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Rosangela Estel Ziech

CARACTERIZAÇÃO MOLECULAR E ANTIGÊNICA DE *Clostridium chauvoei* ISOLADOS DE CASOS DE CARBÚNCULO SINTOMÁTICO EM BOVINOS

Santa Maria, RS
2018

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração Medicina Veterinária Preventiva, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Doutor em Medicina Veterinária**

Orientadora: Profª. Agueda Castagna de Vargas

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É exatamente disso que a vida é feita, de momentos.
Momentos que temos que passar, sendo bons ou ruins, para o nosso aprendizado.
Nunca se esquecendo do mais importante:
Nada nessa vida é por acaso.
Absolutamente nada. Por isso, temos que nos preocupar em fazer a nossa parte, da melhor forma possível.
A vida nem sempre segue a nossa vontade, mas ela é perfeita naquilo que tem que ser.

Chico Xavier

"Science never solves a problem without creating ten more."

George Bernard Shaw

RESUMO

CARACTERIZAÇÃO MOLECULAR E ANTIGÊNICA DE *Clostridium chauvoei* ISOLADOS DE CASOS DE CARBÚNCULO SINTOMÁTICO EM BOVINOS

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As clostridioses formam o grupo de infecções e intoxicações causadas por micro-organismos anaeróbios do gênero *Clostridium*. Dentre estas enfermidades, a principal doença que determina mionecrose é o carbúnculo sintomático, uma infecção endógena e aguda que acomete principalmente os bovinos, cujo agente etiológico é *Clostridium chauvoei*. O controle desta enfermidade se baseia em medidas adequadas de manejo e vacinações sistemáticas de todo o rebanho. As vacinas contra o carbúnculo sintomático comercializadas no Brasil são, no geral, compostas de múltiplos antígenos e seguem as normas para o controle da qualidade e eficiência definidas na legislação publicada pela Secretaria de Defesa Agropecuária (SDA) do Ministério de Agricultura, Pecuária e Abastecimento (MAPA). No entanto, são escassos os trabalhos com evidências científicas que comprovem a eficiência da vacina, assim como trabalhos que indiquem o grau de similaridade entre cepas de *C. chauvoei* utilizadas na produção destas vacinas, cepas de maior ocorrência no campo e a cepa padrão utilizada no teste de eficiência da vacina pelo MAPA. Da mesma forma os relatos acerca de falhas vacinais são informais e as circunstâncias destas falhas não são claramente elucidadas. Os principais fatores de virulência de *C. chauvoei* são a neuraminidase, citotoxina A (CctA) e a flagelina, sendo que a CctA parece ser o principal. Esta tese teve por objetivo avaliar a eficácia protetiva de vacinas comerciais frente ao desafio com *C. chauvoei*, bem como realizar a comparação genômica das cepas utilizadas no desafio vacinal (manuscrito 2). A diversidade molecular foi investigada, a partir do sequenciamento parcial dos genes da neuraminidase (*nanA*), CctA (*cctA*) e flagelina (*fliC*) de dezessete cepas de *C. chauvoei* isoladas de casos clínicos em bovinos (manuscrito 3). Também pretendeu à comparação genômica de seis cepas isoladas de casos de carbúnculo sintomático no Brasil com vistas a pesquisar diferenças moleculares na única cepa com apresentação clínica visceral (manuscrito 4). Dessa forma, discutir as implicações da caracterização molecular e antigênica no entendimento do carbúnculo sintomático, e subsidiar futuras pesquisas a fim de melhorar o controle da doença. Concluímos que o desempenho equivalente das duas cepas de *C. chauvoei* após o desafio *in vivo* e a incapacidade de infectar os animais vacinados estão correlacionados com a homologia genética das cepas, sugerindo que as falhas vacinais não estão relacionadas à variabilidade antigênica. O sequenciamento parcial dos genes *nanA* e *cctA* mostrou alta homologia entre as cepas. Por essa razão, estes antígenos solúveis são bons candidatos a antígenos vacinais. Além disso, três alelos foram detectados no gene *fliC*. Na comparação genômica as seis cepas brasileiras se mostraram altamente conservadas, tal como foi observado no sequenciamento parcial dos genes e na comparação das cepas utilizadas no desafio vacinal. Os resultados apresentados nesta tese podem ser um indicativo de que neste momento evolutivo *C. chauvoei* não está sendo desafiado a desenvolver mutações.

Palavras-chave: Clostridioses, flagelina, neuraminidase, CctA, genoma.

ABSTRACT

MOLECULAR AND ANTIGENIC CHARACTERIZATION OF *Clostridium chauvoei* ISOLATES FROM BLACKLEG IN CATTLE

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Clostridioses are the group of infections and intoxications caused by anaerobic microorganisms of the genus *Clostridium*. Among these diseases, the main disease that causes myonecrosis is blackleg, an endogenous and acute infection that mainly affects cattle, whose etiological agent is *Clostridium chauvoei*. Control of this disease is based on adequate management measures and systematic vaccinations of herd. Vaccines against blackleg marketed in Brazil are, in general, composed of multiple antigens and follow the standards for quality and efficiency control defined in the legislation published by the Ministry of Agriculture, Livestock and Food Supply (MAPA). However, there are few studies with scientific evidence to prove the effectiveness of the vaccine, as well as studies indicating the degree of similarity between strains of *C. chauvoei* used in the production of these vaccines, strains occurring in the field and the standard strain. Likewise reports of vaccine failures are informal and the circumstances of these failures are not clearly elucidated. The main virulence factors of *C. chauvoei* are neuraminidase, cytotoxin A (CctA) and flagellin, and CctA appears to be the main. This thesis aimed to evaluate the protective efficacy of commercial vaccines against the challenge with *C. chauvoei*, as well as to carry out the genomic comparison of the strains used in the vaccine challenge (manuscript 2). The molecular diversity, from the partial sequencing of the neuraminidase (*nanA*), CctA (*cctA*) and flagellin (*fliC*) genes of seventeen strains of *C. chauvoei* isolated from clinical cases in cattle was investigated (manuscript 3). It also aimed to the genomic comparison of six strains isolated from cases of blackleg in Brazil with a view to investigate molecular differences in the only strain with visceral clinical presentation (manuscript 4). Thus, to discuss the implications of molecular and antigenic characterization in the understanding of blackleg, and to support future research with the objective of improving disease control. We concluded that the similar performance observed between the strains in challenge *in vivo* and the inability to infect the vaccinated animals are correlated with the genetic homology of the strains, suggesting that vaccine failures are not related to antigenic variability. Partial sequencing of *nanA* and *cctA* genes showed high homology between strains. For this reason, these soluble antigens are good candidates for vaccine antigens. Moreover, three different *fliC* alleles were detected among the strains studied. In the genomic comparison, the six Brazilian strains were highly conserved, as observed in the partial sequencing of the genes and in the comparison of the strains used in the vaccine challenge. The results presented in this thesis may be indicative that at this evolutionary time *C. chauvoei* is not being challenged to develop mutations.

Key-words: Clostridiosis, flagellin, neuraminidase, CctA, genome.

