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Julia Pires Espíndola

DIVERSIDADE GENÉTICA DE RECEPTORES DE LACTOFERRINA E TRANSFERRINA EM *Moraxella bovis* E *Moraxella bovoculi*

Santa Maria, RS
2021

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de **Doutora em Medicina Veterinária.**

Orientador: Profª. Drª. Agueda Castagna de Vargas
Co-Orientador: Prof. Dr. Rafael Frandoloso

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RESUMO

DIVERSIDADE GENÉTICA DE RECEPTORES DE LACTOFERRINA E TRANSFERRINA EM *Moraxella bovis* E *Moraxella bovoculi*

AUTORA: Julia Pires Espíndola
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A ceratoconjuntivite infecciosa bovina (CIB) é a doença ocular de maior relevância em bovinos e possui grande impacto econômico e os principais agentes etiológicos associados a CIB são *Moraxella bovis* (*M. bovis*) e *Moraxella bovoculi* (*M. bovoculi*). Para o controle desta doença a vacinação é imprescindível, entretanto, as vacinas atualmente disponíveis apresentam baixa eficiência. Espécies de *Moraxella* possuem sistemas capazes de extrair ferro das glicoproteínas, lactoferrina e transferrina presentes no hospedeiro. Cada receptor é composto por uma proteína integral da membrana externa, a proteína de ligação à lactoferrina ou transferrina A (LbpA ou TbpA), e uma lipoproteína de superfície amplamente exposta, a proteína de ligação à lactoferrina ou transferrina B (LbpB ou TbpB). Esses receptores são conhecidos por serem funcionalmente e geneticamente relacionados, sendo essenciais para a manutenção dos patógenos na superfície da mucosa do trato respiratório superior e para o desenvolvimento da doença. Estudos envolvendo a imunização com antígenos derivados desses receptores demonstram grande capacidade de prevenir infecções, bem como de eliminar a colonização do trato respiratório superior. Além disso, a posição privilegiada na superfície celular e a presença esperada desses receptores em todos os isolados de *Moraxella* spp. sugerem um grande potencial destes como antígenos vacinais. Porém, até o momento não existem estudos sobre a diversidade desses receptores nos patógenos responsáveis pela CIB e tampouco foram investigados como potenciais compostos vacinais. Neste contexto, esta tese foi elaborada visando investigar a diversidade genética e a distribuição das sequências alinhadas na estrutura das proteínas TbpA e TbpB de *M. bovis* e *M. bovoculi* (manuscrito 1) e a diversidade de LbpA, bem como desenvolvimento de antígenos híbridos (manuscrito 2). Para o manuscrito 1, trinta e sete sequências de *M. bovis* e *M. bovoculi* foram utilizadas, sendo traduzidas para aminoácidos e alinhadas. Posteriormente, árvores filogenéticas foram construídas para cada proteína, e os alinhamentos foram mapeados nas estruturas preditas das proteínas. Na análise filogenética, as sequências de TbpB foram separadas por espécies e mostraram-se mais variáveis que as de TbpA. Também, foram selecionadas duas cepas representativas de TbpB que provavelmente são capazes de cobrir toda a variabilidade encontrada nessas cepas. No manuscrito 2, análise semelhante a anterior foi realizada com trinta e seis sequências de LbpA, além disso, foram construídos cinco antígenos híbridos utilizando quatro combinações diferentes dos *loops* de LbpA em um esqueleto da lipoproteína de superfície de *Vibrio cholerae* (VcSLP). LbpA mostrou-se bastante conservada, inclusive em toda sua estrutura. Os antígenos híbridos foram expressos em pequena escala de forma eficiente e apresentaram potencial para serem produzidos em grande escala para análise de imunogenicidade e de reatividade cruzada como antígenos vacinais. Em conclusão, foi demonstrado em dois estudos diferentes a diversidade de TbpA, TbpB e LbpA e as abordagens que podem ser feitas para produzir antígenos vacinais a partir destas proteínas pensando em abranger toda diversidade genética destes receptores em ambas as espécies.

Palavras-chave: *Moraxella bovis*. *Moraxella bovoculi*. Bovinos. TbpA. TbpB. LbpA.

ABSTRACT

GENETIC DIVERSITY OF LACTOFERRIN AND TRANSFERRIN RECEPTORS IN *Moraxella bovis* AND *Moraxella bovoculi*

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Infectious bovine keratoconjunctivitis (IBK) is the most important eye disease in cattle, which has a great economic impact due to eye injuries and loss of vision, resulting in pain, reduced milk production, and decreased weight gain. The main etiological agents associated with IBK are *Moraxella bovis* (*M. bovis*) and *Moraxella bovoculi* (*M. bovoculi*). Vaccination is essential for the disease control; however, currently available vaccines may present limited efficiency. *Moraxella* spp. have systems capable of extracting iron from the host glycoproteins named lactoferrin and transferrin. Each receptor is composed of an integral outer membrane protein, named lactoferrin or transferrin binding protein A (LbpA or TbpA), and of exposed surface lipoprotein, named lactoferrin or transferrin binding protein B (LbpB or TbpB). These receptors are known to be functionally and genetically related and are known for being essential in the maintenance of pathogens on the mucosal surface leading to disease development. Studies involving immunization with antigens derived from these receptors demonstrate a great capacity to prevent infection, as well as to eliminate colonization of the upper respiratory tract. A great potential of these receptors as vaccine antigens is expected due to their privileged position on the cell surface, and their presumed ubiquity in all isolates of *Moraxella* spp. However, to date, there have been no studies on the diversity of the pathogens responsible for IBK, or they have been investigated as potential vaccine compounds. In this context, this thesis was designed to investigate the genetic diversity and its distribution in the structure of TbpA and TbpB proteins of *M. bovis* and *M. bovoculi* (manuscript 1) and the diversity of LbpA, as well as the development of hybrid antigens (manuscript 2). For manuscript 1, DNA sequences of thirty-seven *M. bovis* and *M. bovoculi* strains were translated into amino acids to build phylogenetic trees for each protein. The alignments were then mapped on the predicted structures of the proteins. In the phylogenetic analysis, TbpB sequences were separated by species and more variable than TbpA. Also, two representative strains of TbpB were selected that are likely to be able to cover all the variability found in these strains. In manuscript 2, a similar analysis was performed with thirty-six LbpA sequences, and five hybrid antigens were constructed using four different combinations of LbpA loops in a scaffold of the surface lipoprotein from *Vibrio cholerae* (VcSLP). LbpA was very conserved also throughout its whole structure. Hybrid antigens were expressed on a small scale efficiently and had the potential to be large scaled for analysis of immunogenicity and cross-reactivity as vaccine antigens. In conclusion, it was demonstrated in two different studies the diversity of TbpA, TbpB, and LbpA and the approaches that can be taken to produce vaccine antigens derived from these proteins intending to cover all the genetic diversity of the species.

Keywords: *Moraxella bovis*. *Moraxella bovoculi*. Cattle. TbpA. TbpB. LbpA.

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1 INTRODUÇÃO

A ceratoconjuntivite infecciosa bovina (CIB) é a doença ocular de maior importância na criação de bovinos em todo o mundo e a segunda causa de perdas produtivas em bovinos jovens (POSTMA et al., 2008). O impacto econômico decorrente das lesões oculares e consequente perda da visão é expressivo, resultando em diminuição do ganho de peso, redução na produção de leite, dificuldades de manejo dos animais e elevados custos com tratamentos, além de afetar o bem-estar e a depreciação dos animais acometidos (SLATTER et al., 1982).

Altamente contagiosa, a CIB é considerada uma doença que ocorre mais comumente nos meses quentes, e geralmente, com prevalência elevada, ocorrendo principalmente na forma de surtos. Esta doença possui distribuição mundial e é encontrada em países com rebanhos bovinos expressivos, como o Brasil, e está amplamente disseminada no estado do Rio Grande do Sul (GIL-TURNES; DE ARAUJO, 1982; CONCEIÇÃO; TURNES, 2003).

Moraxella bovis (*M. bovis*) é considerado o agente etiológico primário da CIB por ser o único micro-organismo capaz de reproduzir a doença de acordo com os postulados de Koch (CHANDLER; BAPTISTA; TURFREY, 1979). *M. bovis* exibe dois principais fatores de virulência, sendo determinantes para efetividade da infecção e são considerados pré-requisitos para o estabelecimento da doença: presença de fimbria (pili) tipo IV na superfície da célula bacteriana e secreção da citotoxina beta-hemolítica (POSTMA; CARFAGNINI; MINATEL, 2008). A fímbria permite ao micro-organismo a ligação nas células epiteliais e a citotoxina promove poros através da lise das células epiteliais da córnea e recruta neutrófilos, resultando na liberação de enzimas que degradam o estroma corneal (ANGELOS; BALL, 2007).

Porém, Recentemente, *Moraxella bovoculi* (*M. bovoculi*) foi descrito (2007) e é considerado um patógeno associado a CIB (ANGELOS; BALL, 2007; O'CONNOR et al., 2012; GOULD et al., 2013; SOSA; ZUNINO, 2013).Foi distinguida dos outros membros do gênero *Moraxella* pela sua caracterização bioquímica, sustentada pela análise do gene constitutivo 16S-23S (ANGELOS; BALL, 2007). No entanto, apesar do isolamento dessa espécie a partir de lesões oculares de bovinos e das mesmas possuírem fatores de virulência semelhantes aos encontrados em *M. bovis*, seu envolvimento com a ocorrência natural de CIB não tem sido comprovado e em desafios clínicos não foi capaz de reproduzir as lesões (ANGELOS; BALL, 2007; GOULD et al., 2013; LIBARDONI et al., 2012; O'CONNOR et al., 2012; SOSA; ZUNINO, 2013). Então, ainda que as evidências circunstanciais sugiram um papel de *M. bovoculi* na patogênese da doença, a contribuição precisa deste organismo para a

CIB, seja como patógeno primário, oportunista, ou fator predisponente adicional, ainda não foi estabelecida (ANGELOS, 2010). Entretanto, geralmente são encontradas infecções mistas por *M. bovis* e *M. bovoculi* e bacterinas produzidas com as duas espécies juntas tem melhorado a eficácia vacinal. Em propriedades que vacinam apenas frente a *M. bovis*, há o aumento do número de casos de infecção unicamente por *M. bovoculi* e vice-versa (ANGELOS et al., 2010).

As medidas preventivas e terapêuticas disponíveis para o controle da CIB têm limitado sucesso: a prevenção através de descarte dos portadores continua sendo impraticável, o controle de vetores é difícil na maioria das regiões e o tratamento com antimicrobianos gera altos custos e muitas vezes não apresenta resultados eficientes e necessita repetição. Desta forma, a principal estratégia para prevenção da CIB é a vacinação frente aos patógenos envolvidos no desenvolvimento da enfermidade (CONCEIÇÃO, F.; TURNES, 2003; MCCONNEL; HOUSE, 2005).

Neste sentido, inúmeras vacinas já foram testadas, desde bacterinas, vacinas recombinantes baseadas nos fatores de virulência pili tipo IV e citotoxina, bem como a produção de vacinas autógenas (DAVIDSON; STOKKA, 2003; MCCONNEL; HOUSE, 2005; BURNS; O'CONNOR, 2008). Algumas destas estão disponíveis comercialmente, no entanto, sua eficácia limitada tem sido atribuída, principalmente, a grande diversidade antigênica de isolados de *M. bovis* e em sua maioria a não inserção de *M. bovoculi* (CONCEIÇÃO, F. R. et al., 2003; O'CONNOR et al., 2011; ANGELOS et al., 2016). Estas vacinas apresentaram proteção homóloga contra determinado sorogrupo e os resultados de proteção heteróloga são geralmente insatisfatórios (PUGH; HUGHES, 1976; ANGELOS; BALL, 2007; BURNS; O'CONNOR, 2008). Além disso, demonstraram reduzida reatividade cruzada quando foram avaliadas bacterinas frente a isolados de *M. bovis*, *M. bovoculi* e *M. ovis* (KOWALSKI et al., 2017). Vacinas autógenas de *M. bovis* também tem se mostrado ineficazes, possivelmente devido à alta diversidade de antígenos em cepas circulantes em um mesmo um rebanho (O'CONNOR et al., 2012). Além disso, no Brasil, existe uma vacina disponível incluindo *M. bovoculi* desde 2015 e em 2017 o Departamento de Agricultura dos Estados Unidos (USDA) aprovou uma vacina autógena frente a este patógeno, mas ainda não se consegue observar o impacto dessas vacinas ou análise da eficiência delas.

Membros da família *Moraxellaceae* expressam receptores de superfície especializados na captação de ferro, através de duas proteínas de união à transferrina (Tbps) e em duas proteínas de união à lactoferrina (Lbps), capazes de captar as glicoproteínas trasferrina (Tf) e a lactoferrina (Lf), respectivamente, do hospedeiro e extrair o ferro carreado por elas. Este processo é essencial à sobrevivência desses patógenos na mucosa e necessário para a

manutenção da infecção (GRAY-OWEN; SCHRYVERS, 1996). Estes receptores são conhecidos por serem funcionalmente e geneticamente relacionados e desempenharem um papel essencial na captação de ferro (CORNELISSEN; ANDERSON; SPARLING, 1997).

