800			100		800									100	200							
Canicola Tande	100						400	1600		800	100	100						100		100				200				100		
Castellonis Castellon 3					100																				100	100				
Ballum Mus 127						100		100		1600											100									
Opossum isolate		200				100		100		100	100			400		800	200				100			400		100	100		100	

[a] Serum samples previously positive by MAT randomly selected from the sera bank of CCZ–UFPel. Antibody titer against a panel of *Leptospira* strains, including the white-eared opossum isolate.

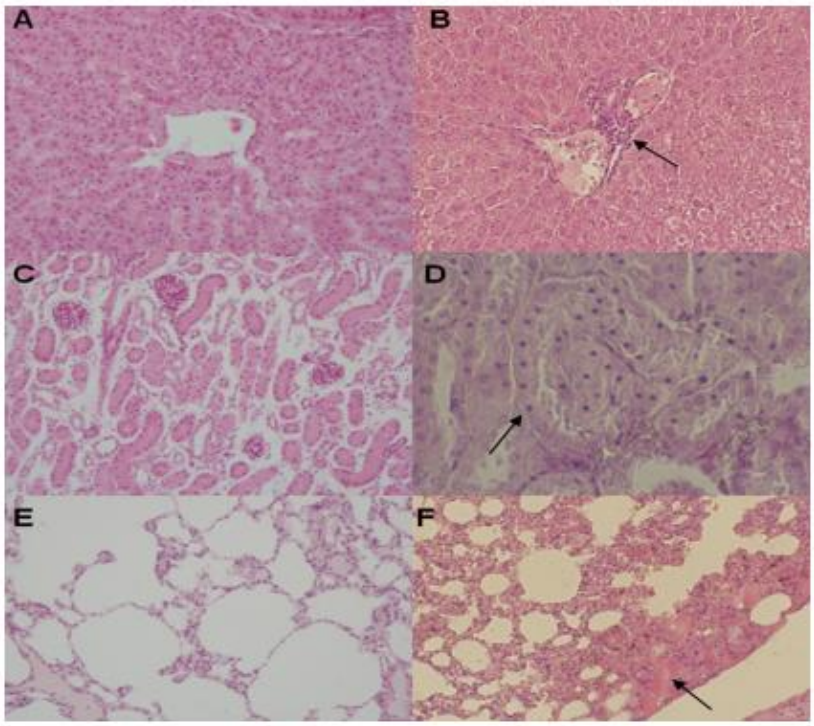

Fig. 2. Histopathological analysis of hamster inoculated with the white-eared opossum isolate (*L. borgpetersenii* serovar Castellonis). Note pathologic changes in tissues stained with hematoxylin and eosin: (B) lymphocytes in liver infiltration in lobar vein center (20×), (D) pyknosis nuclei in cells of renal tubules (40×) and (E) hemorrhage of the lung (40×). Normal hamster tissue (negative control): (A) liver, (C) lung architecture and (E) tubular epithelium in kidney.

samples reacted against the isolate, with titers of 200 and 100, respectively (data not shown). The MAT of the white-eared opossum serum samples revealed that the prevalence of leptospirosis is high in this species. Out of the 33 serum samples tested, 12 samples (36.4%) had specific antibody titers against the isolate, ranging from 100 to 200.

The white-eared opossum isolate was virulent in the hamster model, all of the animals inoculated with the isolate developed acute lethal infection as characterized by hepatic, renal and pulmonary lesions. Macroscopic pulmonary hemorrhaging with widespread bleeding was observed and microscopic foci of alveolar hemorrhage occurred in all animals. Renal, hepatic and pulmonary tissues presented infiltration with lymphocyte cells and pyknosis was observed in the renal tubule cells. None of these features were observed in the healthy control animals (Fig. 2).

4. Discussion

The epidemiology of leptospirosis is influenced by the existence of maintenance hosts, which ensure the perpetuation of the organism (Levett, 2001). Mammals such as marsupials and didelphidae contribute to the maintenance of *Leptospira* spp. in the environment (Day et al., 1998; Liceras de Hidalgo and Sulzer, 1984). During an outbreak, several domestic and wild animal species living in biocenosis can be infected and transmission tends to occur through direct contact with urine containing leptospires. Leptospirosis transmission may also occur occasionally from maintenance hosts to other species as a result of contact with environments contaminated with infected urine (Faine et al., 1999).