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LISTA DE ABREVIATURAS E SIGLAS

ATCC	*American Type Culture Collection*
BCIP	*5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt*
BHI	*Brain Heart Infusion*
bp	*Base pairs*
$CaCl_2$	Cloreto de Cálcio
CBM	*Carbohydrate binding module*
CctA	*Clostridium chauvoei* citotoxina A
ccta	Gene que codifica a CctA
CFU	*Colony forming unit*
DNA	Ácido Desoxirribonucleico
dNTP	Desoxirribonucleotídeos Fosfatados
ELISA	*Enzyme Linked Immunosorbent Assay*
FAPERGS	Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul
FliC	Flagelina
fliC	Gene que codifica a FliC
g	Grama
h	Hora
HCl	Ácido clorídrico
IgG	Imunoglobulina G
IL	Interleucina
kbps	Quilobit por segundo
kDa	Quilodalton
LABAC	Laboratório de Bacteriologia
LANAGRO	Laboratório Nacional Agropecuário
LD	Lethal dose
log	Logaritmo decimal
M	Molar
MAPA	Ministério de Agricultura, Pecuária e Abastecimento
meso-DAP	*meso-diaminopinelic acid*
$MgCl_2$	Cloreto de Magnésio
MIC	*Minimal inhibitory concentration*
mL	Mililitro
mmol	Milimol
mM	Milimolar
MT	Manguinhos-Teixeira
N	Normal
NaCl	Cloreto de Sódio
Nag	Hialuronidase
NanA	Neuraminidase
nanA	Gene que codifica a NanA
NBT	*Nitro Blue Tetrazolium*
nm	Nanômetro
OD	*Optical Density*
ORF	*Open Reading Frame*
PAMP	*Pathogen-associated molecular pattern*
PBS	*Phosphate buffered saline*
PCR	Reação em cadeia da polimerase
pH	Potencial hidrogeniônico

PPGMV	Programa de Pós Graduação em Medicina Veterinária
PVDF	*Polyvinylidene diflouride*
rDNA	DNA ribossomal
rpm	Rotações por minuto
rRNA	RNA ribossomal
RNA	Ácido Ribonucleico
s	Segundos
SB	Setor de Bacteriologia
SBP	Setor de Bacteriologia/ Protocolo de Pesquisa
SDA	Secretaria de Defesa Agropecuária
SIF	Serviço de Inspeção Federal
SNP	*Single nucleotide polymorphisms*
TBS/TRIS	Tris-(hidroximetil)-aminometano
TGF	*Transforming growth factor*
TLR5	*Toll-like receptor 5*
UFRGS	Universidade Federal do Rio Grande do Sul
UFSM	Universidade Federal de Santa Maria
v/v	Volume/volume
w/v	*Weight/volume*
µg	Micrograma
µl	Microlitro
°C	Graus Celsius

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1 INTRODUÇÃO

O Brasil possui o segundo maior rebanho bovino do mundo e o primeiro rebanho comercial (ALVIM & SOARES-FILHO, 2013). O país conta com cerca de 226 milhões de cabeças, que em 2017 produziram 9,26 milhões de toneladas de carne. Quando traçado um comparativo com o rebanho dos Estados Unidos com 93 milhões de cabeças e 11,38 milhões de toneladas de carne produzidas, pode-se observar que o sistema produtivo brasileiro possui uma produtividade inferior ao americano (FORMIGONI, 2017). Algumas causas desta diferença são alimentação, manejo e sanidade, melhorias nestas áreas poderiam trazer um incremento da produtividade e consequente produção.

Dentre as principais enfermidades que causam prejuízos à pecuária de corte estão as clostridioses. O carbúnculo sintomático é uma clostridiose de distribuição mundial que gera significativas perdas econômicas (DUTRA; SOUZA; BORSANELLI, 2011), como por exemplo, US$ 43 milhões/anuais na criação bovina da Nigéria (USEH; NOK; ESIEVO, 2006). No Brasil, embora os prejuízos econômicos causados à bovinocultura sejam de difícil mensuração, em razão da escassez de dados consistentes de ocorrência, estima-se que estes sejam elevados (BALDASSI et al., 1985). O impacto econômico estimado para os surtos da doença no Centro-Oeste brasileiro foi de 0,75 a 27,50% nas fazendas que não utilizavam a vacinação, de 0,50 a 33,33% nas fazendas sem informação sobre a realização de vacinação e de 0,72 a 6,00% em fazendas onde o rebanho foi vacinado (HECKLER et al., 2018). Dados retrospectivos do Setor de Patologia Veterinária da UFRGS apontam que dos 135 casos suspeitos (2,88% do total de necropsias realizadas no período) de enfermidades clostridiais em herbívoros no período de janeiro de 1996 a dezembro de 2011, 5,93% foram identificadas como carbúnculo sintomático (RAYMUNDO et al., 2014). No Centro-Oeste brasileiro de 1994 a 2014, 59 casos oriundos de 51 surtos foram relatados, o que corresponde a 1,1% das 5375 mortes de bovinos investigadas no período. Somente em cinco desses casos, amostras do músculo afetado foram positivas no cultivo para *C. chauvoei* (HECKLER et al., 2018). Dentre as espécies do gênero *Clostridium*, *C. chauvoei* representou a segunda mais isolada no Laboratório de Bacteriologia do Departamento de Medicina Veterinária da UFSM (LABAC) entre os anos de 1988 a 2007, foram 24 isolamentos em bovinos (28,45%) (MABONI; ASSIS; VARGAS, 2010). Nos anos subsequentes de 2009 a 2018 foram recebidas no LABAC 40 amostras suspeitas de carbúnculo sintomático, a partir destas foram isoladas 12 (30%) cepas de *C. chauvoei*, cuja identidade foi confirmada pela reação em cadeia da polimerase (PCR) (dados não publicados). *C. chauvoei* é sensível ao oxigênio, outro fator que

torna o isolamento fastidioso é a possibilidade de contaminação da amostra por outros micro-organismos (ASSIS et al., 2005), inclusive pertencentes ao mesmo gênero.

O gênero *Clostridium* compreende mais de 160 espécies, em sua maioria comensais e não patogênicas. Os membros deste gênero são encontrados no solo, pastagens, esgoto, sedimentos marinhos, trato intestinal de humanos e animais e em animais em putrefação (CATO; GEORGE; FINEGOLD, 1986). As bactérias do gênero *Clostridium* são bacilos, Gram positivos com parede celular usualmente constituída por *meso*-DAP (*meso-diaminopinelic acid*) e possuem a capacidade de esporular, o que as torna potencialmente infectantes no ambiente por longos períodos (MAINIL et al., 2006). Reconhecido como o gênero bacteriano mais toxigênico, *Clostridium* spp. determina a ocorrência de infecções e intoxicações que apresentam altas taxas de letalidade e mortalidade tanto em seres humanos como em animais (LOBATO et al., 2013).