Fenwick et al. (1996) e Brown et al. (1998) sugeriram que *M. bovis* possuía um eficiente sistema de aquisição de ferro, constituído por sideróforos e receptores de membrana externa, que reconhecem lactoferrina e transferrina bovinas, possibilitando assim, a utilização dessas duas importantes proteínas como fontes de ferro para sua sobrevivência e multiplicação (BROWN et al., 1998; FENWICK et al., 1996). Yu e Schryvers (1994) demonstraram utilizando a técnica de *Dot blot* que os receptores de *M. bovis* ligam-se especificamente a transferrina bovina e depois em 2002, confirmaram a capacidade de *M. bovis* de captar ferro a partir da Lf e Tf (YU; SCHRYVERS, 1994).

A Tf é uma glicoproteína carreadora de Fe, presente na circulação sanguínea, fluido cérebro-espinhal e nas mucosas dos mamíferos. Possui a função de transportar o Fe para células que compõem diferentes tecidos e é capaz de sequestrar qualquer molécula de Fe livre na circulação. Estruturalmente a Tf é de aproximadamente 75-80 kDa, composta por dois lóbulos, um denominado C e o outro N, permitindo a ligação de uma molécula de Fe em cada (NOINAJ; BUCHANAN; CORNELISSEN, 2012). Apenas um terço da transferrina é saturada com Fe, assim consegue se ligar a todo Fe livre encontrado prevenindo o crescimento de patógenos invasores (YANG et al., 2011). A Lf é mais encontrada nas mucosas, nas secreções e nos locais de inflamação e se liga ao ferro com maior afinidade que a Tf em pH ácido, que ocorre em alguns locais de infecção. Existe um alto nível de semelhança na identidade entre Tf e Lf, principalmente na estrutura de ambas (YU; SCHRYVERS, 2002).

A proteína de união à transferrina A (TbpA) consiste em uma proteína integral de membrana de aproximadamente 100 kDa e dependente energeticamente do complexo TonB. Esta proteína forma um poro transmembrana que conecta o espaço extracelular com o periplasmático. TbpA liga-se a transferrina também no C-lóbulo, com uma superfície bastante grande de 81 resíduos aminoácidos. Essa superfície é altamente eletropositiva, o que complementa a superfície eletronegativa da transferrina (NOINAJ; BUCHANAN; CORNELISSEN, 2012).

A proteína de união à transferrina B (TbpB) tem menor tamanho (60 - 80 kDa) e está ancorada na membrana externa por uma sequência de 18 aminoácidos hidrofóbicos. A TbpB é responsável por captar a transferrina e transportá-la até a superfície da TbpA, a qual extrai o ferro da transferrina e transporta este elemento para o espaço periplasmático. Estruturalmente, a TbpB está constituída por dois lóbulos denominados de N e C, arquitetonicamente muito

parecidos. Funcionalmente, o lóbulo N (altamente eletropositivo) tem alta afinidade pela transferrina (Tf), já o lóbulo C (eletronegativo) apenas consegue fixá-la (fracamente). Adicionalmente, o lóbulo C apresenta-se, de forma geral, bem mais conservado que o lóbulo N nos micro-organismos *H. parasuis*, *A. pleuropneumoniae* e *A. suis* (NOINAJ; BUCHANAN; CORNELISSEN, 2012; YANG et al., 2011).

Ao contrário da TbpA, que liga com igual afinidade a holo-Tf e a apo-Tf, a TbpB tem grande preferência de ligação pela a holo-Tf (carregada), sendo a função primária desta proteína a seleção da Tf a ser capturada. A interação entre a TbpB e a Tf ocorre pela ligação do N-lóbulo da TbpB com o C-lóbulo da Tf, assim como a TbpA também interage somente com o C-lóbulo da Tf, porém os sítios que cada uma das Tbps utilizada para a ligação, são distintos dentro do C-lóbulo da Tf (RETZER et al., 1998). Além disso, foi observada a necessidade da interação entre TbpA e TbpB para captação de ferro (FULLER et al., 1998). Para a formação do complexo, é necessária a presença da transferrina, e sem ela, sabe-se que as duas proteínas do sistema de captação de ferro não interagem. Foi sugerido que elas não constituem um complexo sistema de captação de ferro na membrana sem a presença da transferrina (POWELL et al., 1998; YANG et al., 2011).

O lóbulo N-terminal da TbpB liga-se nos dois domínios do lóbulo C-terminal da transferrina, mantendo uma conformação fechada que não permite a liberação do ferro antes de ser entregue a TbpA (CALMETTES et al., 2012). O peptídeo âncora é necessário para formação do complexo ternário no qual TbpB carrega a transferrina para a TbpA e provavelmente, é ele com sua variabilidade e mudança de estrutura que interage com o C-lóbulo da TbpB e modula essa interação. Durante a formação do complexo ternário o C-lóbulo da transferrina se modifica de uma conformação fechada para uma parcialmente aberta, facilitando a remoção do ferro pela TbpA e seu futuro transporte através da membrana (NOINAJ; BUCHANAN; CORNELISSEN, 2012).

As duas proteínas são expressas coordenadamente em um *operon* bicistrônico, semelhante a maioria dos patógenos, o gene *tbpB* está na frente do *tbpA* (MYERS et al., 1998; YU; SCHRYVERS, 2002). A variabilidade nas sequências e na antigenicidade é maior na TbpB, sendo TbpA é bem mais conservada porém nada como alguns outros antígenos de superfície que possuem fases ou variações antigênicas (exemplo, pili na *M. bovis*) (FULLER et al., 1998).

Pela exposição da TbpB na superfície, ela apresenta um alto nível de variação interespécies (<15% de identidade), demonstrando a necessidade do patógeno de evadir o sistema imune do hospedeiro, entretanto existem relatos de epítopos conservados entre essas

proteínas (GRAY-OWEN; SCHRYVERS, 1996). Enquanto o lóbulo-C e parte do centro do lóbulo-N são bastante conservadas, a parte do N-lóbulo que interage com a transferrina é onde ocorrem as maiores variabilidades na TbpB (CALMETTES et al., 2012; NOINAJ et al., 2012). Porém, alguns estudos demonstraram que o C-lóbulo da TbpB também consegue interagir com a transferrina, mas com menor afinidade (RENAULD-MONGÉNIE et al., 2004)

Em um estudo, demonstrou-se que mutantes de *Neisseria gonorrhoeae* sem TbpA (incapacitadas de utilizar transferrina como fontes de ferro) são avirulentas em humanos (CORNELISSEN et al., 1998), comprovando a importância desse sistema de captação de ferro para iniciar e proliferar a infecção (NOINAJ et al., 2012). Isolados sem TbpB continuaram utilizando ferro a partir da transferrina, porém de forma menos eficiente, aproximadamente 50% menos de eficiência (RENAULD-MONGÉNIE et al., 2004). Corroborando com isso, TbpB demonstrou sua importância na sobrevivência e estabelecimento da doença em um modelo de infecção suíno por *Actinobacillus pleuropneuomoniae*, onde um isolado sem TbpB foi avirulento e incapaz de colonizar o sistema respiratório do hospedeiro (BALTES; HENNIG-PAUKA; GERLACH, 2002).

Em relação as proteínas de união à lactoferrina (Lbps), elas apresentam alta homologia com as Tbps entre *Moraxella* sp. e *Neisseria* sp., e foi observado que compartilham a mesma ligação e funcionamento entre as proteínas e a Lf, que as Tbps com a Tf em *Neisseria meningitidis* (BROOKS; ARUTYUNOVA; LEMIEUX, 2014; OSTAN et al., 2017).

Essas proteínas têm sido apontadas como potenciais antígenos vacinais desde sua identificação devido sua acessibilidade de superfície e até o momento, a maioria dos esforços para avaliar a eficácia potencial desses antígenos de superfície tem sido focada nas Tbps (SCHRYVERS et al., 1998).

A capacidade protetora de TbpB foi demonstrada em *Glaesserella parasuis* (FRANDOLOSO et al., 2015) e a proteína recombinante induziu anticorpos que reconheceram TbpBs de diversos isolados, com epítopos conservados distribuídos pelos dois lóbulos, reafirmando o grande potencial desta proteína (BARASUOL *et al.*, 2017). Além disso, verificou-se, em um estudo com *Moraxella catarrhalis*, um peptídeo derivado de LbpA altamente conservado em todas as cepas testadas e altamente imunogênico em camundongos, resultado na eliminação de *M. catarrhalis* dos pulmões de camundongos previamente imunizados (YASSIN; AMIN; ATTIA, 2016). Além disso, para *Neisseria meningitidis* LbpA foi imunogênica e capaz de induzir anticorpos bactericidas, mas a reatividade cruzada desses anticorpos foi limitada (PETTERSSON et al., 2006).

No entanto, em alguns estudos com *M. catarrhalis* e *N. meningitidis*, LbpA e TbpA foram relatados como não imunogênicos ou incapazes de induzir anticorpos bactericidas em humanos (MYERS et al., 1998; YU et al., 1999). Adicionalmente, observam-se alguns entraves apresentaram muitas para a produção de um antígeno vacinal de LbpA e TbpA em larga escala, como a localização destas na célula e a demanda por algum detergente para extrair e purificar as proteínas (FEGAN et al., 2019). Com isso, são necessárias novas abordagens frente a essas proteínas com o intuito de produzir antígenos mais eficientes.

Nesse contexto, para iniciar a investigação dessas proteínas como potenciais antígenos vacinais frente a CIB, a análise de sua diversidade genética se faz essencial. Desta forma, este estudo teve o objetivo de investigar a diversidade das proteínas LbpA, TbpA e TbpB entre e dentro das espécies de *M. bovis* e *M. bovoculi* e sua distribuição nas respectivas estruturas para auxiliar no entendimento e desenvolvimento de uma vacina eficaz frente a CIB, com hipótese de que as proteínas que compõem os receptores de lactoferrina e transferrina serão geneticamente conservados o suficiente para serem considerados como bons potenciais antígenos vacinais.

Diante do exposto acima, a seguir serão apresentados dois manuscritos, os quais abordam a diversidade dos receptores de transferrina e lactoferrina de *M. bovis* e *M. bovoculi*, e o desenvolvimento de potenciais antígenos vacinais. Manuscrito 1 intitulado de “Genetic diversity of transferrin binding proteins A and B in *Moraxella bovis* and *Moraxella bovoculi*” e o manuscrito 2 “Diversity and development of hybrid LbpA antigen from *Moraxella bovis* and *Moraxella bovoculi*”. Além disso, o manuscrito 3, que está aceito na revista Ciência Rural, aborda um assunto diferente da tese e uma nova metodologia de diagnóstico molecular utilizando papel filtro com *Clostridium chauvoei,* o qual foi desenvolvido no período do doutorado.

2 MANUSCRITO 1

Genetic diversity of transferrin binding proteins A and B in *Moraxella bovis* and *Moraxella bovoculi*

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Genetic diversity of transferrin binding proteins A and B in *Moraxella bovis* and *Moraxella bovoculi*

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Highlights

TbpB presented higher variability than TbpA between the thirty-seven strains of *M. bovis* and *M. bovoculi* analyzed.

TbpB variability was mostly restricted to the N-lobe of the predicted structure.

Phylogenetic tree of TbpB clustered the amino acid sequences according to the species.

The approach of selecting two TbpB representative strains presents the potential to cover all variability and to be further investigated as vaccine antigens for IBK.

Abstract

Infectious bovine keratoconjunctivitis (IBK) is a disease that impacts cattle production due to eye lesions and loss of vision, resulting in pain, reduction of milk yields, and decreased weight gain. The etiologic agents associated with IBK are *Moraxella bovis* (*M. bovis*) and *Moraxella bovoculi* (*M. bovoculi*). Vaccination is essential in the disease control; however, there are no efficient vaccines available. This work aimed to analyze the sequence diversity of receptor proteins transferrin binding protein A (TbpA) and transferrin binding protein B (TbpB) in *M. bovis* and *M. bovoculi*. To achieve this goal, phylogenetic analysis was performed based on *tbpA* and *tbpB* genes from 37 *M. bovis* and *M. bovoculi* strains. Subsequently, the alignments

were mapped to the structure models of the proteins selected as representative. In the phylogenetic analysis, TbpB sequences shown higher variability and were separated by species. Strains from North America clustered apart of strains from South America in TbpA. The sequences of TbpB mapped into the respective structure displayed N-lobe as the most diverse region, which is the site of interaction with transferrin. TbpA was highly conserved throughout all the structure. These results show the transferrin receptor proteins are potential considerable conserved vaccine antigens and present the possibility of using two representative strains from TbpB that could cover all variability found in this study.