A wide diversity of *Leptospira* spp. constitutes the etiological agents of leptospirosis. In this study, a *Leptospira* isolate was recovered from a captured white-eared opossum. This isolate was characterized by partial *rpoB* gene sequencing and MLVA (Salaun et al., 2006), allowing the isolate to be identified as *L. borgpetersenii* based on the *rpoB* sequence identity (100%) to a reference strain deposited in GenBank. The isolate was identified as serovar Castellonis based on the MLVA profile. Although rodents represent the largest population of synanthropic animal carriers of *Leptospira* spp., opossums are commonly found in urban and suburban areas of tropical and subtropical regions. Marsupials have been reported to harbor serovars such as *Leptospira kirschneri* serovar Grippotyphosa (Bharti et al., 2003) and *L. interrogans* sorovar Canicola (Brihuega et al., 2007). Our data suggests that *Didelphis albiventris* is a host of *L. borgpetersenii* serovar Castellonis in the study area. This finding indicates that the white-eared opossum can be a reservoir for different pathogenic *Leptospira* spp.

To determine the virulence of the new isolate it was used to inoculate hamsters, the animal species that mimics the pathology associated with acute lethal forms of human and experimental leptospirosis, as previously described (Arean, 1962; Pereira et al., 2005; Silva et al., 2008). Lesions were observed in liver, lung and kidney tissue. The isolate caused clinical signs of leptospirosis and lesions in all of the hamsters inoculated, attesting to its virulence. The presence of microscopic pulmonary hemorrhaging in the absence of gross features has also been reported in humans and in guinea pigs

(Arean, 1962; Nally et al., 2004). The pattern of acute cell swelling in fulminant disease and a picture of interstitial nephritis in more prolonged illness have been reported in humans (Arean, 1962).

The new isolate was included in the MAT battery for testing animal and human serum samples to investigate the presence of antibodies against this isolate. The inclusion of serovars isolated from local leptospirosis cases is recommended in order to increase MAT sensitivity (Faine et al., 1999; Levett, 2001; WHO, 2003). In this study, the isolate from a white-eared opossum improved the sensitivity of the MAT for canine samples. In a sample of 30 serum samples that were previously characterized as negative, 4 were found to be positive. In addition, 14 out of 30 positive samples also recognized the isolate in the MAT. This data suggests that there is contact between dogs and white-eared opossums in suburban areas.

The white-eared opossums presented with low antibody titers in the MAT (data not shown), which suggests the opossum is only moderately susceptible to infection (Babudieri, 1958), a characteristic of reservoir hosts (Faine et al., 1999). Low antibodies titers against *Leptospira* serovars were previously described when opossums of the genus *Didelphis* were inoculated intraperitoneally with serovar Grippotyphosa and clinical signs were not detected, although lesions attributed to leptospirosis were observed in liver and kidney tissues (Reilly, 1970).

Leptospiral shedding in urine has been reported in several species of wild animals such as capybaras (*Hidrochaerys hidrochaerys*) experimentally infected with *L. interrogans* serovar Pomona (Marvulo et al., 2009). Similar results were described in other species infected with various serovars, including the striped skunk (*Mephitis mephitis*) that was infected with serovar Pomona (McGowan and Karstad, 1965); coyotes (*Canis latrans*) infected with serovars Pomona, Canicola and Copenhageni (Marler et al., 1979); marmoset monkeys (*Callithrix jacchus*) infected with serovar Copenhageni (Pereira et al., 2005); the common opossum (*Didelphis marsupialis*) infected with serovar Grippotyphosa (Reilly, 1970) and the common brush-tail opossum (*Trichosurus vulpecula*) infected with serovar Balcanica (Hathaway et al., 1981).

In conclusion, the findings of this study contribute to the epidemiology of leptospirosis, identifying *D. albiventris* as a host reservoir for *L. borgpetersenii* serovar Castellonis. In addition, the inclusion of the isolate in the MAT battery resulted in a significant increase in the detection rate, mainly in canine populations, an animal species that has contact with white-eared opossum in suburban areas.