Somente 25 a 30 espécies são patogênicas, destacando-se 13 espécies classificadas pelas lesões e sinais clínicos: I. neurotóxicas (*C. botulinum, C. tetani*), II. enteropatogênicas (*C. perfringens, C. difficile, C. colinum, C. spiroforme*), III. histotóxicas mionecrosantes (*C. perfringens tipo A, C. novyi* tipo A, *C. sordellii, C. septicum, C. chauvoei*) e IV. histotóxicas hepatotóxicas (*C. novyi* tipo B, *C. haemolyticum*) (TITBAL et al., 2006; SATHISH; SWAMINATHAN, 2009; PIRES, 2015). Nesta tese a espécie de estudo inclui *Clostridium chauvoei.*

Com ampla distribuição, principalmente em áreas de produção de bovinos (SATHISH; SWAMINATHAN, 2009; HANG'OMBE et al., 2000) *C. chauvoei* contamina o solo, bebedouros e currais por meio da decomposição de carcaças ou eliminação de esporos pelas fezes (SATHISH; SWAMINATHAN, 2008; BAGGE; LEWERIN; JOHANSSON, 2009), contribuindo para perpetuação do agente, em especial em propriedades onde a doença é endêmica (SOJKA et al., 1992).

C. chauvoei mede 0,5-1,7 a 1,6-9,7 μm, ocorre separadamente ou aos pares. Os endosporos formados são ovais, sub-terminais ou terminais causando deformação na célula mãe (LIMA, 1992). O esporo possui no cerne apenas DNA, RNA, ribossomos, enzimas e moléculas pequenas como ácido dipicolínico e cálcio (LEGGETT et al., 2012). A esporulação é um mecanismo de resistência que permite a sobrevivência dos clostrídios em condições adversas (GALPERIN et al., 2012). Este processo na maioria das espécies de *Clostridium* spp. possivelmente está associado as restrições nutricionais (PAREDES-SABJA & SARKER, 2009).

O primeiro *draft* do genoma de uma cepa desta espécie foi depositado no GenBank em 2013 (FALQUET; CALDERON-COPETE; FREY, 2013), e em 2017 foram publicados dois artigos com as sequências completas de *C. chauvoei* (RYCHENER et al., 2017; THOMAS et al., 2017). A análise genômica demonstrou a presença de um único cromossomo circular com mais de 2,8 milhões de pares de bases, que é relativamente pequeno quando comparado aos demais *Clostridium* spp., como por exemplo, com a primeira sequência do genoma de *C. difficile* publicada na literatura, que possui 4,2 milhões de pares de bases (SEBAIHIA et al., 2006). O material genético compacto de *C. chauvoei* é justificado em parte pelo pequeno número de espécies animais susceptíveis, bovinos, ovinos e caprinos (FREY; FALQUET, 2015). Das mais de duas mil *Open Reading Frames* (ORF) analisadas, apenas 13 estão relacionadas aos fatores de virulência primários, como a produção de toxinas. O restante do genoma é responsável por codificar elementos estruturais, como cápsula e parede celular, elementos de resistência, como esporulação/dormência e resistência a antimicrobianos, e metabolismo bacteriano. Além disso, *C. chauvoei* possui apenas um plasmídeo, do tipo críptico, cuja função ainda não foi elucidada (FREY; FALQUET, 2015; PIRES, 2015).

Dessa forma, os objetivos desse estudo foram determinar o grau de similaridade de isolados de *C. chauvoei* a partir do sequenciamento parcial de genes da neuraminidase, citotoxina A e flagelina. Ademais, avaliar a eficácia protetiva de vacinas comerciais frente ao desafio com *C. chauvoei* e adicionalmente realizar a comparação genômica das cepas utilizadas no desafio vacinal. Também visou à comparação genômica de seis cepas isoladas de casos de carbúnculo sintomático no Brasil com ênfase nas diferenças moleculares da única cepa isolada de um bovino com apresentação clínica visceral, que manifestou lesão cardíaca (SB 87/13 286). Por conseguinte, discutir as implicações da caracterização molecular e antigênica no entendimento do carbúnculo sintomático, e subsidiar futuras pesquisas com o objetivo de aperfeiçoar o controle da doença.

A presente tese é composta por um artigo de revisão e três manuscritos descritos a seguir:

ARTIGO 1: Blackleg in cattle: current understanding and future research needs (artigo de revisão). Publicado no periódico Ciência Rural, classificação B1 no quadriênio 2013-2016.

MANUSCRITO 2: Protective efficacy of commercial vaccines against a virulent field strain of *Clostridium chauvoei*.

MANUSCRITO 3: Molecular characterization of virulence genes *cctA*, *nanA*, and *fliC* in *Clostridium chauvoei*.

MANUSCRITO 4: Genomic comparison of Brazilian *Clostridium chauvoei* isolates from blackleg.

2 ARTIGO 1

Blackleg in cattle: current understanding and future research needs

Carbúnculo sintomático: compreensão atual e futuras necessidades de pesquisa

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ABSTRACT

Blackleg is an endogenous acute infection that principally affects cattle, whose etiologic agent is the anaerobic bacterium *Clostridium chauvoei*. In recent years, the major virulence factors of *C. chauvoei* have been discovered and described. However, the pathogenesis of blackleg in cattle, and in particular, the movement of the pathogen from the point of entry to the affected tissues is not yet fully elucidated. Disease control is based on appropriate management and vaccination. This review summarizes the latest research findings that contribute toward the understanding of the disease in cattle, provide a foundation to preventive strategies, and identify future research needs.

Key words: *Clostridium chauvoei*, sudden death, myonecrosis.

RESUMO

O carbúnculo sintomático é uma infecção endógena, aguda, que acomete principalmente bovinos, cujo agente etiológico é a bactéria anaeróbica *Clostridium chauvoei*. Recentemente, os principais fatores de virulência do *C. chauvoei* foram descobertos e descritos. Contudo, a patogênese do carbúnculo sintomático em bovinos, especialmente a circulação do patógeno desde o ponto de entrada até os tecidos acometidos ainda não está completamente elucidada. O controle desta enfermidade é baseado em medidas adequadas de manejo e vacinação. Esta revisão reúne as mais recentes descobertas que contribuíram para o entendimento da doença em bovinos, fornece embasamento para medidas preventivas e aponta futuras necessidades de pesquisa.

Palavras-chave: *Clostridium chauvoei*, morte súbita, mionecrose.

INTRODUCTION

Blackleg, also known as black quarter, is a generally fatal form of myonecrosis usually observed in young cattle (USEH et al., 2006b). *Clostridium chauvoei*, the etiologic agent of blackleg, is a gram-positive, motile, histotoxic, and sporulating anaerobic bacterium (QUINN et al., 2011). This infectious disease is acute and globally spread among ruminants, causing significant loss in livestock production (FREY & FALQUET, 2015). Although blackleg vaccination has been carried out since 1930, sporadic outbreaks are still recorded annually worldwide (USEH et al., 2006b).

In 1782, Chabert differentiated between blackleg and anthrax on the basis of symptomatic and pathological features (KRIEK & ODENDAAL, 2004). Although blackleg is

one of the oldest known diseases affecting cattle, there are important gaps in the understanding of this disease, especially with respect to its pathogenesis. Focusing on the cattle disease, this article aims to offer an overview of the current knowledge about the etiology, virulence factors, epidemiology, pathogenesis, diagnosis, and prevention of blackleg and to identify areas for further research and development.

Epidemiology and clinical and pathological manifestation

Blackleg is an endemic disease in both developed and developing countries and a well-known cause of financial loss to cattle breeders (USEH et al., 2006b). Most cases of blackleg occur during the warm months, or after soil excavation, or during very high annual rainfall that can expose and activate latent spores. In addition, the disease is enzootic in areas with a history of flooding (USEH et al., 2006a; HUANG et al., 2013). The type of soil and water permeability might represent an important indicative factor in surveillance programs, which was once associated with an increased risk of blackleg cases in Zambia (HANG'OMBE et al., 2000) and Austria (WOLF et al., 2017).