Keywords: infectious bovine keratoconjunctivitis, cattle, TbpA, TbpB, sequence diversity

1. Introduction

Infectious bovine keratoconjunctivitis (IBK, or "pink eye"), is the most common and highly contagious ocular disease in cattle worldwide (Brown et al., 1998), with enormous economic impact due to loss of vision, and therefore, decreased weight gain, reduction in milk production, costs with antibiotics and harm to the welfare of the animals infected (Snowder et al., 2005). *Moraxella bovis* is the primary agent of this disease and can be isolated from nasal or conjunctival secretions and it is believed to be transmitted by direct contact, through animal handlers or flies (Brown et al., 1998). *Moraxella bovoculi* shares the same pathogenic mechanisms as *M. bovis* and it is found in pure culture of a high number of animals affected, however, is only recognized as an associated pathogen to IBK (Postma et al., 2008).

IBK prevention through vaccination still relies upon commercially available vaccines composed of whole-cell or purified pili of *Moraxella* spp., which usually only shows homologous protection, attributed to a high antigenic diversity of pili and the presence of different *Moraxella* species (Davidson and Stokka, 2003; Burns and O'Connor, 2008; O'Connor et al., 2011).

Moraxella spp. expresses surface transferrin binding proteins (Tbps) under iron-limiting conditions as an adaptation to living in the upper respiratory tract, these receptors are mechanisms of survival for the pathogens and are responsible for the maintenance of the infection in the host (Yu and Schryvers, 1994). The bacterial transferrin (Tf) receptor is composed of transferrin binding protein A (TbpA) consists of an integral membrane protein and energy-dependent on the TonB complex, this protein forms a transmembrane pore that connects the extracellular space with the periplasm (RETZER et al., 1998). The transferrin binding protein B (TbpB) is anchored in the outer membrane by a sequence of 18 hydrophobic amino acids and it is responsible for capturing transferrin and transporting it to the surface of TbpA, which extracts iron from transferrin and transports this element to the periplasmic space. Structurally, TbpB is composed by two lobes called N-lobe and C-lobe, which are architecturally similar (YANG et al., 2011).

Studies comprehending immunization with antigens derived from Tbps demonstrate a great ability to prevent infection as well as to eliminate colonization of the upper respiratory tract in bovine (*Mannheimia haemolytica*) and swine (*Glaesserella parasuis)* infections (Potter et al., 1999; Frandoloso et al., 2011; Barasuol et al., 2017). A great potential of these receptors as vaccine antigens is also expected due to their privileged position on the cell surface, and their presumed ubiquity in all isolates of *Moraxella* spp.

Knowledge about the genetic diversity of *M. bovis* and *M. bovoculi* is scarce. Understanding sequence variation and conserved regions of proteins are essential to place these proteins as candidates for the formulation of an efficient and broad-spectrum vaccine against IBK. In this context, the present study aimed to analyze the sequence and structural diversity of the transferrin receptor proteins in *M. bovis* and *M. bovoculi*.

2. Materials & methods

2.1 Strains and DNA extraction

M. bovis and *M. bovoculi* field strains (n = 29) used in this study were sampled with swabs from the eyes of cattle presenting clinical signs of IBK. After growth in 5% defibrinated sheep blood agar the colonies were characterized by morphology, Gram staining, and biochemical tests (Macfaddin, 2000). The *Moraxella* species were differentiated and confirmed through the polymerase chain reaction (PCR) technique as described by Angelos and Ball (2007).

The samples were stored in a freezer -20°C at the Laboratory of Bacteriology from the Federal University of Santa Maria, Brazil, from 1986 to 2018, comprising strains from Brazil, Uruguay, and Argentina. Additional strains (n = 3) were obtained from Schryvers Laboratory from the University of Calgary, Canada, isolated in 1993. Also, ATCC reference strains from *M. bovis* and *M. bovoculi* (n = 2) were used. The total of strains, that represents one single animal each, are all described in Table 1.

For DNA extraction the bacteria were grown in blood agar and incubated for 36-48 hours at 35°C and then a loop of pure culture was suspended in 5 ml of brain heart infusion (BHI) and growth overnight. Genomic DNA was extracted from these cultures using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's guidelines. The genomic DNA quantity and quality were assessed by spectrophotometry (NanoDrop, Thermo Scientific, USA) and stored at -20ºC until use.

2.2 PCR amplification and sequence analysis

Polymerase chain reaction (PCR) was used to amplify *tbpA* and *tbpB* genes from *M. bovis* and *M. bovoculi*. The PCR master mix consisted of 10μL PCR reaction buffer, 1μL of 10 mM dNTPs, 2.5μL of each primer at 5 μM, 1μL of Phire Hot Start II DNA Polymerase (Waltham, Massachusetts, EUA), and 1μL of genomic DNA template.

Primers were designed as part of this study using the Clone Manager 9 software (Sci Ed Software LLC, 2016) to amplify the whole *tbpA* gene (TbpAMbov-F and TbpAMbov-R) and *tbpB* gene (TbpBMbov-F and TbpBMbov-R). An additional set of primers (TbpAMbov-IF and TbpAMbov-IR) was used to amplify an internal region of the *tbpA* gene to complete the full gene. The primer sets include the sequencing primers M13F and M13R at their 5_ end.

PCR was carried out in a BioRad Thermocycler (Mississauga, Ontario, Canada) and the cycling conditions were, initial denaturation of 98°C for 30s; 35 cycles of 98°C for 5s; 61°C (TbpAMbov-F and TbpAMbov-R); 62°C (TbpAMbov-IF and TbpAMbov-IR) and 55 °C (TbpBMbov-F and TbpBMbov-R) for 5 s and 72°C for 30s and a final extension at 72°C for 2 min. The amplified products were electrophoresed and visualized on a 2% agarose gel stained with ethidium bromide (0.5 μg) to confirm amplification.

Sanger sequencing was performed by Macrogen USA (Rockville, MD) or the University of Calgary DNA Services. Three complete sequences (n = 3) were also obtained from annotated genomes found at National Center for Biotechnology Information (NCBI) databases from IBK clinical cases. The total DNA sequences (n = 37) were then translated into amino acids using the ExPASy bioinformatics resource portal online (https://web.expasy.org/translate/) (Gasteiger et al., 2003). Alignment of the amino acid sequences was conducted using MEGA, version 7.0 (Kumar et al., 2016). Phylogenetic analysis was also performed using MEGA, version 7.0, and the unrooted phylogenetic trees were generated from cleaned alignments using the maximum likelihood method, with 1000 Bootstraps, employing the JTT model with standard settings.

2.3 Representative strains and structural models

The phylogenetic trees were analyzed with the software NaVARgator (https://github.com/dave-the-scientist/navargator/blob/master/navargator.py) (D Curran, 2018,

unpublished data), which was used to classify the strains into clusters and to select the representative ones for each protein. The *ab initio* modeling software I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the structures of TbpA and TbpB from the representative strains reflecting each cluster identified through the phylogenetic analysis. The sequence alignments were mapped onto the structural models using the MolecularToolbox software (D Curran, 2014, unpublished data) and the structures were viewed with PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

3. Results

3.1 Moraxella strains identification

All 29 clinical strains analyzed in this study were confirmed through microbiological culture and PCR (Angelos and Ball, 2007) and identified as 6 *M. bovis* and 23 *M. bovoculi*.

3.2 Sequence diversity

The genes *tbpA* and *tbpB* were amplified in all *M. bovis* and *M. bovoculi* strains investigated in this study, with gene sizes of 2800 bp and 1800 bp, respectively. Phylogenetic trees were generated, and, in both genes, two distinct clusters were identified, which were supported by bootstrapping scores. However, within each clade, the support values vary greatly (Figures 1 and 2).

The TbpA phylogenetic tree separated the amino acid sequences into two major clades, one with only *M. bovoculi* strains and the other one with all of the *M. bovis* and two *M. bovoculi* (186V and SB31/18 H3) strains. The maximum likelihood unrooted phylogenetic tree of the 37 TbpA sequences is shown in Figure 1. TbpA *M. bovis* strains were separated into two clades according to geographic region, where strains originated from South America clustered apart

of North America (USA + Canada) sequences. In the TbpB phylogenetic tree, all amino acid sequences were exactly separated by species, *M. bovis* strains were in a clade and *M. bovoculi* in another. The maximum likelihood unrooted phylogenetic tree of the 37 TbpB sequences is shown in Figure 2. Inside the *M. bovis* strains cluster, they were also separated by origin South America and North America, except for SB246/04. For both TbpA and TbpB the ATCCs were separated from both groups.

The amino acid sequence alignment showed 93.77% of conserved sites among the TbpA sequences and 79.79% among the TbpB. Considering the groups *M. bovis* and *M. bovoculi*, TbpA analysis presented low distances inside both groups (0.009) and between groups (0.021). TbpB showed similar results but higher distances within groups *M. bovis* (0.012) and *M. bovoculi* (0.014) and between groups (0.183). Also, mean *p*-distance in TbpA ranged from 0.0 to 0.031 and in TbpB from 0.0 to 0.173. This analysis of the diversity of TbpA and TbpB from strains of *M. bovis* and *M. bovoculi* indicated that TbpB presented more variability than TbpA, especially between the two different species.

3.3 Mapping of the alignments into structural models

The phylogenetic trees were analyzed by NAVargator software and strains SBP126/17 901 (*M. bovis*) and JUR3 (*M. bovoculi*) were selected as representative ones for TbpA and strains SBP13/18 H1 (*M. bovis*) and GF9 (*M. bovoculi*) were chosen for TbpB. Then, these representative strains were successfully used for the prediction of structural models.

Mapping of the alignments into the structural models in TbpA showed high conservation throughout the whole structure as shown in a preponderance of blue in Figure 3 (A and B). In the TbpB structures, the conserved regions painted in blue were mostly located in the C-lobe region, as shown in Figure 3 (C and D). Colorful regions showed the variability in the N-lobe, in both handle domain and β-barrel of TbpB. These findings revealed that the

higher variability in TbpB was located in the N-lobe region of the protein structure, and TbpA showed less variability throughout the whole protein. There was almost no significant variation in the map alignments of both proteins in *M. bovis* and *M. bovoculi*.

4. Discussion

A considerable number of research groups have been investigating potential vaccines against *M. bovis* and *M. bovoculi* to prevent IBK since commercially available vaccines present limited efficacy. Transferrin binding proteins have been considered as potential vaccine antigens for many diseases with importance in veterinary medicine, such as Glasser's disease (*G. parasuis*) and bovine respiratory disease (*M. haemolytica*). These proteins have shown promising results in situations of experimental challenge, were able to prevent infection, and also to reduce or eliminate the colonization of the upper respiratory tract (Potter et al., 1999; Frandoloso et al., 2011; Barasuol et al., 2017).

Nevertheless, the development of a transferrin receptor-based vaccine is still challenging due to sequence and antigenic diversity among transferrin receptor proteins. This highlights the importance of this study, which provides further insights into the sequence diversity of TbpA and TbpB in *M. bovis* and *M. bovoculi* species and the distribution throughout the protein structure, which is an essential step in the evaluation of these antigens as potential vaccine components.

Strains from IBK clinical cases analyzed in this study were from a considerable representative collection, limited geographically but diverse temporally. Consisting of 37 strains, 11 *M. bovis*, and 26 *M. bovoculi*, distributed in 32 years, most of them from Brazil, with also some strains from Uruguay, Argentina, Canada, and the USA, comprehending South American and North American strains.

The *tbpA* and *tbpB* genes were detected in all *M. bovis* and *M. bovoculi* strains analyzed. The transferrin receptor was already identified in *M. bovis* and now also in *M. bovoculi* (Yu and Schryvers, 1994). Yu and Schryvers (1994) also showed that the *M. bovis* receptors bind specifically to the bovine transferrin and that *M. bovis* can capture iron from the Tf (Yu and Schryvers, 1994.; Yu and Schryvers, 2002). Also, it was demonstrated in *M. catarrhalis* that when TbpA is deleted the iron capture is blocked, and when TbpB is not present, occurred a decrease in the efficiency of the system, showing these proteins are essential in the caption of iron (Luke and Campagnari, 1999).

Phylogenetic trees of TbpA and TbpB amino acid sequences mostly clustered them into two main genetically different groups, basically according to the species, differently from a study with *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, and *G. parasuis* where the transferrin receptor protein sequences clustered independently of the species (Curran et al., 2015), showing that a potential vaccine for IBK using this proteins should be composed by the two species to cover all variability presented here.

As shown in this study, TbpA is highly conserved within the *M. bovis* and *M. bovoculi* groups. On the other hand, TbpB presented higher variability and distances inside and between the species. While the TbpB phylogenetic tree clustered all protein sequences exactly by species, *M. bovis* strains were in a clade and *M. bovoculi* in another, for TbpA there were two exceptions, where one clade comprised only *M. bovoculi* strains and the other comprised all *M. bovis* and two *M. bovoculi* (186V and SB31/18 H3). The two strains that did not follow the species grouping, were closely related to *M. bovis* TbpA, with similarities higher than 95% with the ATCC strain. This analysis presents that both proteins presented advantages to be approached as vaccine antigens.