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Anexo 2 - Artigo publicado durante o período de desenvolvimento da tese

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Detection of Virulence Factors and Molecular Typing of Pathogenic *Leptospira* from Capybara (*Hydrochaeris hydrochaeris*)

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Abstract Leptospirosis is a globally prevalent zoonosis caused by pathogenic *Leptospira* spp.; several serologic variants have reservoirs in synanthropic rodents. The capybara is the largest living rodent in the world, and it has a wide geographical distribution in Central and South America. This rodent is a significant source of *Leptospira* since the agent is shed via urine into the environment and is a potential public health threat. In this study, we isolated and identified by molecular techniques a pathogenic *Leptospira* from capybara in southern Brazil. The isolated strain was characterized by partial *rpoB* gene sequencing and variable-number tandem-repeats analysis as *L. interrogans*, serogroup Icterohaemorrhagiae. In addition, to confirm the expression of virulence factors, the bacterial immunoglobulin-like proteins A and B expression was detected by indirect immunofluorescence using leptospiral specific monoclonal antibodies. This report identifies capybaras as an important source of infection and provides insight into the epidemiology of leptospirosis.

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Short Communication

Leptospirosis is a worldwide zoonosis usually transmitted to humans through contaminated water or direct exposure to urine of infected animals, and has a high mortality and morbidity rate [4, 5, 13, 15]. The disease is caused by pathogenic bacteria of genus *Leptospira*, which is classified into 12 pathogenic species that affect a wide variety of animals [2, 15], *L. interrogans* and *L. borgpetersenii* being the main cause of human disease [7, 12, 29]. Tropical and subtropical developing countries offer opportunities to exposure of the human population since infected wild and domestic animals pose a persistent public health threat [9, 15]. For *Leptospira* isolates identification and characterization, molecular and antigenic methods have been used [5, 26], such as variable-number tandem-repeats (VNTR) analysis of *L. interrogans* sensu stricto identification [16], *rpoB* gene sequencing for *Leptospira* species identification [14], as also recently developed monoclonal antibodies (mAbs), to detect bacterial virulence factors [18].

The capybara (*Hidrochaerys hidrochaerys*) is the largest living rodent in the world. It requires few ambient conditions which are an important aspect in promoting wide geographic distribution in Central and South American countries [8]. The ideal habitat of the capybara includes pasture and abundant and permanent water supply for drinking, reproduction, regulation of body temperature, and for safety [3, 11]. Synanthropic and wild rodents have been considered important *Leptospira* reservoirs [26], thus capybaras have a potential in leptospires maintenance and their gregarious habits could promote the transmission of

bacteria among individuals of the same social group. In this work, we report the molecular typing of a *Leptospira* isolate from capybara by partial *rpoB* gene sequencing and VNTR analysis. Furthermore, an indirect immunofluorescence was performed to verify the virulence factors expression using specific mAbs against leptospiral immunoglobulin-like proteins (Ligs) A and B conserved regions.

A juvenile female capybara captured by the Environmental Protection Police in the city of Pelotas, RS, Brazil (31°46″S, 52°20″W) was admitted to the Wildlife Rehabilitation Nucleus/Center Screening of Wild Animals (NURFS/CETAS) of the Federal University of Pelotas for veterinary medical care. After the animal's death, the kidneys were aseptically removed and macerated under sterile conditions in 5 mL of Ellinghausen–McCullough–Johnson–Harris (EMJH) liquid medium supplemented with *Leptospira* Enrichment EMJH (Difco, BD Diagnostics, Sparks, MD, USA) and the cultures were incubated at 30 °C for 1 h. After, 500 µL inoculum was transferred to three tubes containing 5 mL of EMJH medium resulting in 10^{-1}, 10^{-2}, and 10^{-3} dilutions. Cultures were incubated at 30 °C and examined weekly by dark-field microscopy over 2–4 months. The procedures used in the present study were approved by the federal authorities (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis—IBAMA), authorization for scientific activities number 24501-1.