Cattle aged between 6 to 24 months, in good health, are mostly affected (UZAL, 2012). The clinical signs of the hyperacute form of this disease are usually not observed because of sudden death. The acute form of the disease is often reported with swelling and crepitus of affected muscles (SINGH et al., 1993).

The most commonly reported findings in classical blackleg are acute neutrophil necrotizing myositis that affects the skeletal muscle, and visceral myonecrosis, which is rarely diagnosed, but can affect the heart, sublingual muscles, and diaphragm (ASSIS et al., 2010; CASAGRANDE et al., 2015). Other unusual findings include fibrinous pleuritis, pericarditis, epicarditis (DALY et al., 2009), and severe acute necrotizing enteritis (HARWOOD et al., 2007) as well as the highly uncommon meningoencephalitis (MALONE et al., 1986; SAC, 2016).

Etiology and virulence factors

Blackleg is caused by an anaerobic, highly pathogenic, endospore-forming, gram-positive bacterium called *C. chauvoei*, which produces lemon-shaped endospores and requires an enriched medium for growth (QUINN et al., 2011). The first draft genome sequence of a virulent *C. chauvoei* strain became available in 2013; it consists of 2.8 million base pairs (bp) (FALQUET et al., 2013). Moreover, it contains a cryptic plasmid, about 5.5 kbps in size

(FREY & FALQUET, 2015). Recently, the full genome sequences of 20 strains of *C. chauvoei*, isolated from four different continents over a period of 64 years, were determined and analyzed. The results of this study showed that the strains analyzed were highly conserved, which further indicates that the evolution of *C. chauvoei* has reached a dead end (RYCHENER et al. 2017).

The relative small genome of *C. chauvoei*, compared to other *Clostridium* species such as *C. difficile* (4.2 million bp) (SEBAIHIA et al., 2006), reflects its adaptation to a restricted host range (bovine, caprine, and ovine), where *C. chauvoei* is able to replicate and cause disease (FREY & FALQUET, 2015). However, a comparative analysis of the circular genome sequences of the *C. chauvoei* type strain (ATCC 10092) and the field strain 12S0467 isolated in Germany showed novel variations in the regulatory genes, indicating that *C. chauvoei* has specific control of the regulatory events, unlike the genomes of other *Clostridium* species (THOMAS et al., 2017).

C. chauvoei has 69 genes dedicated to the mechanisms involved in sporulation and dormancy (FREY & FALQUET, 2015), which might be considered as virulence factors that enable the pathogen to resist adverse environmental conditions and remain potentially infectious over years (GALPERIN et al., 2012). Recently, it was observed that the genes related to sporulation and germination in *C. chauvoei* are homologous to those in *Clostridia* cluster I group, that includes *C. botulinum, C. haemolyticum, C. novyi, C. perfringens, C. tetani, C. septicum*, and *C. chauvoei* (THOMAS et al., 2017). Furthermore, *C. chauvoei* produces several cellular (somatic and flagellar) (CRICHTON et al., 1990) and soluble antigens associated with virulence.

Cellular antigens

Somatic antigens are associated with the bacterial cell. Such antigens are considered crucial immunogenic components involved in the protection against *C. chauvoei* infection, and therefore, current and old vaccines contained bacterins or were solely composed of bacterins. In this sense, some studies aiming to improve vaccine quality have described important characteristics of somatic antigen expression. The amount of somatic antigens varies with bacterial growth as well as environmental conditions such as pH and carbohydrate availability, showing increased expression during the stationary phase of growth in axenic culture medium (CORTINAS et al., 1994). However, the expression of these antigens does not seem to vary among strains from different origins (MATTAR et al., 2002), which

underlines the very high genetic similarity observed among strains from all over the globe (RYCHNER et al., 2017).

Flagellar antigens have been studied extensively, highlighting flagellin, which is encoded by the *fliC* gene. Flagellin has a pathogen-associated molecular pattern (PAMP) that is recognized by toll-like receptor 5 (TLR5) expressed by monocytes and fibroblasts. The receptors at the surface of intestinal epithelial cells bind the conserved regions of flagellin (N and C terminals), resulting in the activation of cytokine secretion (YOON et al., 2012). Flagellin was found to be important for protective immunity by opsonic activity, resulting in the clearance of *C. chauvoei* by polymorphonuclear leukocytes in mice (TAMURA & TANAKA, 1984; TAMURA & TANAKA, 1987). Flagellar expression and motility are reversible in *C. chauvoei* and are associated with complete expression of virulence (TAMURA et al., 1995). Further studies characterized flagellin and evaluated its protective activity by using a recombinant flagellin protein (KOJIMA et al., 1999; 2000). These authors reported poor protective immunity induced by the recombinant flagellin in mice, suggesting that a conformation-dependent epitope plays an important role in the development of immunity against blackleg. The poor protective activity of the recombinant flagellin protein observed previously (KOJIMA et al., 2000) can be attributed to the fact that these authors did not considered that there are a minimum of two copies of *fliC* gene on the chromosome of *C. chauvoei* (SASAKI et al., 2002). RYCHENER et al., (2017) found three copies of the allelic variants *fliC1*, *fliC2*, and *fliC3* of flagellin in most strains studied, thus showing 91.8% amino acid identity with each other in a given strain and 82–96% identity between the paralogues of different strains. THOMAS and collaborators (2017) also revealed the presence of three *fliC* genes.

The cell surface-associated antigens of *C. chauvoei*, other than flagellin, have not yet been explored. USHARANI et al., (2016) identified some important cell surface-associated proteins of *C. chauvoei*, such as enolase, chaperonin, ribosomal protein L10, flavoprotein, and glycosyl hydrolase, which showed protective antigenicity in other bacteria. However, further studies are necessary to evaluate the role of these surface-associated proteins in protection against blackleg.

Soluble antigens and toxins

The soluble antigens, mainly represented by toxins, are deeply involved in the pathogenesis of blackleg. At present, five *C. chauvoei* toxins are known: the hemolytic

leukocidin CctA, oxygen-labile hemolysin D (or hemolysin III), DNase (β-toxin), hyaluronidase Nag (previously called γ-toxin), and neuraminidase/sialidase NanA.

The pore-forming, oxygen-stable leukocidin hemolysin called *C. chauvoei* cytotoxin A (CctA) confers strong hemolytic activity, which is observed as a halo around the colonies on blood agar growth medium (FREY et al., 2012). CctA as a mature protein has a molecular mass of 32.2 kDa. It is a major toxin and hemolysin produced by *C. chauvoei*, which is shown to be highly cytotoxic to the bovine epithelial cell line ECaNEp (FREY et al., 2012). In addition, FREY et al., (2012) used the conventional assay for testing the potency of blackleg vaccine, which contains purified recombinant CctA as the sole antigen, and protects 80% guinea pigs from the challenge with virulent *C. chauvoei*. The antibodies directed against CctA play the main role in the protective immunity exerted against blackleg; thus, it is a valuable candidate for blackleg vaccines and for the potency testing of current vaccines.