The clustering patterns of TbpA were similar but did not match exactly the TbpB distribution, suggesting they were capable of evolution independently of the other protein as it

occurs in other pathogens (Curran et al., 2015). *M. bovoculi* strains from the same outbreak were closely related and most of them located in the same branches, like SB31/18 NH1 and SB31/18 NH2, that shows that even with different strains inside an outbreak a potential vaccine based on these antigens could cover all of them. Showing better prospects than type IV pili, one of the most used antigens for IBK vaccines, although pili-based immunity is specific to serogroup and highly variable according to the geographic region and inside each outbreak (Sosa et al., 2015).

Therefore, most of the *M. bovis* vaccines until now are composed of antigens with high variability compared to the proteins described here. Both TbpA and TbpB display potential for the composition of a vaccine for IBK, especially with the addition of *M. bovoculi*, which is reported to be involved in a high number of cases and is absent in the composition of the most commonly used vaccines.

Although most of the older strains have shared the same branches, strains from 2018 did not cluster, suggesting years may not influence their variability. The geographic region seems to significantly influence the genetic variability of both proteins in *M. bovis* once they were separated into two clades (i. e. South America cluster vs. North America cluster). An exception was ATCC *M. bovis* and SB246/04 TbpBs sequence, which did not follow that distribution.

Mapping of the alignments into the structural models of TbpA showed high conservation throughout almost the whole structure, especially in the β-barrel region. It was observed that there is variability in the TbpA predicted structure located in the extracellular loop regions, belonging to the handle domain, those known to be most accessible to the host immune system (Noinaj et al., 2012a). On contrary, TbpB, because of the exposure in the surface, displayed way higher diversity and the major variability was located in the N-lobe region, handle domain and β-barrel, called the cap region, that interacts with the transferrin and thus interacting with host immune system (Adamiak et al., 2015). Interesting, the C-lobe, which

also interacts with transferrin but with less affinity, was fairly conserved as already observed in other species and it is especially displayed in the large loop regions (Renauld-mongénie et al., 2004; Calmettes et al., 2012).

Even with the substantial variability in the cap region of TbpB, it was already shown that the mutation of individual amino acids did not affect binding, that there is somehow conservation in the binding region between *Glaesserella parasuis* TbpB and swine transferrin despite the sequences diversity (Frandoloso et al., 2015). This suggests that the variation in residues in the binding region among different TbpBs may control the exchange from different clusters, so the variability showed here in TbpB probably does not affect the protein capacities. Also, it was demonstrated high cross-protective antibody induced by TbpB and TbpB derived antigens in *Neisseria meningitidis* (Calmettes et al., 2012; Noinaj et al., 2012b).

TbpA presented less variability, however, it is still not considered a good and practicable option for large-scale development (Fegan et al., 2019). Due to its location in the outer membrane as an integral membrane protein, with not all surface is available, its production as vaccine antigen is challenging, it demands detergent to be extracted and purified, and several reagents to maintain its stability and solubility after processing, requiring more steps than TbpB based antigen, and usually producing lower yields (Fegan et al., 2019). To overcome this challenge, TbpA as a vaccine target may require novel approaches for generating antigens like the development of hybrid antigens displaying conserved loops from the receptor protein (Fegan et al., 2019).

Although the overall diversity, especially in the N-lobe region of TbpB, the approach of selecting representative strains through NAVargator, may make the challenge of sequence diversity for vaccine design more manageable, with the possibility of covering the variability. Since it was demonstrated that the receptor protein sequences clustered according to the

Moraxella species, the suggested vaccine based on this receptor should logically target both species.

5. Conclusion

This study shows that the TbpA and TbpB receptor proteins are considerably conserved in *M. bovis* and *M. bovoculi*. The approach for a vaccine based on these receptors should logically target the two *Moraxella* species involved in the IBK and potentially be derived from the two representative strains selected in this study for each protein, which would cover all the variability among the isolates analyzed.

Conflicts of interest

The authors have no conflict of interest to declare.

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Table 1 –*Moraxella bovis* and *Moraxella bovoculi* strains used in this study.

Strain ID	Species	Year	Origin
SB31/18 H3	*M. bovoculi*	2018	Brazil
SBP13/18 I	*M. bovoculi*	2018	Brazil
SB31/18 NH1	*M. bovoculi*	2018	Brazil
SB31/18 NH2	*M. bovoculi*	2018	Brazil
SBP13/18 1091 F	*M. bovis*	2018	Brazil
SBP13/18 H	*M. bovis*	2018	Brazil
SBP13/18 M	*M. bovoculi*	2018	Brazil
SBP13/18 P	*M. bovis*	2018	Brazil
SBP126/17 636	*M. bovis*	2017	Brazil
SBP126/17 901	*M. bovis*	2017	Brazil
SB15/13	*M. bovoculi*	2013	Brazil
SB57/12 8127	*M. bovoculi*	2012	Brazil
SB15/10	*M. bovoculi*	2010	Brazil
246/04	*M. bovis*	2004	Brazil
SB150/02 5	*M. bovoculi*	2002	Brazil
SB151/01	*M. bovoculi*	2001	Brazil
186V	*M. bovoculi*	1999	Argentina
56/96	*M. bovoculi*	1996	Brazil
n388	*M. bovis*	1993	Canada
n113	*M. bovis*	1993	Canada
n114	*M. bovis*	1993	Canada
Jackson	*M. bovoculi*	<1990	Brazil
2419	*M. bovoculi*	<1990	Uruguay
JUR 3	*M. bovoculi*	<1990	Brazil
Alegrete	*M. bovoculi*	<1990	Brazil
2439	*M. bovoculi*	<1990	Uruguay
173	*M. bovoculi*	<1990	Brazil
Viviane	*M. bovoculi*	<1990	Brazil
290	*M. bovoculi*	<1990	Brazil
JUR 5	*M. bovoculi*	<1990	Brazil
3486	*M. bovoculi*	<1990	Brazil
GF9	*M. bovoculi*	1986	Brazil
ATCC 17949 *M. bovis*	*M. bovis*	-	US
ATCC BAA1259 *M. bovoculi*	*M. bovoculi*	-	US
Epp63 (NZ_CP030241*)	*M. bovis*	-	US
Strain 58069 (NZ_CP011374.1*)	*M. bovoculi*	-	US
Strain 58086 (NZ_CP011381*)	*M. bovoculi*	-	US

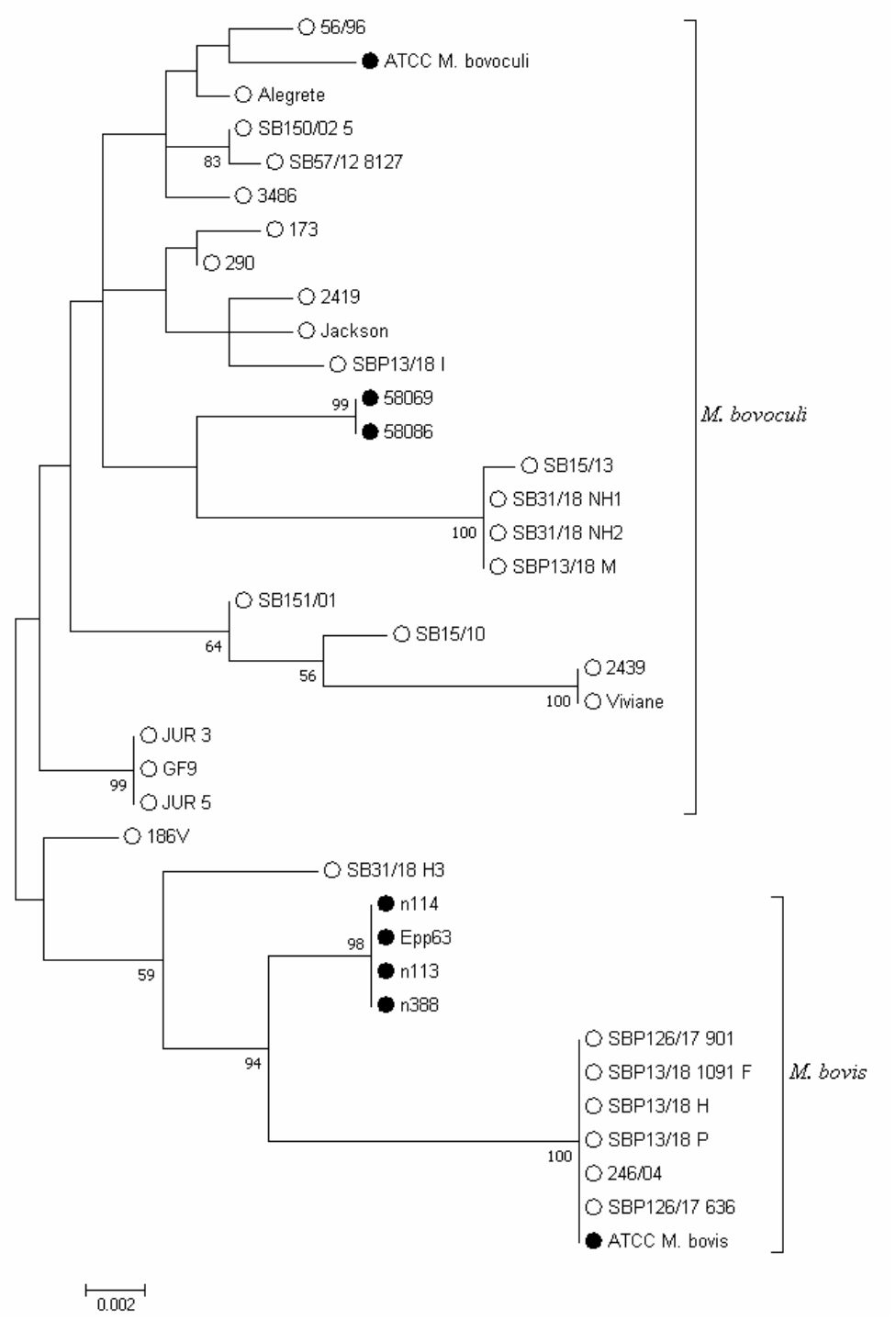

Figure 1. Phylogenetic tree constructed by Maximum Likelihood method based on the JTT matrix-based model of 37 amino acid sequences of transferrin binding protein A (TbpA) from *Moraxella bovis* (11) and *Moraxella bovoculi* (26). Only bootstrap values higher than 60% of 1000 replicates are shown. Black circles marks strains from North America and white circles strains from South America. Evolution analysis were conducted in MEGA 7.0.

Figure 2. Phylogenetic tree constructed by Maximum Likelihood method based on the JTT matrix-based model of 37 amino acid sequences of transferrin binding protein B (TbpB) from *Moraxella bovis* (11) and *Moraxella bovoculi* (26). Only bootstrap values higher than 60% of 1000 replicates are shown. Black circles marks strains from North America and white circles strains from South America. Evolution analysis were conducted in MEGA 7.0.

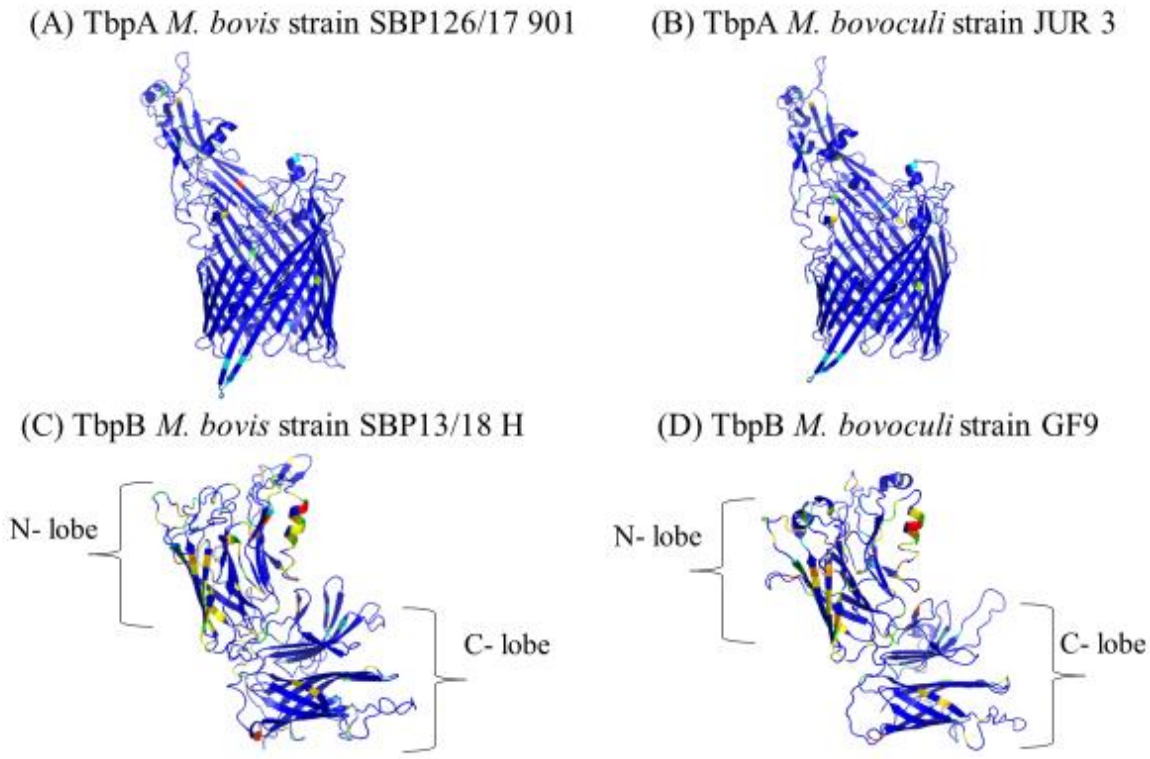

Figure 3. Sequence alignments mapped in the structural model of TbpA (transferrin binding protein A) *Moraxella bovis* strain SBP126/17 901 (A) and *Moraxella bovoculi* strain JUR 3 (B), and TbpB (transferrin binding protein B) *Moraxella bovis* strain SBP13/18 H (C) and *Moraxella bovoculi* strain GF9 (D). Regions colored in blue indicates conserved amino acids between the amino acid sequences and colorful (yellow to red) parts indicate the variable regions. Structures were predicted using I-TASSER and the sequences were mapped in the structures with MolecularToolbox.