For DNA extraction, a 7-day culture was harvested by centrifugation (15,000×*g*, for 30 min) at 4 °C, and the cell pellet was frozen at −20 °C. Genomic DNA was extracted using the Illustra Bacteria GenomicPrep Mini Spin Kit (GE Healthcare®) following the protocol designed for Gram negative bacteria. The extracted DNA was submitted to agarose gel electrophoresis in order to be quantified and evaluated with regard to its integrity, and stored at −20 °C. The partial *rpoB* gene was amplified and sequenced using primers previously described [14]. PCR amplification was carried out with 1 cycle at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 51 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 7 min. Aliquots were evaluated by agarose gel electrophoresis. Before sequencing, PCR products were purified using GFX PCR DNA and Gel Band purification kit (GE Healthcare®) according to manufacturer instructions. The sequencing was performed in a MegaBACE 1000 DNA sequencer (GE Healthcare®) using Dynamic ET-terminator technology. Chromatograms were assembled and analyzed using ContigExpress® module of Vector NTI 10.0 suite (Invitrogen®). The assembled sequence was submitted to BLAST alignment (www.ncbi.nlm.nih.gov/BLAST) against other *rpoB* sequences available in GenBank. For VNTR analysis, seven discriminatory primers were used: VNTR4, VNTR7, VNTR9, VNTR10, VNTR11, VNTR19, and VNTR23 [16, 24]. The PCR reaction was performed using a cycle of 94 °C for 5 min, followed by 35 cycles of amplification at 94 °C for 30 min, 55 °C for 30 s, 72 °C for 1 min, and a final extension of 72 °C for 30 s. Aliquots were evaluated by agarose gel electrophoresis and the size of amplified products was estimated by comparison [16]. DNA from previously characterized *L. interrogans* strain Fiocruz L1-130 was chosen as positive control [13, 19]. mAb anti-Ligs A and B conserved region (LigBrep) were used to identify bacterial virulence [18]. Briefly, 15 µL of a 10^8 leptospiral culture was added into the slide chambers (ICN Biomedicals Inc., CA, USA) and incubated at 30 °C for 1 h. The slides were then blocked with 10 % of bovine fetal serum diluted in PBS and mAb anti-Ligs was added. Goat anti-mouse FITC conjugate (Invitrogen, USA) was used to detect the complex antigen:antibody. A drop of mounting medium was then added and labeling was visualized by fluorescence microscopy (Olympus BX 51) with excitation wavelength of 450 nm. Confirmation of bacteria in the microscopic field was achieved by DNA staining with Hoechst 33258. The *L. interrogans* strain Fiocruz L1-130 in low and high passages [13] were used as control.

A PCR product of approximately 600 bp correspondent to *rpoB* gene was amplified and sequenced in order to perform the taxonomic classification of the isolate. The BLAST analysis (www.ncbi.nlm.nih.gov/BLAST) demonstrated 100 % identity with the *rpoB* gene from *L. interrogans* (data not shown). In VNTR results, the *Leptospira* isolate obtained from the capybara kidney presented amplification for the seven discriminatory primers used (Fig. 1). Analysis of the electrophoresis profile of amplified VNTR fragments revealed an identical pattern of our isolate with the *L. interrogans* serogroup Icterohaemorrhagiae serovar Icterohaemorrhagiae or Copenhageni, according to the dendrogram proposed by Majed et al. [16]. To confirm the expression of Ligs A and B repetitive region in the isolate, an indirect immunofluorescence using mAb anti-Ligs was performed. The Fig. 2A illustrates the reaction of mAb with native Ligs expressed in the capybara isolate and in *L. interrogans* Fiocruz L1-130 low passage used as control (Fig. 2a).

The epidemiology and clinical features of leptospirosis are usually associated with the serovars and serogroups of *Leptospira* [16] involving more than 250 serovars and numerous maintenance hosts [25] that act as a source of infection and can excrete leptospires in urine for prolonged intervals [9]. Currently, 12 species of pathogenic *Leptospira* have been identified [2]; however, approximately half of all pathogenic serovars described at the moment were classified as *L. interrogans* and *L. borgpetersenii* [1]. *L. interrogans* sensu stricto is responsible for the most frequent and severe cases of human and animal leptospirosis [28], and its mammals' isolates identification has been based

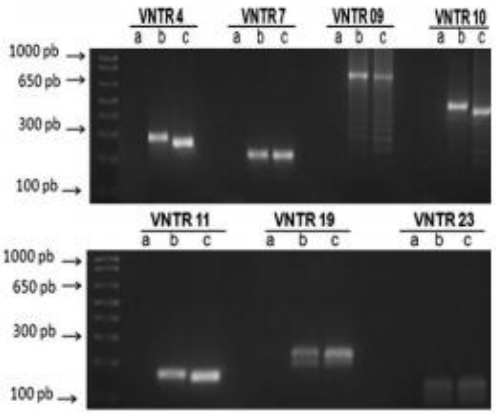

Fig. 1 Electrophoresis in 2 % agarose gel. Molecular marker (1 kb plus DNA ladder). (*a*) Negative control (water template), (*b*) positive control (genomic DNA of *L. interrogans* serovar Copenhageni strain Fiocruz L1-130), and (*c*) genomic DNA isolated from capybara

on molecular typing since this approach provides rapid typing as well as a highly discriminating assay useful for epidemiological studies of pathogenic bacteria [13, 16, 30].