The previously described oxygen-stable necrotizing hemolysin (α-toxin) (HANG'OMBE et al., 2006) might be CctA, although the reported molecular mass of this α-toxin hemolysin is 25 kDa, which is significantly lower. Alternatively, this α-toxin could represent the putative hemolysin III, also called hemolysin D or δ-toxin (protein #276) found on the genome of *C. chauvoei* (FREY & FALQUET, 2015), whose molecular mass is around 25 kDa. However, the latter might correspond to a weak hemolysin that is oxygen labile and potentially thiol-activated (GILBERT, 2002). It must be noted that hemolysin III is not specific to *C. chauvoei* as several pathogenic, commensal, and environmental gram-positive bacteria express it or carry genes coding for this class of hemolysin. Although there is no clarity about hemolysin III in *C. chauvoei*, it is reported to be similar to the θ-toxin produced by *C. perfringens* and the tetanolysin produced by *C. tetani* (HATHEWAY, 1990). Using monospecific antibodies directed against CctA, FREY et al., (2012) fully neutralized all the hemolytic activity expressed by *C. chauvoei*, showing that other than CctA, this pathogen does not produce any entity with measurable hemolytic activity.

DNase (β-toxin) is an enzyme of the deoxyribonuclease type; it is a thermostable protein responsible for the nuclear degradation of muscle cells (HATHEWAY, 1990). This toxin actively participates in clostridial myonecrosis (CORTINAS et al., 1999). It was found in >80% *C. chauvoei* strains isolated from cattle (CARLONI et al., 2005), although the strains showed different capacities of toxin production. Full genome analysis of *C. chauvoei* revealed the presence of two genes encoding the large and small subunits of exo-deoxyribonuclease VII that most likely represent the DNase activity of *C. chauvoei*

(FALQUET et al., 2013). The genes encoding exo-deoxyribonuclease VII are present and fully conserved in all 20 strains of *C. chauvoei* (RYCHENER et al., 2017).

Hyaluronidase (γ-toxin) is an enzyme inactivated by heat and capable of breaking down hyaluronic acid. It is assumed to be responsible for the destruction of the loose connective tissue that surrounds the muscles, thus favoring the spread of *C. chauvoei* in the tissues of the infected host (HATHEWAY, 1990). In addition, the end products of hyaluronate degradation are disaccharides, which might be a source of nutrients for the pathogen (HYNES & WALTON, 2000). The genome of *C. chauvoei* has two different hyaluronidase genes, namely, *nagH* and *nagJ*. Currently, the functional activity of *nagH* has been confirmed (FREY & WÜTHRICH, unpublished data).

Neuraminidase/sialidase (NanA) was purified and characterized by HEUERMANN et al., (1991). They showed that the enzymatic activity cleaves N-acetylneuraminic acid (sialic acid) in carbohydrate polymers, present in many mammalian cell membranes as well as many microorganisms (USEH et al., 2003). NanA was characterized in detail as an 81-kDa protein that is secreted as a dimer (VILEI et al., 2011). It is encoded by the *nanA* gene, which is fully conserved across the *C. chauvoei* strains isolated over 60 years from various geographical locations across four different continents (RYCHENER et al., 2017). A recombinant molecule derived from *nanA* containing the sialic acid-binding domain (CBM40) is able to fully neutralize the sialidase activity of *C. chauvoei* (VILEI et al., 2011). Thus, NanA can also be used as a potential antigen to aid protective immunity.

The findings about the protective immunity exerted by soluble antigens against blackleg leave no doubt about the importance of these antigens in the pathogenesis of this condition. Interestingly, the sialidase *NanA*, hyaluronidase *NagH* and *NagI*, and leukocidin *CctA* are well conserved in *C. chauvoei* strains (VILEI et al., 2011; FREY et al., 2012; RYCHENER et al., 2017). Thus, the failures in vaccine development cannot be explained by the variations in the genes encoding major soluble antigens. A schematic illustration of blackleg pathogenesis, along with the major virulence factors, is shown in Figure 1.

Pathogenesis

Although blackleg is a clinically well-known disease, there is currently no consensus on the mechanisms underlying the pathogenesis of *C. chauvoei*. *C. chauvoei* spores are found in cattle gut as well as in pasture soil, which indicates that the infection is acquired by the ingestion of *C. chauvoei* spores. The ingested spores or those produced after germination

cycles in the gut are transported from the intestine or lesions in the oral cavity to the muscles and tissues by macrophages across Peyer's patches (JUBB et al., 1991; PIRES et al., 2012; UZAL, 2012). After reaching the tissues, the spores remain dormant until specific conditions are generated, such as anaerobiosis, resulting in their germination, multiplication, and consequently production of exotoxins (UZAL et al., 2012).

After a traumatic injury, the oxygen levels of the muscle tissue reduce, and the lactic acid concentration increases anaerobically during glycolysis (conversion of pyruvate to lactate), leading to the germination of spores, multiplication of bacteria, and consequent toxin production (MINETT, 1948a; UZAL et al., 2003). However, these hypotheses are not enough to explain why only young animals are affected or why the diaphragm or heart is the only affected area at times. In addition, it is not known whether the conditions allow the germination of latent spores in cases where there is no muscular injury, possibly because of the higher concentration of muscle glycogen caused by the high degree of muscle synthesis, which can serve as a substrate for *C. chauvoei* (VAN VLEET & VALENTINE, 2007). Latent spores of *C. chauvoei* can be found in healthy cattle carcass, in organs such as the liver and spleen (MINETT, 1948b; KERRY, 1964; SATHISH & SWAMINATHAN, 2009). In a surveillance study involving two slaughterhouses of Sao Paulo, Brazil, *C. chauvoei* was identified by microbiological culture in 7.5% of muscle samples and 1.7% of liver samples (SCHOCKEN-ITURRINO et al., 2000).

A recent study showed that the vegetative and sporulated forms of *C. chauvoei* are able to resist the microbicidal effects of macrophages in murine and bovine monocyte-derived macrophages, supporting the importance of macrophages during the early pathogenesis of blackleg (PIRES et al., 2017). These authors also noted a pro-inflammatory cytokine profile such as IL-12 and IL-23 transcription in bovine macrophages after infection with vegetative *C. chauvoei*. Conversely, in bovine macrophages infected with the spores of *C. chauvoei*, an anti-inflammatory cytokine profile such as induction of IL-10 and TGF-beta transcription was observed (PIRES et al., 2017). The anti-inflammatory profile induced by spores might explain their latency after macrophage internalization. Future research should, thus, investigate the possible role of genetic susceptibility in the occurrence of blackleg. A starting point could be the genetic characterization of the ability of phagocytic cells, especially macrophages, to clear *C. chauvoei* post internalization.

Diagnosis

Very few affected animals survive, and death usually occurs within 48 h of clinical manifestation. Once the animals die on the pasture, postmortem decomposition complicates an accurate diagnosis (DALY et al., 2009). A preliminary diagnosis of blackleg can be undertaken on the live animal on the basis of clinical signs and the presence of typical muscle emphysema. Postmortem findings include dark, discolored, swollen, and rancid muscle upon incision of the affected area. The affected muscle will contain excess fluid and gas bubbles and smell like rancid butter because of bacterial production of butyric acid. Body cavities will also contain excess fluid. Overall, tissue decomposition is rapid, assumingly by the action of potent toxins. In cardiac myositis, fluid accumulation is observed around the heart, with large amounts of fibrin too (SULTANA et al., 2008).