3 MANUSCRITO 2

Diversity and development of hybrid LbpA antigen from *Moraxella bovis* and *Moraxella bovoculi*

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(Submetido a Veterinary Microbiology)

Diversity and development of hybrid LbpA antigen from *Moraxella bovis* and *Moraxella bovoculi*

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Highlights

LbpA was very conserved between the thirty-six strains of *M. bovis* and *M. bovoculi* analyzed.

Phylogenetic tree clustered LbpA amino acid sequences according to species of *Moraxella*.

Five hybrids displaying LbpA loops of *Moraxella* spp. in the VcSLP scaffold were successfully designed and expressed in small scale.

LbpA presents potential to be further investigated as a vaccine antigen for IBK.

Abstract

Infectious bovine keratoconjunctivitis (IBK) is the most important ocular disease in cattle and the main agents involved are *Moraxella bovis* and *Moraxella bovoculi*. Prevention through vaccination is the main control method, but the currently available vaccines have no proof of efficiency. Lactoferrin binding protein A (LbpA) is a lipoprotein located in the outer membrane that belongs to a receptor complex that captures iron, essential for the development of the disease. So far, this lipoprotein was not investigated as a potential vaccine antigen for IBK. This study aimed to investigate the sequence diversity of this protein and design hybrid

antigens with a surface lipoprotein (SLP) from *Vibrio cholerae* as a scaffold. Thirty-six sequences of *M. bovis* and *M. bovoculi* strains were used in this study. DNA was extracted and the *lbpA* gene amplified by PCR. Following this, the amplicons were sequenced, and the DNA sequences were translated into amino acids and aligned. Subsequently, a maximum likelihood phylogenetic tree was constructed, and the alignment was mapped in the LbpA *M. bovis* structure. After this analysis, five hybrid antigens using the LbpA loops were constructed using the VcSLP scaffold. LbpA was highly conserved between the strains, and the phylogenetic tree clustered the amino acid sequences according to the *Moraxella* species. LbpA was shown to be very conserved between the different strains and the hybrids displaying the LbpA loops in the VcSLP scaffold were successfully designed and produced, presenting the potential to be further investigated as a vaccine antigen.

Keywords: LbpA, *Moraxella bovis*, *Moraxella bovoculi*, sequence diversity, hybrid

1. Introduction

Infectious bovine keratoconjunctivitis (IBK, or “pink eye”) is the most common and highly contagious ocular disease in cattle worldwide and the second most important in calves (Brown et al., 1998) with substantial economic impact due to loss of vision, decreased weight gain, reduction in milk production, costs with antibiotics and impact in the welfare of the animals infected (Snowder et al., 2005). The pathogens *Moraxella bovis* and *Moraxella bovoculi* are involved in the development of the disease (Postma et al., 2008), and IBK prevention through vaccination relies upon commercially available vaccines composed of whole-cell or *Moraxella bovis* pili purified, which presents a high antigenic diversity and seems to confer only homologous protection (Davidson and Stokka, 2003; Burns and O’Connor, 2008; O’Connor et al., 2011).

The bacterial lactoferrin receptor complex that *Moraxella* spp. express consists of a TonB dependent integral membrane protein, a lactoferrin binding protein A (LbpA), that transfer iron across the outer membrane, and a peripheral lipoprotein, lactoferrin binding protein B (LbpB) that extends away from the bacterial surface to bind iron-loaded lactoferrin (Gray-Owen and Schryvers, 1996; Wong and Schryvers, 2003). The lactoferrin receptor is responsible for the adaptation of the bacteria to survive and maintenance of the infection in the upper respiratory tract. Furthermore, the inactivation of LbpA interrupts the iron uptake process as LbpB lowers the efficiency of iron capture, confirming their essential role in the pathogenesis of the disease (Cornelissen et al., 1992; Pettersson et al., 1994; Yu and Schryvers, 2002).

Studies comprehending immunization with transferrin binding proteins (Tbps), which is known to be functionally and genetically related to LbpA and LbpB (Gray-Owen and Schryvers, 1996), demonstrate great ability to prevent infection as well as to eliminate colonization of the upper respiratory tract in cattle and swine (Potter et al., 1999; Frandoloso et al., 2011; Barasuol et al., 2017; Guizzo et al., 2018). However, there is still little information about lactoferrin binding proteins (Lbps) production and immune response but in some studies with *M. catarrhalis* and *N. meningitidis*, LbpA were related as non-immunogenic or not able to produce bactericidal antibodies (Myers et al., 1998; Yu et al., 1999) and to present some barriers for antigen production (Fegan et al., 2019).

To overcome these challenges, it was recently described with TbpA, a hybrid antigen approach in which surface epitopes from the *Neisseria meningitidis* TbpA receptor protein were displayed on a derivative of the C-lobe of the TbpB, the loopless C-lobe (LCL). The engineered hybrid antigen provided stable and immunogenic antigens and was able to produce anti-TbpA bactericidal antibodies that inhibit transferrin-dependent growth (Fegan et al., 2019).

It is essential to know the genetic diversity of the *lbpA* gene from *M. bovis* and *M. bovoculi* and to identify conserved regions to access the LbpA protein as a candidate for the

formulation of an efficient and broad-spectrum vaccine against IBK. Taking this into consideration, this study aimed to discover the sequence diversity of the LbpA receptor protein and then engineer hybrid antigens derived from this protein.

2. Materials and methods

2.1 Strains and DNA extraction

M. bovis and *M. bovoculi* field strains (n = 28) used in this study were sampled with swabs from the eyes of cattle presenting clinical signs of IBK. After growth in 5% defibrinated sheep blood agar, the colonies were characterized by morphology, Gram staining, and biochemical tests (MacFaddin, 2000). The *Moraxella* species were differentiated and confirmed through the polymerase chain reaction (PCR) technique as described by Angelos and Ball (2007).

The samples were stored in a freezer -20°C at the Laboratory of Bacteriology from the Federal University of Santa Maria from 1986 to 2018, comprising strains from Brazil, Uruguay, and Argentina. Additional strains (n = 3) were obtained from Schryvers Laboratory from the University of Calgary, Canada, isolated in 1993. Also, two ATCC reference strains (n=2) were used. All 36 strains are described in Table 1, consisting of 11 *M. bovis* and 25 *M. bovoculi*.

For DNA extraction, the bacteria were grown in blood agar and incubated for 36-48 hours at 35°C, and then a loop of pure culture was suspended in 5 ml of brain heart infusion (BHI) and growth overnight. Genomic DNA was extracted from these cultures using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's guidelines. The genomic DNA quantity and quality were assessed by spectrophotometry (NanoDrop, Thermo Scientific, USA) and stored at -20ºC until use.

2.2 Sequencing and phylogenetic analysis

PCR was used to amplify the *lbpA* gene from *M. bovis* and *M. bovoculi* strains. The PCR master mix consisted of 10µL PCR reaction buffer, 1µL of 10 mM dNTPs, 2.5µL of each primer at 5 µM, 1µL of Phire Hot Start II DNA Polymerase (Thermo Scientific, Ottawa, ON, Canada), and 1µL of genomic DNA template. Primers were designed with Clone Manager 9 software (Sci Ed Software LLC, 2016) as part of this study to amplify the whole *lbpA* gene (LbpAMbov-F; LbpAMbov-R and LbpAMbov-IF; LbpAMbov-IR), and the sequencing primers M13F and M13R were added at their 5_ end.

PCR was carried out in a BioRad Thermocycler (Mississauga, Ontario, Canada) and the cycling conditions were, initial denaturation of 98°C for 30s; 35 cycles of 98°C for 5s; 56.6°C for 5 s and 72°C for 30s and a final extension at 72°C for 2 min. The amplified products were electrophoresed and visualized on a 2% agarose gel stained with ethidium bromide (0.5 µg) to confirm amplification.

Sanger sequencing was performed by Macrogen USA (Rockville, MD) or the University of Calgary DNA Services. Three sequences (n=3) were also obtained from annotated genomes found at National Center for Biotechnology Information (NCBI) databases. The total DNA sequences (n=36) were then translated into amino acids using the ExPASy bioinformatics resource portal online (https://web.expasy.org/translate/) (Gasteiger et al., 2003). Alignment of the amino acid sequences was conducted using MEGA, version 7.0 (Kumar et al., 2016). Phylogenetic analysis was also performed using the program MEGA, version 7.0, and the unrooted phylogenetic tree was generated from a clean alignment using the Maximum Likelihood method, with 1000 Bootstraps, employing the JTT model with standard configuration.

2.3 Mapping of the alignment in the structure

The phylogenetic tree was analyzed with the software NaVARgator (https://github.com/dave-the-scientist/navargator/blob/master/navargator.py) (D Curran, 2018, unpublished data) to select a representative strain between the analyzed. Consecutively, the modeling software I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/) was used to predict the structure of the LbpA Epp63 strain (*M. bovis*) based on the PDB model 3V89. Consecutively, the sequence alignment was mapped onto the structural model using the MolecularToolbox software (D Curran, 2014, unpublished data) and the structure was viewed with PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC).

2.4 Design and construction of hybrid antigens

The modeling software I-TASSER was also used to predict the structure of the surface lipoprotein from *Vibrio cholerae* (VcSLP) (Figure 1A) and then the predicted structures of LbpA Epp63 strain (*M. bovis*) (Figure 1B). VcSLP was employed as the basis for the antigen design, LbpA Epp63 was used for the selection of the loops, and VcSLP for the scaffold. This "foreign" scaffold was chosen since it is unlikely to induce antibodies against *M. bovis* and *M. bovoculi* and any serum reactivity would be attributed only to the displayed loops from LbpA in future experiments.

The sequence alignment of the eleven LbpA loops was realized with ESPript 3.0 and the conserved sites between the strains were considered (Robert and Gouet, 2014). Loop 1 was too small with 5 amino acids and was not considered. The other ones presented a variable number of amino acids, going from 15 to 90 residues, specifically loop 2 (90), loop 3 (55), loop 4 (26), loop 5 (44), loop 6 (14), loop 7 (33), loop 8 (17), loop 9 (21), loop 10 (30) and loop 11 (40).

For the hybrid gene construction, the loops were randomly selected, four for each construction. The fully automated BioXp 3200 system (SGI-DNA) was used to create the

constructs for transformation. Loops were removed from the VcSLP scaffold and these were replaced by DNA sequence encoding the desired LbpA Epp63 strain loops. The resulting hybrid genes were synthesized and cloned into customized expression vector p15 based with a tetR promoter (e5460) encoding an N-terminal polyhistidine-tagged. After they were transformed into chemically competent cells E. cloni ® 10G (the Lucigen *Escherichia coli* transformation strain) and plated on LB-chloramphenicol plates, the cloning was confirmed, and the plasmids were extracted. Five antigens were designed, and the resulting constructions were CMbVcSLPLbploops115106, EMbVcSLPlbploops2378, FMbVcSLPLbploops3489, GMbVcSLPLbploops45910, and HMbVcSLPLbploops21167.