For *Leptospira* from capybara molecular typing, we performed analysis of VNTRs. Also called multiple-locus VNTR analysis, it has proved to be a highly powerful and discriminant method to study the population structure of bacteria [20] and characterize isolates even from monomorphic bacterial populations [10]. The genome of *L. interrogans* serovar Lai was sequenced [21] and that permitted the definition of pairs of primers flanking some VNTR-like loci to determine whether VNTR analysis will be used to differentiate serogroup and serovar from *L. interrogans* sensu strictu [16]; furthermore, we performed *rpoB* gene sequencing to confirm the *Leptospira* species [14, 22]. In this work, by means of both molecular techniques, we characterized a *Leptospira* isolate from capybara as *L. interrogans* serogroup Icterohaemorrhagiae serovar Icterohaemorrhagiae or Copenhageni. These two serovars cannot be differentiated by molecular techniques; however, the serogroup identification should be useful in leptospirosis outbreaks and epidemiological surveys [15].

In addition, the bacterial expression of Ligs A and B proteins was used as an approach to isolate strain virulence characterization since the presence of some genes does not insure the expression by bacteria. The mAbs anti-Ligs used in this work were developed by our group and are useful specific tools for *Leptospira* virulence characterization [18]. Ligs are proteins that interact with the extra-cellular matrix and play a role as adhesins for both *L. interrogans* and *L. borgpetersenii* for specific attachment (anchoring) to the host tissue [6]. Despite animal model importance in experimental challenge infections, in vitro identification of new *Leptospira* isolates virulence can be done using mAbs anti-Ligs prior to animal model studies.

The capybara is the largest rodent in the world and is a widespread species in Central and South America. However, reports regarding the importance of this animal in the epidemiology of leptospirosis are rare. In a previous serologic survey of capybaras in southern Brazil, high titers of agglutinating antibodies found in MAT against a serovar Bratislava and serovar Australis (local isolate from dog characterized by molecular techniques as *L. noguchii*) suggested the possibility of circulation of *L. interrogans* and *L. noguchii* in capybaras [23]. In experimental infection of capybaras with *L. interrogans* serovar Pomona, it was demonstrated that the capybara is susceptible, and the species experiences the classical leptospiremic and leptospiruric phases; agglutinins against *Leptospira* serovar Pomona were initially detected between days 2 and 7, peaked between days 9 and 27, and persisted until day 83 [17]. These results suggest that the capybara is susceptible to infection and shedding pathogenic *Leptospira* in urine. These animals could be efficient reservoirs for

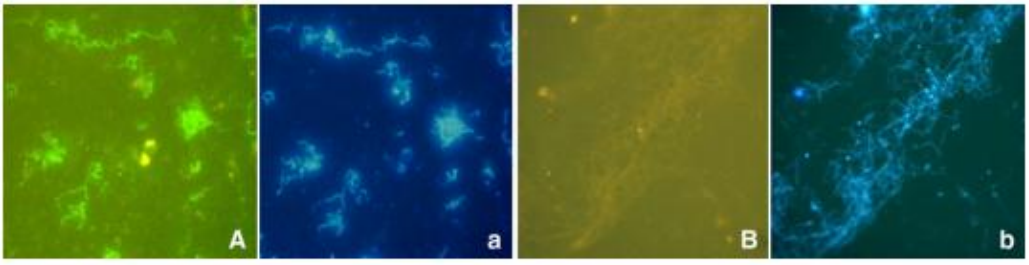

Fig. 2 Indirect immunofluorescence demonstrating the expression of LigBrep in surface of *L. interrogans* isolated from capybara. **A** *L. interrogans* reacting with antibodies anti-Ligs, **B** *L. interrogans* confronted with a normal mouse serum (negative control). **a** and **b** Leptospiral DNA stained with Hoechst 33258. Visualization was performed with a ×100 objective on an Olympus BX 51 fluorescence microscope and photographed with digital camera Olympus BP 72

L. interrogans as the isolate reported here because they live in areas with abundant water, an important environmental factor for disease transmission [9, 17, 27]. Continuous surveillance should be carried out in order to acquire further knowledge of the maintenance of this animal species. The findings of this work indicate that capybaras could be hosts for *L. interrogans* and may play an important role as a source of infection for animals and humans, providing insights into the epidemiology of leptospirosis.