Traditionally, the diagnosis of blackleg is confirmed by microbiological culture and isolation of the causative microorganism. However, this is not always successful because of the difficulties in obtaining, submitting, and processing the samples in the laboratory. FARIAS et al., (2012) proposed the use of direct polymerase chain reaction (PCR) using common filter paper as an alternative to collecting, storing, and shipping material to the laboratory for the diagnosis of blackleg; the sensitivity and specificity of this approach was 100%. In addition, *C. chauvoei* is sensitive to oxygen, and it tends to be overgrown easily by other microorganisms in the samples (ASSIS et al., 2005).

Routine clinical microbiology laboratory tests might falsely identify *C. septicum*, instead of *C. chauvoei* (NAGANO et al., 2008). It is difficult to differentiate between *C. chauvoei* and *C. septicum* because of morphological and biochemical similarities. Based on 16S rDNA sequence analysis, *C. chauvoei* and *C. septicum* have been identified as closely related species with a high level of similarity (99.3%) (KUHNERT et al., 1996). THOMAS et al., (2017) also showed high relatedness (74%) between *C. chauvoei* and *C. septicum* by phylogenomic analysis. It should be noted that *C. septicum* is a microorganism capable of initiating malignant edema, a highly lethal exogenous infection (ASSIS et al., 2012). In humans, *C. septicum* is an important cause of death from spontaneous, nontraumatic gas gangrene in both adults and children (ABELLA et al., 2003; SMITH-SLATAS et al., 2006). BARNES et al., (1975) suggested that some of the deaths related to *C. chauvoei* are caused by *C. septicum* or a co-infection with *C. chauvoei* and *C. septicum*.

Further diagnostic methods for the identification and differentiation of *C. chauvoei* and *C. septicum* include immunofluorescence assays (SEISE et al., 2014),

immunohistochemistry (ASSIS et al., 2005), and MALDI-TOF MS technology (GROSSE-HERRENTHEY et al., 2008). Molecular assays such as conventional PCR on the 16S rRNA genes (KUHNERT et al., 1997, 1996; UZAL et al., 2003), 16S and 23S rDNA spacer regions (SASAKI et al., 2001; IDREES et al., 2014), and flagellin genes (KOJIMA et al., 2001; SASAKI et al., 2002) and a multiplex real-time PCR (GAROFOLO et al., 2011; HALM et al., 2010; LANGE et al., 2010) have also been used as diagnostic methods. A recombinant flagellin-based ELISA assay was developed for the detection of *C. chauvoei* and could detect up to 10^4 CFU/mL of *C. chauvoei*, in addition to its high specificity (USHARANI et al., 2015).

Prevention and control

Although published studies about the ability of vaccination to prevent morbidity and mortality by blackleg in cattle are scarce (UZAL, 2012), vaccination is the principal prophylactic measure that can be used to control the disease (CHANDLER & GULASEKHARAM, 1974). ARAUJO et al., (2010) evaluated the serological response of beef calf tissue subjected to different vaccination regimens. This study showed that booster shots significantly increased the serological response at 30 days post immunization. The serum IgG levels against *C. chauvoei* were significantly higher in calves that were first vaccinated at four months, compared to calves vaccinated at eight months of age. The serological response of calves that were vaccinated twice was found to be satisfactory, independent of the first vaccination being given at four or eight months of age. According to ARAUJO et al., (2010), cattle should be vaccinated at four months of age, followed by a booster dose one month later, and then annually repeated. For disease control, it is important to highlight that the carcasses of animals suffering from blackleg must be burned to restrain the contamination of pastures.

Some authors consider that the use of native strains can improve commercial vaccine preparations, by increasing bovine immune response (ORTIZ-ORTEGA et al., 2012). However, a recent study involving whole genome analysis of 20 strains of *C. chauvoei* isolated across four continents over 64 years (RYCHENER et al., 2017) indicated virtually no genomic variations between the strains used in vaccines and those obtained from outbreaks, suggesting that the reason for vaccine failure could be a factor other than the lack of similarity between the two sets of strains. Given that vaccination is quite common, and so are disease outbreaks, essential questions continue to remain about the causes for vaccine failure. Most

likely, practical issues such as management failures, inconsistent vaccination, delayed application of the first dose, incorrect timing of booster doses, and vaccination of dams are the causes for vaccine failure. In addition, cattle breeders tend to stop vaccinating herds when the outbreaks reduce, and as a result, the disease recurs.

Because *C. chauvoei* survives within macrophages, inactivated vaccines might not elicit the best type of immune response (cellular immunity). In Brazil, a live attenuated vaccine was patented by the Institute Oswado Cruz in 1908 (GODOY, 1910). This was the only live vaccine against *C. chauvoei* available in the world. However, since 2014, the production of this vaccine was stopped despite personal observations that this widely used live vaccine promoted adequate protection against blackleg in Brazil.

The potential underestimation of blackleg incidence is a limiting factor for prevention and control measures. Farmers form an important bridge between the cases of blackleg and official surveillance programs. Offering compensation for reporting a case might motivate the farmers to report every case. Additionally, a subsidized vaccination program could help control blackleg occurrence and thus reduce environmental contamination with *C. chauvoei* spores, especially in high-risk areas. Alternative measures to prevent and control blackleg can focus on specific pasture management practices, like artificial drainage of pastures. In fact, a recent study pointed out that blackleg cases are usually clustered within geographic areas with poor water permeability (WOLF et al., 2017).

CONCLUSION

Blackleg is an acute and often fatal infection occurring in cattle that continues to remain endemic worldwide despite large vaccination programs. Studies characterizing cellular and soluble antigens are necessary to improve the chances of developing a protective vaccine. We also highlighted that the commercial vaccines are bacterins that are probably ineffective in extending immunity against *C. chauvoei* spores, sialidase, and CctA.

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DECLARATION OF CONFLICT OF INTEREST