2.5 Protein expression

The plasmids were then transformed into ER2566 cells and cultured in 20 mL of LB media overnight. Following, the overnight culture was transferred to 1L of LB broth, incubated at 37°C under rotation (150 rpm) to reach an optical density (OD) of 0.6 at 600 nm, at this time the protein expression was induced with IPTG 0.1M (Isopropyl-beta-D-thiogalactopyranoside) for 18 hours at 37°C. The culture was centrifuged at 4000 x rpm for 30 minutes at 4°C and the cell pellets were subsequently resuspended in resuspension buffer containing protease inhibitor (Roche), lysozyme (Sigma Aldrich), and DNase (Sigma Aldrich). The cells were then lysed by sonication and then the lysate was centrifuged at 14 000 x rpm for 90 minutes at 4°C. The resulting pellet was resuspended in 8M urea wash buffer and incubated under shaking overnight at 4°C.

The suspension was centrifuged at 14 000 x rpm for 50 min at 4°C and the supernatant was syringe-filtered (0.45 μm) and added to a Histrap FF 5 ml column for affinity capture using AKTA püre (GE healthcare). The column was washed with decreased concentrations of urea (8M-0M) wash buffer for refolding and after, the protein was eluted from the column using

elution buffer (300mM imidazole). The expression of the recombinant proteins was verified using SDS-PAGE. Next, a 50K Amicon Pro Affinity Concentrator (Millipore) was used to concentrate the proteins.

3. Results and discussion

All the strains analyzed in this study were from IBK clinical cases forming a considerable representative collection, the ones that were identified in this study were confirmed through microbiological culture and PCR (Angelos and Ball, 2007) as 6 *M. bovis* and 22 *M. bovoculi.*

The pursuit of an effective vaccine for the prevention of IBK has been a long-standing goal, with a lot of potential vaccines and even commercially available ones. However, there is no proof of good efficiency, and outbreaks of IBK are still a problem in cattle production. The considerable sequence and antigenic diversity among *M. bovis* and *M. bovoculi* strains continue to be a challenge (Burns and O'Connor, 2008; Kowalski et al., 2017).

The LbpA receptor protein has not yet been considered as a potential vaccine antigen in these species, even being highly conserved, as confirmed in this study (Figure 2), and essential for the disease development and maintenance of the pathogen in the mucosal surface (Yu and Schryvers, 2002). Besides, the *lbpA* gene was found in all clinical isolates from *M. bovis* and *M. bovoculi* of this study, as expected, with a size of 2751 bps (Yu and Schryvers, 2002; Pettersson et al., 2006).

The phylogenetic tree of the LbpA receptor protein clustered the sequences into two main genetically different groups, according to the species, one with *M. bovoculi* and the other with *M. bovis*, as shown in Figure 2, and is supported by bootstrapping scores; however, within each branch, the values vary greatly.

Strains were from 32 years apart, most of them from Brazil, with also some strains from Uruguay and Argentina, Canada, and the USA, comprehending South American and North American strains, respectively. But they were not especially separated by each outbreak, geographic region, or year of isolation. In general, the amino acid sequences were highly genetically conserved among them, which is shown by the short branches and the low distances within groups (0.011) and between the *M. bovis* and *M. bovoculi* groups (0.026). Also, the *p* distance ranged from 0.000 to 0.049, which shows the number of different sites between the strains was low.

LbpA had 93% of conserved sites between all strains from both species and the chosen representative strain Epp63 LbpA was highly similar to LbpA from *M. bovoculi* (98.69%), *Moraxella ovis* (96.50%), and even with the transferrin binding protein (TbpA) from *M. bovis* (96.68%), TbpA from *M. bovoculi* (95.85%) and *M. ovis* (96.47%). This demonstrates that the selection of this strain could cover practically all of the variability and even *M. ovis* strains, that was already reported to be isolated from mixed infections with *M. bovis* and/or *M. bovoculi* (Libardoni et al., 2012; O'Connor et al., 2012).

The structure of LbpA consists of a β-barrel domain and eleven loops extending out from the barrel, and when the sequences were mapped in the structure (Figure 3) displayed conserved regions throughout the whole protein, with some variable regions located in the β-sheets and on the loops. LbpA is believed to bind to the C-lobe of the lactoferrin, with the extended loops and the removal of iron is thought to be analogous to the TbpA: Transferrin complex (Yu and Schryvers, 2002).

The loops when aligned individually also showed high conservation. Loops 4,6,7,8,9, and 10 were 100% conserved between the 36 strains and the more variable were loop 5 with 88.6% of conserved sites, followed by loop 2 with 89.14%. The plug domain and the loop 3 helix (94.55%), which is important for binding, were also highly conserved. Some of these

loops in previous studies were confirmed to be surface-exposed and immunogenic like loops 3, 4, 5, 7, and 10 from *Neisseria meningitidis* LbpA (Prinz et al., 1999; Pettersson et al., 2006). Because of the high conservation and since they are mostly all surface exposed, they appeared to be good options for the design of the hybrid antigens in this study.

Interestingly, it was already observed in a study with *Moraxella catarrhalis* that a peptide derived from LbpA was very conserved across all the tested strains, highly immunogenic in mice, and effectively cleared *M. catarrhalis* from mice lungs. Besides, antibodies against the peptide were bactericidal against heterogeneous *M. catarrhalis* strains (Yassin et al., 2016). Furthermore, *Neisseria meningitidis* LbpA was immunogenic and able to induce bactericidal antibodies, but the cross-reactivity of these antibodies was limited (Pettersson et al., 2006).

However, in some studies with *M. catarrhalis* and *N. meningitidis*, LbpA and TbpA were related as non-immunogenic or not able to produce bactericidal antibodies (Myers et al., 1998; Yu et al., 1999) and besides that, presents some barriers to the production as an antigen on a large-scale basis, like the demand for detergent to extract and purify the proteins, needing new approaches for production and induction of a protective response (Fegan et al., 2019).

To overcome these challenges, it was recently described with TbpA, a hybrid antigen approach in which surface epitopes from the *Neisseria meningitidis* TbpA receptor protein were displayed on a derivative of the C-lobe of the TbpB, the loopless C-lobe (LCL). The engineered hybrid antigen provided stable and immunogenic antigens and was able to produce anti-TbpA bactericidal antibodies that inhibit transferrin-dependent growth (Fegan et al., 2019). Furthermore, surface loops from *Acinetobacter baumannii* ZnuD were presented in the same LCL scaffold, and immunization with this hybrid displaying four loops protected mice completely from infection (Qamsari et al., 2020).

Taking this into consideration, engineered hybrid antigens enter as a potential solution for the TbpA and LbpA possible problems of expression, and in this study, this approach was followed and presented a combination of different four loops of LbpA in a “foreign” scaffold. It was tested a different scaffold than the LCL since it previously interfered in the immune response and a surface lipoprotein from *Vibrio cholerae* was selected, so it would not produce antibodies against *M. bovis* or *M. bovoculi* (Qamsari et al., 2020).

All five LbpA hybrids designed were successfully synthesized, cloned, and expressed in small-scale. The proteins were expressed with sizes hybrid C (41.2 KDa), E (50.6 kDa), F (42.1 kDa), G (40.7 kDa), and H (48.1kDa). Protein purification was achieved with an AKTA purifier, with an average concentration of 2mg/ml of each protein, and did not present precipitation after dialysis with PBS, showing it is all probably very stable proteins. However, the proteins produced inclusion bodies, and 8M urea was required for solubilization. Also, refolding with decreasing concentrations of urea was performed, to restore the natural conformation of the protein (Creighton, 1980; Santos et al., 2012).

The designed hybrids here appeared as good candidates to be analyzed in future experiments of immunogenicity and cross-reactivity as potential vaccine antigens. Immunizations in animals would answer more questions and cross-reactivity analysis against *M. bovis* and *M. bovoculi* strains would discover the capacity of covering all different clusters.

4. Conclusion

This study highlights that the LbpA receptor protein is a considerable conserved protein between all isolates from *M. bovis* and *M. bovoculi* studied, and the approach of constructing hybrid antigens displaying the LbpA *M. bovis* loops in a foreign scaffold is probably a good option as a vaccine candidate, requiring some improvements for large-scale production.

Conflicts of interest

The authors have no conflict of interest to declare.

Acknowledgments

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Table 1 – *Moraxella bovis* and *Moraxella bovoculi* strains used in this study.

Strain ID	Species	Year	Origin
SB31/18 H3	*M. bovoculi*	2018	Brazil
SB31/18 NH1	*M. bovoculi*	2018	Brazil
SB31/18 NH2	*M. bovoculi*	2018	Brazil
SBP13/18 1091 F	*M. bovis*	2018	Brazil
SBP13/18 H1	*M. bovis*	2018	Brazil
SBP13/18 M	*M. bovoculi*	2018	Brazil
SBP13/18 P	*M. bovis*	2018	Brazil
SBP126/17 636	*M. bovis*	2017	Brazil
SBP126/17 901	*M. bovis*	2017	Brazil
SB15/13	*M. bovoculi*	2013	Brazil
SB57/12 8127	*M. bovoculi*	2012	Brazil
SB15/10	*M. bovoculi*	2010	Brazil
246/04	*M. bovis*	2004	Brazil
SB150/02 5	*M. bovoculi*	2002	Brazil
SB151/01	*M. bovoculi*	2001	Brazil
186V	*M. bovoculi*	1999	Argentina
56/96	*M. bovoculi*	1996	Brazil
n388	*M. bovis*	1993	Canada
n113	*M. bovis*	1993	Canada
n114	*M. bovis*	1993	Canada
Jackson	*M. bovoculi*	<1990	Brazil
2419	*M. bovoculi*	<1990	Uruguay
JUR 3	*M. bovoculi*	<1990	Brazil
Alegrete	*M. bovoculi*	<1990	Brazil
2439	*M. bovoculi*	<1990	Uruguay
173	*M. bovoculi*	<1990	Brazil
Viviane	*M. bovoculi*	<1990	Brazil
290	*M. bovoculi*	<1990	Brazil
JUR 5	*M. bovoculi*	<1990	Brazil
3486	*M. bovoculi*	<1990	Brazil
GF9	*M. bovoculi*	1986	Brazil
ATCC 17949 *M. bovis*	*M. bovis*	-	US
ATCC BAA1259 *M. bovoculi*	*M. bovoculi*	-	US
Epp63 (NZ_CP030241*)	*M. bovis*	-	US
Strain 58069 (NZ_CP011374.1*)	*M. bovoculi*	-	US
Strain 58086 (NZ_CP011381*)	*M. bovoculi*	-	US

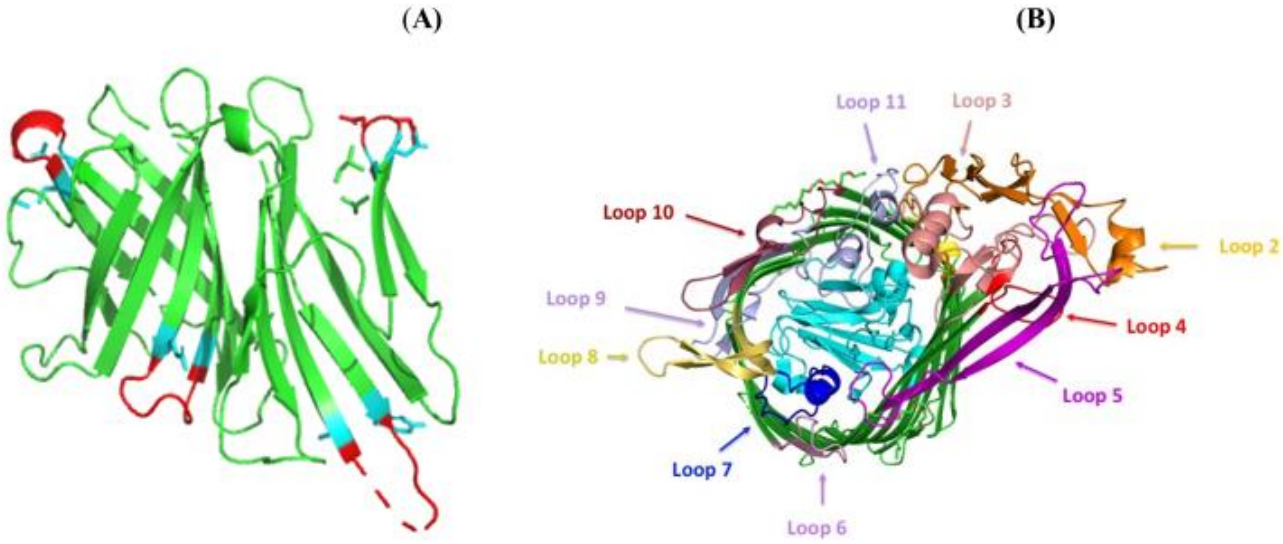

Figure 1. LbpA (lactoferrin binding protein A) hybrid design. Surface lipoprotein scaffold from *Vibrio cholerae* (A) and LbpA *Moraxella bovis* Epp63 strain (B). Protein structure models were predicted in I-TASSER and colored in PyMOL. Red represents the four regions where the loops were inserted in the scaffold (A) and the ten loops were identified with colorful arrows (from Loop 2 to Loop 11) (B).