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Conflict of Interest No competing financial interests exist.

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Anexo 3 - Artigo publicado durante o período de desenvolvimento da tese

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Artigo Cientifico
Medicina Veterinária

Seropositivity to *Leptospira interrogans* Canicola local isolate associated to tongue necrosis in dog without significant hematological alterations

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ABSTRACT: Leptospirosis is a global zoonosis caused by infection with the spriochetal bacterium of the genus, *Leptospira*. Dogs may be exposed to leptospires in the environment by contact with urine of an infected host, contaminated water or moist soil, where the bacteria may survive for several months. In this work, we report the clinical case of a dog suffering tip of the tongue necrosis caused by pahogenic *L. interrogans* serovar Canicola strain Tande. Laboratorial examinations demonstrated urinalysis and blood chemistry changes without significant hematological alterations. The 3.200 antibodies titers against Tande strain were detected on Microscopic Agglutination Test (MAT), confirming the diagnosis of leptopirosis. Aggressive fluid therapy and β-lactâmic antibiotics were used for treatment and to prevent and treat acute kidney damage. The dog had a complete clinical recovery after treatment.

Keywords: leptospirosis, serology, local isolate

Soropositividade para um isolado local de *Leptospira interrogans* Canicola associado à necrose de lingua em cão sem a presença de alterações hematológicas

RESUMO: A leptospirose é uma zoonose de distribuição mundial causa por bactérias espiroquetas do gênero *Leptospira* spp. Cães são expostos ao agente por meio de urina de hospedeiros infectados, água ou solo contaminados, onde bactéria pode sobreviver por muitos meses. Neste trabalho, relatamos o caso clínico de um cão apresentando como sinal clínico necrose da ponta da língua associada a uma infeção aguda pela patogênica *L. iterrogans* Canicola cepa Tande. A infecção aguda por esta cepa patogênica causou mudança nos parâmetros da bioquímica sérica e na urinálise, sem causar alterações hematológicas. Título de anticorpos de 3.200 contra cepa Tande foram detectados pelo teste de soroaglutinação microscópica (MAT), confirmando o diagnóstico de leptospirose. A associação de antibióticos β-lactâmicos foram utilizados para combater a infecção aguda e fluidoterapia agressiva foi adotada para previnir insuficiência renal. O cão obteve recuperação clínica após tratamento.

Palavras-chave: leptospirose, sorologia, isolado local

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Introduction

Canine leptospirosis is caused by infection by pathogenic bacteria of genus *Leptospira* which is classified into 12 pathogenic species and with antigenically distinct serovars (LEVETT, 2001). The infection in dogs known as Stuttgard disease, is a zoonotic emerging bacterial disease (ALTON et al., 2006; RENTKO et al., 1992; LEVETT, 2001). The bacteria gains access to new hosts by passage through mucous membranes or skin abrasions, often from environmental sources, such as urine-contaminated water (FAINE et al., 1999; LEVETT, 2001). The development of the canine leptospirosis depends on age and immunity of the host, environmental factors and virulence of the infecting serovar that is heterogeneous throughout the world (FAINE et al., 1999).

Isolation followed by typing from renal carriers is important and very useful in epidemiological studies to determine which serovars are present within a particular group of animals, animal species, or a geographical region (FAINE et al., 1999). *Leptospira interrogans* serovar Canicola strain Tande was isolated in Southern Brazil from urine samples from a dog with clinical signs, hematological, blood chemistry and urinary changes typical in canine leptospirosis. The Tande strain increased 20 per cent of enhanced MAT sensitivity when it was included in this serologic diagnosis using dog sera samples in the same study area (BROD et al., 2005). In this work, we report a clinical case of canine leptospirosis in an animal suffering from the tip of the tongue necrosis and had urinalysis test and blood chemistry changes caused by acute infection by *L. interrogans* Canicola Tande strain without significant hematological alterations.