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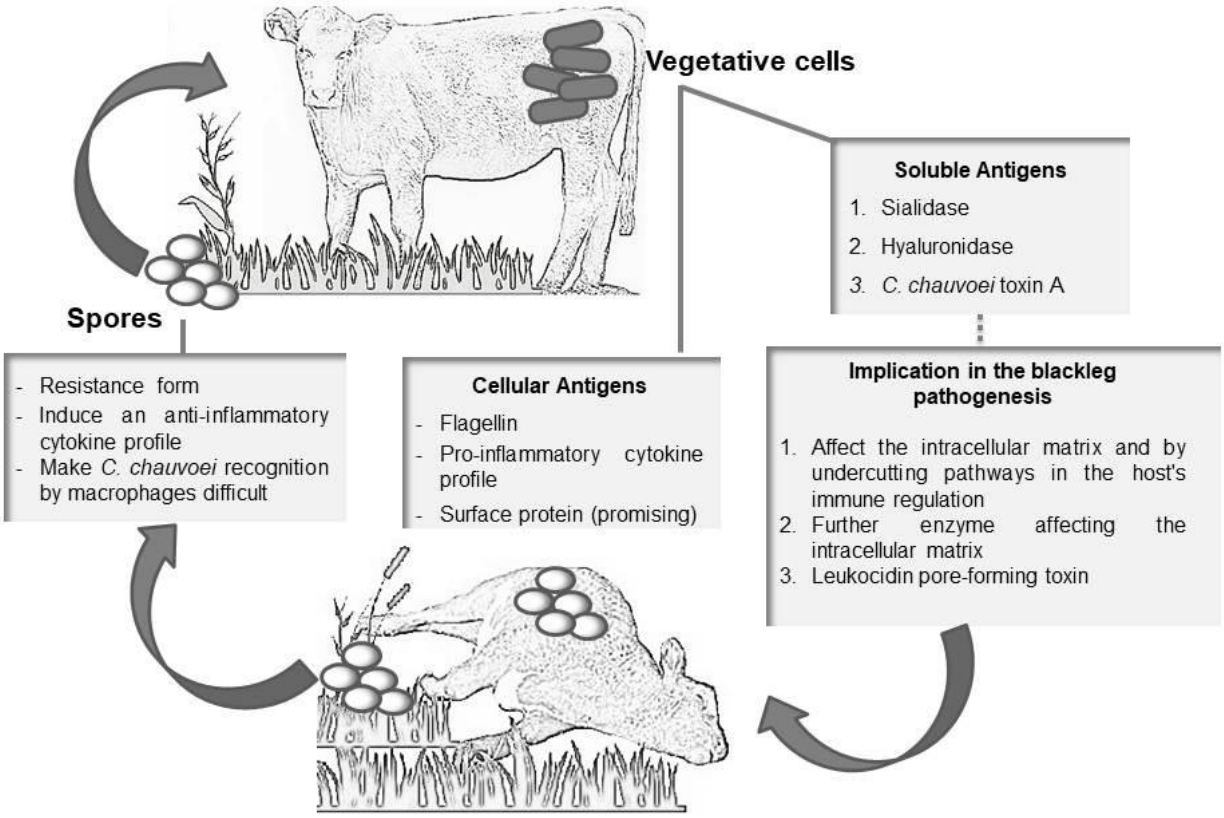

Figure 1. Schematic illustration of blackleg pathogenesis involving currently considered major virulence factors.

3 MANUSCRITO 2

Protective efficacy of commercial vaccines against a virulent field strain of *Clostridium chauvoei*

Eficácia protetiva de vacinas comerciais contra uma cepa de campo virulenta de *Clostridium chauvoei*

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ABSTRACT

Blackleg is an acute and frequently fatal infection that affects mainly cattle, caused by *Clostridium chauvoei*. Administration of formalin-killed, whole-cell vaccines is commonly used to control the blackleg. The aim of this study was to verify the protective efficacy of two commercial vaccines against infection of guinea pigs with two strains of *Clostridium chauvoei*, a virulent field strain (SBP 07/09) and a reference strain used in official tests (MT, Manguinhos-Teixeira). For this purpose, guinea pigs were vaccinated and subsequently challenged with *C. chauvoei*. The two strains were characterized by whole genome sequencing and by determination of the minimal inhibitory concentration for 15 antimicrobials. All vaccinated guinea pigs were protected against infection with both *C. chauvoei* strains. All four vaccinated and challenged groups seroconverted after vaccination, while the control group remained seronegative as determined by the indirect ELISA. The identical performance of the two *C. chauvoei* strains concerning virulence after challenge *in vivo* and inability to infect vaccinated animals is correlated with the genetic homology of the two strains. Furthermore, both commercial vaccines showed protective efficacy against reference and field strains. Although failure of *C. chauvoei* vaccinations have been reported previously, the results from our study and the firstly reported exceptional similarity of *C. chauvoei* strains from all over the world suggest that the vaccine failure seem not to be due to antigenic variability, but to inadequate vaccine management.

Keywords: blackleg, *Clostridium chauvoei*, commercial vaccines, field strain.

RESUMO

O carbúnculo sintomático é uma infecção aguda e frequentemente fatal que afeta principalmente os bovinos, causada por *Clostridium chauvoei*. A administração de vacinas formolizadas compostas pelas células bacterianas inteiras é comumente utilizada no controle do carbúnculo sintomático. O objetivo deste estudo foi verificar em cobaios a eficácia protetiva de duas vacinas comerciais contra duas cepas de *Clostridium chauvoei*, uma cepa de campo virulenta (SBP 07/09) e a cepa de referência utilizada nos testes oficiais (MT, Manguinhos-Teixeira). Para tanto, os cobaios foram vacinados e subsequentemente desafiados com *C. chauvoei*. As duas cepas foram caracterizadas pelo sequenciamento completo do genoma e pela determinação da concentração inibitória mínima frente a 15 antimicrobianos. Todos os cobaios vacinados foram protegidos contra ambas as cepas de *C. chauvoei* testadas. Os quatro grupos vacinados e desafiados soroconverteram, enquanto que o

grupo controle permaneceu soronegativo pelo teste de ELISA indireto. O desempenho idêntico das duas cepas de *C. chauvoei* após o desafio *in vivo* e a incapacidade de infectar os animais vacinados está correlacionado com a homologia genética das cepas. Além disso, ambas as vacinas comerciais demonstraram uma adequada eficácia protetiva contra as cepas de campo e de referência. Embora o insucesso das vacinações contra *C. chauvoei* tenha sido previamente relatado, os resultados deste estudo juntamente com a alta similaridade genética reportada previamente em cepas de diferentes origens do globo sugerem que as falhas vacinais não estão relacionadas à variabilidade antigênica, mas sim ao manejo vacinal inadequado.

Palavras-chave: carbúnculo sintomático, cepa de campo, *Clostridium chauvoei*, vacinas comerciais.

INTRODUCTION

Clostridium chauvoei is a Gram positive, anaerobic and spore forming bacterium that causes blackleg, a highly fatal disease of cattle and sheep. In many countries, this disease causes important economic losses in livestock production. Vaccination against the common clostridial diseases of cattle (including *C. chauvoei* infections) has been practiced for more than 70 years (UZAL, 2012). The first vaccine against this disease was developed by Arloing, Cornevin and Tomas in 1889, based on empirical knowledge of the infectious agent (LOPES, 1977). In contrast to other clostridial vaccines, which consist of toxoids, blackleg vaccines are prepared with formalin-killed whole-cell bacteria (CHANDLER; GULASEKHARAM, 1974). The efficacy of commercialized clostridial vaccines against *C. chauvoei, Clostridium botulinum* and *Clostridium perfringens* is regulated in the Pharmacopoeia of the corresponding countries where the vaccines are used.

In 1977, Reed and Reynolds reported failures of *C. chauvoei* vaccinations against blackleg. The possible reasons for the vaccination failures were thought to be due to differences in the infecting *C. chauvoei* strains. It was therefore suggested that the addition of an inactivated field strain to the vaccine could solve the problems (WOOLCOCK; FROST, 1978). Several studies were carried out evaluating the protective effect and the antigenic variations of the flagella and of a cellular antigen of *C. chauvoei* (CHANDLER; GULASEKHARAM, 1974; KOJIMA et al., 2000; TAMURA et al., 1995, 1992, 1984; TAMURA; TANAKA, 1987; TANAKA et al., 1987). The guinea pig laboratory model was considered to be a valid indicator of field performance for vaccines containing *C. chauvoei*

antigen (CRICHTON et al., 1986). Although many speculative studies have been carried out investigating soluble antigens, in particular toxins, currently only the leucocidin, *C. chauvoei* toxin A (CctA) was shown to induce protective immunity against blackleg (FREY et al., 2012). Still, only two potential virulence factors, the leucocidin CctA and the neuraminidase/sialidase (NanA) have been investigated functionally and genetically (FREY et al., 2012; FREY; FALQUET, 2015; VILEI et al., 2011). Recently, a broad genomic study showed that twenty strains of *C. chauvoei*, isolated from four continents and over a period of 60 years showed high homology, indicating identical phenotypes (RYCHENER et al., 2017). As a consequence, a vaccine made from any given strain should protect universally. This is in contrast to some reports on outbreaks of blackleg in vaccinated cattle, where antigenic variations were expected and were the inclusion of local strains was promoted to improve vaccines (CORPUS et al., 2008; DUTRA et al., 2011; GACEM et al., 2015; MIRANDA et al., 2008; ORTIZ-ORTEGA et al., 2012).