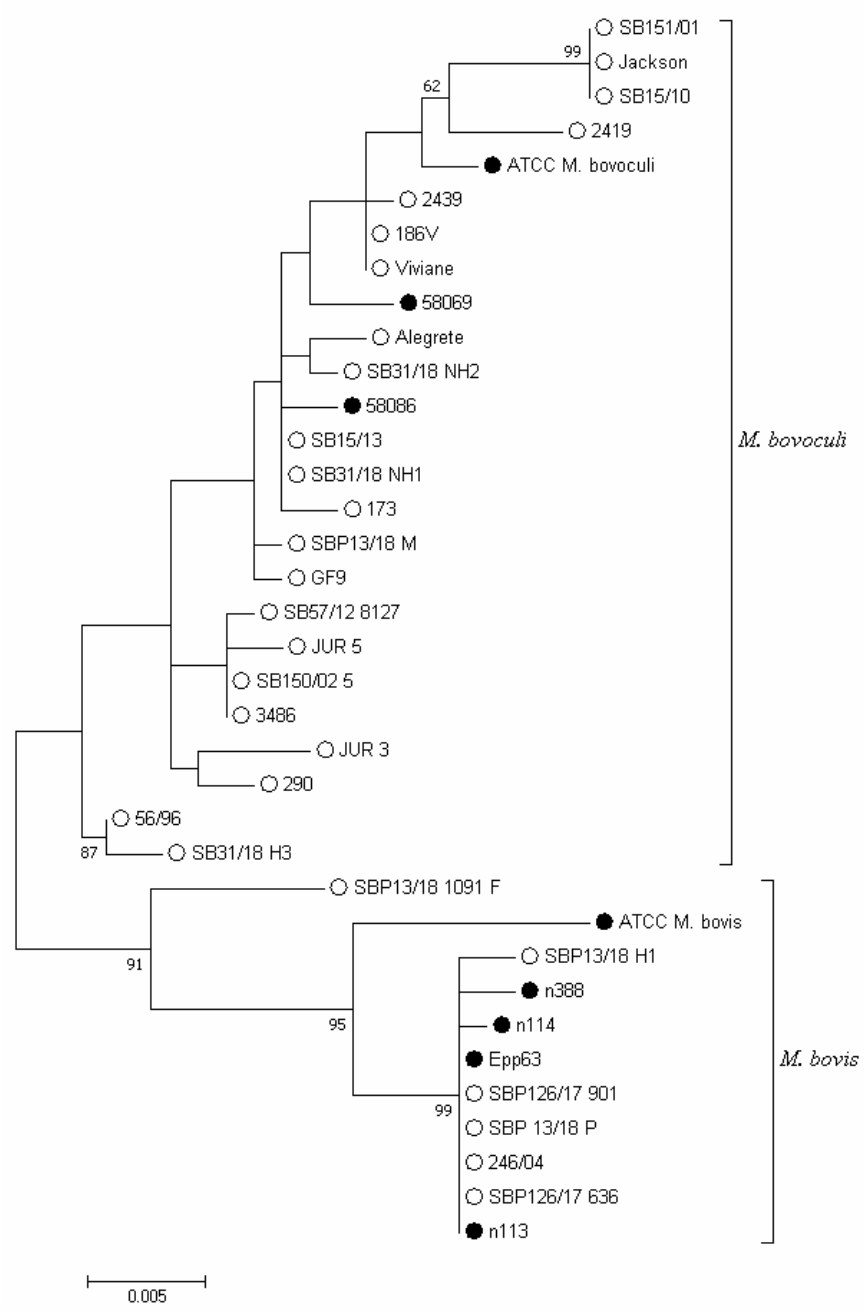

Figure 2. Phylogenetic tree constructed by the Maximum Likelihood method based on the JTT matrix-based model of 36 amino acid sequences of LbpA (lactoferrin binding protein A) from *Moraxella bovis* (11) and *Moraxella bovoculi* (25). Only bootstrap values higher than 60% of 1000 replicates are shown. Black circles mark strains from North America and white circles from South America. Evolutionary analyses were conducted in MEGA 7.0.

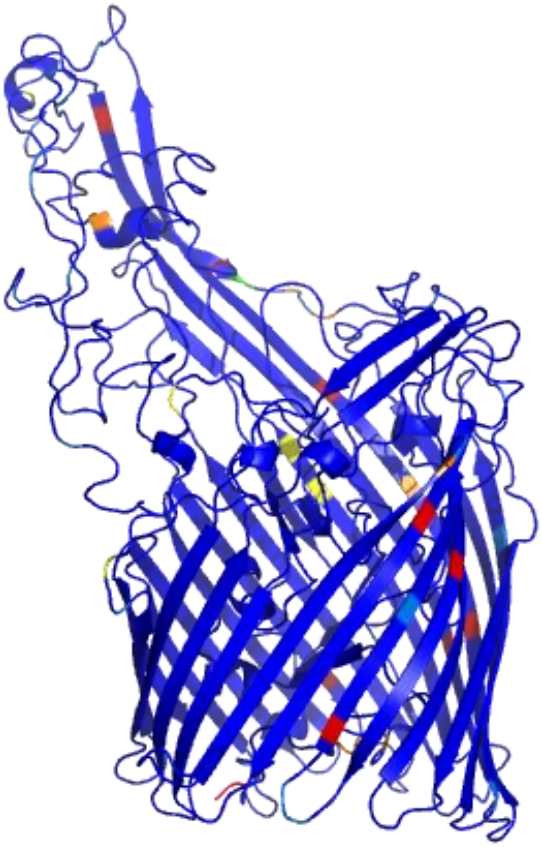

Figure 3. Sequence alignment of 36 strains mapped in the structural model of LbpA (lactoferrin binding protein A) *Moraxella bovis* strain Epp63. LbpA structure model was predicted using I-TASSER and mapping of the sequences were performed with the software MolecularToolbox. Regions colored in blue indicates conserved amino acids between the sequences and colorful parts (yellow to red) indicate variable regions.

4 DISCUSSÃO

Ceratoconjuntivite infecciosa bovina, embora não seja uma doença fatal, é extremamente contagiosa, e destaca-se por sua distribuição mundial e prejuízos econômicos causados na produção de bovinos (SLATTER et al.,1982; CONCEIÇÃO; TURNES, 2003; POSTMA et al., 2008).

Com isso, um número considerável de grupos de pesquisa busca por uma vacina eficaz contra *M. bovis* e *M. bovoculi* para prevenir CIB e diversas vacinas foram e continuam sendo desenvolvidas, vacinas autógenas de *M. bovis* e *M. bovoculi*, vacinas baseadas nos mecanismos de patogenicidade, pili tipo IV e citotoxina, entre outras. Entretanto, ainda não se consegue controlar de forma eficiente esta enfermidade e a eficácia dessas vacinas é questionável, inclusive as disponíveis comercialmente. Os surtos de CIB continuam como um problema na pecuária bovina e a diversidade genética e antigênica entre as cepas de *M. bovis* e *M. bovoculi* continuam a ser um desafio (DAVIDSON; STOKKA, 2003; MCCONNEL; HOUSE, 2005; BURNS; O'CONNOR, 2008; KOWALSKI et al., 2017).

As proteínas de ligação à lactoferrina e à transferrina têm sido consideradas como potenciais antígenos vacinais para muitas doenças com importância na medicina veterinária como a doença de Glasser (*Glaesserella parasuis*) e a doença respiratória bovina (*Mannheimia haemolytica*). Estes antígenos vacinais têm mostrado bons resultados em situações de desafio experimental, capazes de prevenir infecção, e reduzir ou eliminar a colonização no trato respiratório superior (POTTER et al., 1999; FRANDOLOSO et al., 2011; BARASUOL et al., 2017).

Neste contexto, destaca-se a importância deste estudo, onde se avaliou a diversidade genética das proteínas pertencentes aos receptores de lactoferrina e transferrina presentes em *Moraxella bovis* e *Moraxella bovoculi*, com o intuito de desenvolver alternativas de como manejar a diversidade genética esperada e avaliar o potencial destas como antígenos vacinais. As cepas de CIB analisadas neste estudo pertencem a uma coleção consideravelmente representativa, limitada geograficamente, porém diversa em relação ao tempo. Composta por 37 cepas, 11 *M. bovis* e 26 *M. bovoculi*, distribuídas em 32 anos, a maioria do Brasil, como também algumas cepas do Uruguai, Argentina, Canadá e EUA.

Os genes *lbpA*, *tbpA* e *tbpB* foram detectados em todas as cepas de *M. bovis* e *M. bovoculi* analisadas. Estes receptores já haviam sido identificados em *M. bovis* e agora pela primeira vez em *M. bovoculi* (YU; SCHRYVERS, 1994). As árvores filogenéticas das sequências de aminoácidos de LbpA, TbpA e TbpB agruparam-nas principalmente em dois

clusters geneticamente diferentes, basicamente de acordo com a espécie de *Moraxella*. Demonstrando que ao se pensar no desenvolvimento de vacinas baseadas nessas proteínas diferentes abordagens poderão ser consideradas.

O primeiro manuscrito foi desenvolvido abrangendo as proteínas TbpA e TbpB que compõem o receptor bacteriano que capta ferro a partir da transferrina. A análise filogenética demonstrou que as sequências de TbpB foram separadas por espécies, enquanto algumas sequencias de TbpA de *M. bovoculi* estavam mais próximos de *M. bovis*. TbpA mostrou-se menos variável que a TbpB. Além disso, as regiões de maior variabilidade localizaram-se no N-lóbulo da estrutura prevista da TbpB, região onde ocorre a ligação com a transferrina, porém, estudos anteriores já demonstraram que provavelmente essa variabilidade de sequências entre clusters não afeta sua função (FRANDOLOSO et al., 2015).

Com isso, foram selecionadas duas cepas representativas de TbpB que provavelmente são capazes de cobrir toda a variabilidade encontrada nessas cepas. Assim, embora a diversidade geral, especialmente entre o N-lóbulo de TbpB possa ser alta, a abordagem de cepas representativas usada neste estudo, por meio de NAVargator, onde cepas representativas são escolhidas de cada cluster, pode tornar o desafio da diversidade de sequência para desenho de vacinas mais manejável, com possibilidade de cobrir toda a variabilidade. Além disso, uma vez que é demonstrado que as sequências da proteína do receptor se agrupam de acordo com as espécies, uma vacina sugerida com base neste receptor deve ter como alvo as duas espécies.

Análise semelhante a anterior foi realizada no segundo manuscrito, utilizando trinta e seis sequências de LbpA, onde mostrou-se separada também de acordo com as espécies e bastante conservada em toda sua estrutura, especialmente nos *loops*, entre todos os isolados analisados. Adicionalmente, antígenos híbridos projetados entram como uma solução potencial para os possíveis problemas de expressão de LbpA (FEGAN et al., 2019; QAMSARI et al., 2020) e neste estudo, esta abordagem foi seguida, onde cinco antígenos híbridos utilizando quatro combinações diferentes dos *loops* de LbpA em um esqueleto da lipoproteína de superfície de *Vibrio cholerae* (VcSLP) foram desenhados. Os antígenos híbridos foram expressos em pequena escala de forma eficiente e apresentaram potencial para serem produzidos em grande escala para análise de imunogenicidade e de reatividade cruzada como antígenos vacinais.

Mesmo com o número de isolados reduzido, pode ser considerado abrangente devido a possuir isolados de diferentes países e um grande número a partir de uma região bastante importante na pecuária bovina e que é muito acometida pela CIB.

A partir desse estudo é necessário investigar se a diversidade genética encontrada aqui reflete-se na reatividade cruzada do soro dos animais imunizados com essas proteínas frente a estes isolados clínicos e analisar a imunogenicidade das proteínas. Além disso, a produção em grande escala dos híbridos desenvolvidos no estudo e sua análise imunogênica como antígeno vacinal.

5 CONCLUSÃO

Com a realização deste trabalho, conclui-se que as proteínas LbpA, TbpA e TbpB demonstraram considerável baixa variabilidade entre os isolados de *M. bovis* e *M. bovoculi* e as proteínas foram separadas basicamente de acordo com as espécies. Este trabalho sugere duas formas de abordar essas proteínas como antígenos vacinais, sendo que uma vacina baseada nesses receptores deve logicamente abranger as duas espécies envolvidas na CIB. A seleção de dois TbpBs representantes que poderiam cobrir toda a variabilidade dos outros isolados analisados e o desenvolvimento dos cinco antígenos híbridos exibindo diferentes construções de LbpA *loops* no esqueleto de VcSLP foram expressos com sucesso em pequena escala, com potencial para serem ampliados e testados quanto à sua capacidade imunogênica e de reatividade cruzada. Por fim, espera-se que os resultados aqui apresentados possam representar caminhos novos para prevenção da CIB e início da investigação destes antígenos vacinais com grande potencial.