A three years old poodle dog, male, weighing 3.8 kg, unvaccinated against leptospirosis was brought to the Veterinary Hospital of Federal University of Pelotas –

Brazil, where the owner referred to lethargy, emesis, dehydration, anorexia and foul odor in the mouth perceived approximately 4 days earlier. That animal lived near synanthropic rodents. Clinical examination revealed shrunken eyeballs, yellowish discoloration of the mucous membrane of conjunctiva, rectal temperature of 37.2 ℃ and tip tongue necrosis (Figure 1).

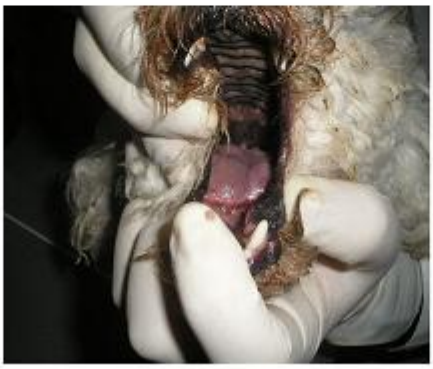

Figure 1. Tip tongue necrosis in dog associated with infection *L. interrogans* Canicola Tande strain.

Blood was collected by cephalic vein puncture with anti-coagulant for a hematological examination and without anticoagulant for biochemical and serological examinations: Complete blood count (CBC), and serum biochemistry: Alanine aminotransferase (ALT), Alkaline phosphatase (ALP), Creatinine and blood urea nitrogen (BUN), a Microscopic Agglutination Test (MAT) were performed to detect antibodies against *Leptospira* spp. using serovars recommended by the World Health Organization (TERPSTRA, 2003) plus canine local isolate (Tande strain). Sera were tested at dilutions of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12,800. A urine sample was collected by catheterization for urinalysis test.

We detected in MAT antibodies titers of 100 against the both serovars Autralis and Bataviae, titers of 800 against the serovar Icterohaemorrhagiae, titer of 1.600 against the serovar Canicola, Hond Utrecht IV strain and titer of 3.200 against serovar Canicola, Tande strain (Table 1). Urinalysis was characterized by proteinuria, pyuria, and bilirubinuria and by increased numbers of granular casts, leukocytes, and hematuria (Table 1).

In the hematological findings, WBC count and WBC differential count (neutrophils and lymphocytes), showed no exceess reference values (Table 2). In relation to biochemical findings, ALT, urea and creatinine exceeded reference values (Table 2). The dog had anacellular urinary sediment, which indicates the kidneys as the source of urine protein.

Canine leptospirosis caused primarily by *L. interrogans* and *L. kirschneri* has been associated with serovars Canicola, Icterohemorrhagiae, Grippotyphosa, Pomona and Bratislava (SYKES et al., 2011). Based on studies that focused on urban populations of dogs, serovars Canicola and Icterohemorrhagiae were considered the most prevalent serovars infecting dogs (FAINE, 1999), however, distinct pathogenic species and serovars had been isolated from dogs worldwide; in southern Brazil *L. noguchii* (SILVA et al., 2009) and *L. interrogans* Canicola, Tande strain (BROD et al., 2005) were isolated from dogs. Criteria for the canine diagnosis of leptospirosis consisted of clinical findings associated with one or more of the following criteria: A single serum MAT titer of greater than or equal to 1600 to a nonvaccinal serogroup in combination with a titer of 800 to serogroups Canicola and Icterohaemorrhagiae or a titer of greater than or equal to 1600 to serogroups Canicola and Icterohaemorrhagiae in a dog that never had been vaccinated against leptospirosis (GREENE, 2006), moreover,

dogs with positive titers on MAT generally have sera that cross-react to a variety of serovars (MILLER et al., 2007; ANDRE-FONTAINE, 2006). In the clinical case reported here, we detected titer of 3.200 against serovar Canicola Tande strain and cross-react to others serovars (titer of 100 against serovars Australis and Bataviae, 800 against serovar Icterohaemorrhagiae and 1.600 against serovar Canicola Hond Utrecht IV) that, associated with the epidemiological data, changes in urinalisys test, serum biochemistry and clinical signs, confirming the diagnosis of acute leptospirosis.