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APÊNDICE A – Diagnosis of blackleg from cattle tissue impregnated in common filter paper

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Diagnosis of blackleg from cattle tissue impregnated in common filter paper

Diagnóstico de carbúnculo sintomático a partir de papel filtro comum impregnado com tecido bovino

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ABSTRACT

Blackleg, an acute myonecrosis caused by *Clostridium chauvoei*, is usually underdiagnosed since the rapid transport of adequate samples for laboratory testing is difficult. This study tested a direct polymerase chain reaction (PCR) technique using common filter paper impregnated with cattle tissue samples obtained from animals suspected of blackleg. Twenty-five samples, belonging to eleven animals from the Rio Grande do Sul State, Brazil, were analyzed. The direct PCR technique identified eight positive animals corroborating with results from microbiological culture. Skeletal muscle was the most common tissue type used in this study and when the animal was positive the pathogen was always detected in this tissue. Storage time of the impregnated filter paper at room temperature did not prove to be a limiting factor for the quality of the results indicating that this procedure can be carried out in the field and samples be sent in regular mail. Our results suggested that direct PCR of common filter paper

impregnated with cattle tissue is a practical and economical alternative for the diagnosis of blackleg.

Keywords: *Clostridium chauvoei*; PCR; cattle; blackleg; diagnosis.

RESUMO

Carbúnculo sintomático, uma mionecrose aguda causada por *Clostridium chauvoei*, costuma ser subdiagnosticada, pois o transporte rápido de amostras adequadas para exames laboratoriais é complicado. O objetivo deste estudo foi testar a técnica de reação em cadeia da polimerase (PCR) direta, utilizando papel filtro comum impregnado com amostras de tecido bovino obtidas de animais suspeitos de carbúnculo sintomático. Foram analisadas 25 amostras, pertencentes a onze animais do estado do Rio Grande do Sul, Brasil. A técnica de PCR direta identificou oito animais positivos, corroborando com os resultados da cultura microbiológica. O músculo esquelético foi o tecido mais utilizado neste estudo e quando o animal foi positivo, o patógeno sempre foi detectado neste tecido. O tempo de armazenamento do papel filtro impregnado, à temperatura ambiente, não se mostrou um fator limitante para a qualidade dos resultados, indicando que esse procedimento pode ser realizado no local e as amostras enviadas por correio normal. Nossos resultados sugerem que a PCR direta usando papel filtro comum impregnado com tecido bovino é uma alternativa prática e econômica para o diagnóstico de carbúnculo sintomático.

Palavras-chave: *Clostridium chauvoei*; PCR; bovino; carbúnculo sintomático; diagnóstico.

Blackleg, an acute, non-contagious myonecrosis caused by *Clostridium chauvoei*, leads to significant losses in the beef cattle industry and is globally responsible for outbreaks and sudden deaths of well-conditioned, young cattle (QUINN et al., 2011). A definitive diagnosis requires the detection of *C. chauvoei* in affected tissues. This is challenging since farmers have

to send tissue samples, correctly and on time, to the laboratory and this is usually economically unfeasible (ABREU et al., 2017). In addition, conventional diagnosis using microbiological culture is difficult and time-consuming leading to underreporting of the disease (UZAL et al., 2003; ZIECH et al., 2018). Thus, alternative molecular techniques and sampling methods are required to optimize the laboratory diagnosis of blackleg.

Polymerase chain reaction (PCR) is a rapid detection strategy for pathogens. When combined with the tissue-impregnated common filter paper samples it eliminates the need for time-consuming procedures associated with DNA extraction (UZAL et al., 2003; MIYASHIRO et al., 2007; ASSIS et al., 2008). Additionally, the filter paper is a low-cost material that is easily reported and enables the shipment of samples without refrigeration (KARIMIAN et al., 2011; SMIT et al., 2014).

Previously, detection of *C. chauvoei* was demonstrated using direct PCR from common filter paper impregnated with bovine liver inoculated with controlled amounts of *C. chauvoei* strain ATCC 10092 (FARIAS et al., 2012). Based on this, the present study evaluated the use of direct PCR and tissue-impregnated common filter paper for the diagnosis of blackleg in cattle using samples obtained from the field.

Twenty-five samples of bovine tissue from 11 animals belonging to the central region of the Rio Grande do Sul state, Brazil, were sent to the Bacteriology Laboratory of the Universidade Federal de Santa Maria between 2011 and 2017. The animals were between 8 and 18 months of age and had experienced sudden death, suspected to be due to blackleg.

Samples arrived refrigerated and were immediately used for microbiological culture, which is the standard diagnostic technique in this laboratory. One gram of tissue was inoculated in Reinforced Clostridial Medium (Oxoid Brazil Ltda) and incubated under anaerobic conditions at 37 °C for 48 h. Ten microlitres were streaked on 5% sheep blood agar and incubated under anaerobic conditions at 37 °C for 48 h. The colony characteristics and

hemolyzing capability of the samples were analyzed. The samples were subjected to Gram staining for morphological determination and biochemical tests (QUINN et al., 2011). Cultures found to be positive for *C. chauvoei* were confirmed with 16S rRNA gene sequencing (LANE et al., 1991). After microbiological culture, all samples were frozen and stored at −20°C.

Direct PCR was performed as described by FARIAS et al. (2012). Common filter paper, 9 cm in diameter, was impregnated with samples only refrigerated (shortly after arrival) and from thawed samples stored frozen (Table 1) and then the filter paper was stored at room temperature. After the storage period, between one week to one year, three small circular pieces, with an area of approximately 3.14 mm^2, were cut out of each impregnated filter paper using Harris Uni-core ™ (Electron Microscopy Sciences, Hatfield, PA). The small circular pieces were placed in sterile 0.2 mL microtubes, covered with 10 µL of absolute methanol, and dried in an oven at 37 °C, with no need for extra steps for DNA extraction.

Molecular identification of *C. chauvoei* involved PCR amplification of the 535 bp *fliC* gene that encodes flagellin (FliC) according to KOJIMA et al. (2001) and SASAKI et al. (2002). The PCR reaction was performed in a final volume of 25µL, containing 2.5µL buffer 10X + 5µM magnesium chloride, 1µL of each primer at 10µM, 1µL of dNTP (Ludwig, Brazil), 1µL of template DNA (approximately 50ng) in the positive control and ultrapure distilled water q.s.p. (Invitrogen, USA). All these PCR components were added to the microtubes and heated to 95ºC for 10 minutes, after that the GoTaq® DNA Polymerase (Promega, USA) was added in the amount of 0.2µl in each microtube. Microtubes were also reserved for positive and negative controls and PCR for each sample was performed in triplicate (FARIAS et al., 2012).

PCR was carried out in a Veriti 96-well thermal cycler (Applied Biosystems, USA) and the cycling conditions were, initial denaturation of 95°C for 5 min; 35 cycles of 95°C for 30 s; 57°C for 30 s and 72°C for 1 min and a final extension at 72°C for 5 min. The amplified products

were electrophoresed and visualized on a 2% agarose gel stained with ethidium bromide (0.5 μg) (FARIAS et al., 2012). To analyze the results descriptive data analysis was carried out.

The presence of the disease was confirmed in eight animals, while the other three were negative using both techniques. Of the twenty-five samples, fifteen were determined positive for *C. chauvoei* using direct PCR (Table 1). Considering microbiological culture, the same samples were also positive; however, one additional was detected resulting in a total of 16 culture-positive samples. Unlike other studies, this one, when compared to the direct PCR microbiological culture shown higher detection of *C. chauvoei* (UZAL et al., 2003; BAGGE et al.,2009).

The tissue that showed a disagreement between the results of both techniques was the splenic tissue (SB 97), which was positive for the pathogen when cultured upon arrival, but negative using direct PCR when performed two months after filter paper impregnation. This could be a result of low numbers of bacteria that were seen only because of the enrichment in the microbiological culture and were below the detection limit of the direct PCR procedure when applied to the impregnated filter paper. Of note, guinea pigs (*Cavia porcellus)* that were infected intramuscularly with *C. chauvoei*, had no bacteria detected in the spleen samples, (ASSIS et al., 2005), suggesting that it is not a tissue where the bacteria are usually found.

The types of tissues used for the diagnosis of blackleg were skeletal muscle, cardiac muscle, and liver tissue. In this study, the most common tissue sample (14/25) consisted of skeletal muscle samples, probably due to the ease of collection. Furthermore, when multiple tissues were sent per animal, the skeletal muscle sample was always positive for the pathogen (SB 97, SB 105, SB 87, SB 131, SB 46, and SBP 121), suggesting that the skeletal muscle may be the preferred tissue sample for this diagnostic test.

Corroborating these results, skeletal muscle samples from dead cattle with blackleg have shown a higher amount of *C. chauvoei* compared to other organs (BAGGE et al., 2009; ASSIS

et al., 2010). The cardiac muscle was also always positive for the pathogen, and the detection of lesions and bacteria in the heart is considered a visceral case. This is not a common or widely reported finding in Brazil. Thus, this sample should preferably be sent, when there are lesions in the myocardium, along with other tissues for laboratory diagnosis (UZAL et al., 2003; ASSIS et al., 2010; CASAGRANDE et al., 2015).

The filter paper impregnated with different samples varied in storage time, from one week to one year, at room temperature. Most of these samples were stored for more than two months, and 96% of the samples showed the same result as the microbiological culture. Until now, FARIAS et al. (2012) analyzed only samples stored for one week. Four samples were positive after one year of storage at room temperature, suggesting that the common filter paper can potentially preserve the DNA of *C. chauvoei* for long periods. Our results were similar to that of KARIMIAN et al. (2011), where filter paper cards were used to store blood and bacteria for up to twelve months. In another study, the filter paper was used to store the African swine fever virus, for up to 9 months, at 20–25 °C, and for 2 months at 37 °C, without a significant loss in detection sensitivity (RANDRIAMPARANY et al., 2016).

A total of 83% of the samples that were only refrigerated before impregnation and 38.46% of samples frozen until impregnation, were positive using PCR. All of these samples were culture positive suggesting that the type of storage before impregnation did not interfere with the results.

Advantages of this technique included the easy impregnation of samples by veterinarians or farm owners, low-cost of common filter paper, and the use of normal postal service, without cold storage, for the shipment of samples to the laboratory (SMIT et al., 2014; MATHEUS et al., 2015). As a result, laboratory diagnosis becomes faster, without the need for other techniques to aid in the survival, growth, and enrichment of the bacteria for PCR (MIYASHIRO et al., 2007; ASSIS et al., 2008).

Finally, it was demonstrated the use of direct PCR on tissue-impregnated common filter paper to identify *C. chauvoei* in bovine tissue samples. This method can serve as a diagnostic technique for blackleg using routine samples of skeletal muscle, cardiac muscle, and liver tissue. Our results suggested that it is a practical, economical, and efficient alternative for the collection and transport of tissue and will help reduce the under-diagnosis of this important disease.

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DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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Table 1 – Sample identification history and results of microbiological culture and direct PCR from filter paper.

Sample ID	Bovine tissue	Year of arrival at the laboratory	Sample condition before impregnation	Storage time in filter paper	Microbiological Culture	Direct PCR
SB 52	SM 1	2011	Frozen	2 mos	+	+
SB 52	SM 2	2011	Frozen	2 mos	+	+
SB 75	SM	2011	Frozen	2 mos	-	-
SB 86	SM 1	2011	Frozen	2 mos	-	-
SB 86	SM 2	2011	Frozen	2 mos	-	-
SB 97	SM	2011	Frozen	2 mos	+	+
SB 97	LT	2011	Frozen	2 mos	-	-
SB 97	ST	2011	Frozen	2 mos	+	-
SB 105	SM	2011	Frozen	2 mos	+	+
SB 105	ST	2011	Frozen	2 mos	-	-
SB 105	LT	2011	Frozen	2 mos	+	+
SB 25	SM	2013	Frozen	2 mos	-	-
SB 25	LT	2013	Frozen	2 mos	-	-
SB 87 285	SM	2013	Refrigerated	1 y	+	+
SB 87 285	CM	2013	Refrigerated	1 y	+	+
SB 87 286	SM	2013	Refrigerated	1 y	+	+
SB 87 286	CM	2013	Refrigerated	1 y	+	+
SB 131	SM 1	2013	Refrigerated	10 mos	+	+
SB 131	SM 2	2013	Refrigerated	10 mos	+	+
SB 131	LT	2013	Refrigerated	10 mos	-	-
SB 46	SM	2014	Refrigerated	2 mos	+	+
SB 46	LT	2014	Refrigerated	2 mos	-	-
SBP 121	SM	2017	Refrigerated	1 wk	+	+
SBP 121	CM	2017	Refrigerated	1 wk	+	+
SBP 121	LT	2017	Refrigerated	1 wk	+	+

y: year; mos: months; wk: week. SB/SBP: laboratory identification; SM: skeletal muscle; LT: liver tissue; CM: cardiac muscle; ST: splenic tissue.