Leukocyte counts fluctuate depending on the stage and severity of leptospirosis infection and cannot show changes such as the clinical case reported here (Table 2). Clinical signs depend on the stage of disease; e.g.: acute infection generally including fever, shivering, muscle tenderness, vomiting, dehydration, peripheral vascular colapse, tachypea, rapid irregular pulse, poor capillary perfusion, hematemesis, hematochezia, melena, espistaxis, widespread petechiae, and Icterus (GREENE, 2006). The extent of damage to organs varies seems to depend on the virulence of the serovar, the inoculums, and host susceptibility (GOLDSTEIN, 2010). In this clinical case, the initial replication mainly damaged the endothelial cells causing the unusual clinical finding of the tip tongue necrosis and alterations in blood chemistry and urinalisys, caused by acute infection from local isolate from *L. interrogans* Canicola local isolate; the Tande strain was previously isolated from urine from a convalescent dog in the same study area suffering hepatic dysfunction, renal failure and a high titers detected in MAT (BROD et al., 2005), indicating that *L. interrogans* Canicola Tande strain is virulent for dogs, causing this unusual clinical tip of the tongue necrosis sign in both cases reported.

Treatment of leptospirosis involves supportive care and depends on the severity of the infection and the presence of renal or hepatic dysfunction and other complicating factors. Aggressive fluid therapy concurrent to the use of antibiotics is crucial to prevent and treat acute kidney damage. The extent of renal damage after treatment may play a key role in determining the long-term prognosis for affected dogs. Antimicrobial therapy should be started as soon as the disease is suspected and usually reduce fever and bacteremia within a few hours after administration. The first goal for terminating bacteremia and sterilizing the urine can be a penicillin derivative (GOLDSTEIN, 2010). The prognosis of leptospirosis in dogs is fair to poor, depending on the clinical state of the patient at initial presentation and on the causative leptospiral serovar. Survival rates of 80% for dogs with acute renal failure due to leptospirosis have been reported (ADIN et al., 2000). In this clinical case, the prognosis was guarded, but recovery was possible. Hospitalization was necessary for aggressive fluid therapy and the use of broad spectrum antibiotics (Ampicilin-Streptomycin). The dog was cured and had a complete clinical recovery after treatment.

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Table 1 – Microscopic Agglutination Test (MAT) and Urinalysis results

Microscopic Agglutination Test (MAT)				
Species	**Serogroup**	**Serovar**	**Strain**	**Titers**
L. interrogans	Australis	Australis	Ballico	100
	Bataviae	Bataviae	Swart	100
	Bataviae	Bataviae	Van Tienem	100
	Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	800
	Canicola	Canicola	Hond Utrecht IV	1.600
	Canicola	Canicola	Tande	3.200

Urinalisys		
Test	**Result**	**Reference Value**
Physical examination		
Urine color	Amber	Straw to amber
Urine appearence	Cloudy	Clear
Specific gravity	1041	1025-1040
Chemical examination		
Ph	6.0	6.0-7.0 (Acidic)
Protein	(+)	Negative
Bilirubin	(++)	Negative
Microscopic examination		
Squamous epithelial	<2	Few
Pus cells	30/HPF	0-5/HPF
Red blood cells	1/HPF	0-2/HPF
Bacteria	Moderate	Few
Cast granular	2/HPF	None

Table 2. Values and reference ranges of the hematologic and biochemical blood results.

Complete Blood Count (CBC)				
	Reference Interval		Results	
White blood cells (WBC)	**(%)**	**(mm^3)**	**(%)**	**(mm^3)**
Basophils	0 – 2	0 - 250	0	0
Eosinophils	5 – 12	500 - 1560	1	115
Monocytes	3 – 7	300 - 910	5	575
Lymphocytes	10 – 22	1035 - 2860	21	2415
Banded neutrophils	0 – 3	0 - 390	0	0
Segmented neutrophils	60 - 75	6000 - 9750	73	8395
Total leukocytes count		10000 - 13000		11500
Red blood cells (RBC)				
Erythrocytes		6 - 8 million/mm^3		7,0
Hemoglobin		14 - 18 g%		16,3
Hematocrit		42 – 55%		49,0
Plasma total protein		6 - 8 g%		9,1

Blood Chemistry			
Tests		**Reference Interval**	**Results**
Blood urea nitrogen	(BUN)	15 a 30 mg/dl	299,0 mg/dl
Creatinine		0,5 a 1,5 mg/dl	1,8 mg/dl
Alanine Aminotransferase	(ALT)	5,3 a 19,8 UI	71,0 UI
Alcaline Phosphatase	(ALP)	8,5 a 60,6 UI	30,0 UI

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