The aim of this paper was to verify the protective efficacy of two commercial vaccines, Coglavax® (CEVA-Phylaxia) and Sintoxan® (MERIAL) against a virulent field strain (SBP 07/09) and the reference strain MT (Manguinhos-Teixeira) that is used for control of blackleg vaccines according to the Brazilian legislation Portaria-SDA n°.49/97 (BRASIL, 1997). Both strains were sequenced and the genomes revealed their almost genetic identity.

MATERIALS AND METHODS

Bacterial strains used

Clostridium chauvoei strain MT is a reference strain to be used according Brazil legislation for vaccine efficacy determinations and was received from LANAGRO – Rio Grande do Sul. The field strain SBP 07/09 (Identification of the Laboratory of Bacteriology in the Research Area) was isolated from natural bovine infection belonging to a vaccinated herd of a farm located at central region of the Rio Grande do Sul state (29° 32′ S, 53° 51′ W), Brazil in 2009. The isolate was confirmed by polymerase chain reaction (PCR) for the *fliC* gene according to the protocol by Sasaki (2002), and *rrs* (16S rRNA) gene sequencing.

Characterization of the strains used in the challenge

Bacterial DNA was extracted according to the protocol by Takeuchi et al. (1997). Primer pairs were designed using Primer-BLAST (NCBI, USA) targeting *C. chauvoei*-specific region of *cctA* and *nanA* genes sequences (Table 1). DNA amplification by PCR was

performed in a reaction volume of 25 µl consisting of 1X enzyme buffer complemented with 1.5 mM magnesium chloride (Promega, USA), 20 mM each dNTP (Ludwig Biotec, Brazil), 1.25 U Taq DNA polymerase (Promega, USA), 10 mmol each primer, ultrapure water qsp (Invitrogen, USA), and 1 µL of template DNA. PCR reactions were carried out in Veriti 96-well Thermal Cycler (Applied Biosystems, USA). The reaction conditions were as follows: 94ºC for 5 minutes; 35 cycles of 95°C for 50s, 61°C for 50s (*cctA*); 62°C for 50s (*nanA*), and 72ºC for 7 minutes, followed by a final extension at 72ºC for 4 min. The amplification products were analyzed on 1.5% agarose gel electrophoresis. The gels were stained with ethidium bromide. PCR products were sequenced by ACTGene Molecular Analysis LTDA (Biotechnology Center, UFRGS, Porto Alegre, RS, Brazil).

Susceptibility of the strains used in the challenge was determined in supplemented Brucella Broth using a commercially available 96-well broth microdilution plates, Sensititre™ ANO2B susceptibility plates for anaerobic organisms (Trek Diagnostics Systems Ltda, England). The minimal inhibitory concentration (MIC) tested was of ampicillin/subactam 0.5/0.25 – 16/8 µg ml^{-1}; amoxillin/clavulanic acid 0.5/0.25 – 16/8 µg ml^{-1}; cefotenan 4 – 64 µg ml^{-1}; penicillin 0.06 – 4 µg ml^{-1}; imipenem 0.12 – 8 µg ml^{-1}; meropenem 0.5 – 8 µg ml^{-1}; clindamycin 0.25 – 8 µg ml^{-1}; cefoxitin 1 – 32 µg ml^{-1}; metronidazole 0.5 – 16 µg ml^{-1}; chloramphenicol 2 – 64 µg ml^{-1}; ampicillin 0.5 – 16 µg ml^{-1}; piperacillin 4 – 128 µg ml^{-1}; tetracycline 0.25 – 8 µg ml^{-1}; mezlocillin 4 – 128 µg ml^{-1}; piperacillin/tazobactam 0.25/4 – 128/4 µg ml^{-1}. The procedure and interpretation of the results were performed according to the manufacturer. The quality control test was based on *Clostridium septicum* ATCC 8065.

Whole genome sequencing assembly and annotation

The full genomes of the two *C. chauvoei* strains MT and SBP 07/09 were sequenced by Illumina® technology HiSeq 2500/3000 (150 bp paired-end reads) platforms performed by GATC-Biotech (Konstanz, Germany) according to the manufacturer's protocols. Genome coverage varied from 50 x to 1000 x. Genome assembly was performed using the Geneious® de novo assembly algorithm (Biomatters Ltd**,** L2, 18 Shortland Street, Auckland, 1010, New Zealand). Annotation of the whole genomes was made using Prokka (SEEMANN, 2014) and MicroScope (VALLENET et al., 2013). Sequence accession numbers are: strain MT: SRR5429445 and strain SBP 07/09: SAMN08623026.

Vaccination and challenge in immunized guinea pigs

Guinea pigs were vaccinated with one of the two polyvalent inactivated vaccines commercialized in Brazil. Vaccine A (Coglavax®, CEVA-Phylaxia), containing seven different clostridial antigens in one single dose: *C. chauvoei* bacterin, *C. perfringens* A/C/D, *C. septicum*, *C. novyi* and *C. tetani* toxins, and Vaccine B (Sintoxan®, MERIAL) containing nine different antigens: *C. chauvoei* and *C. haemolyticum* bacterins, *C. perfringens* B/C/D, *C. septicum*, *C. novyi*, *C. tetani* and *C. sordellii* toxins.

The challenge in immunized guinea pigs was carried out as regulated by MAPA (BRASIL, 1997), as described below. Guinea pigs (*Cavia porcellus*) weighing approximately 400 g were provided by Lanagro (National Agricultural and Livestock Laboratory) – Sao Paulo. The lethal dose (LD_{50}) of field strain SBP 07/09 was calculated by the method of Reed and Muench (1938) and established in $1x10^5$ LD_{50} mL^{-1} (FARIAS, 2011). The sporulated MT reference strain, routinely used for the official efficacy test of commercially available clostridial vaccines in Brazil, was provided by Lanagro-Rio Grande do Sul in titration of $6x10^4$ LD_{50} mL^{-1}.

The guinea pigs were divided into four vaccinated groups and two control groups. Two groups of eight guinea pigs each received vaccine A (0.8 ml) and two groups received vaccine B (0.6 ml). The two control groups, composed of five guinea pigs each, were left unvaccinated. Twenty-one days after the first vaccination, the same animals were revaccinated (FARIAS, 2011). Vaccinations were performed using one-fifth of the bovine dose recommended by the manufacturer subcutaneously in the ventral thoracic area (BRASIL, 1997).

Both the vaccinated and control animals were challenged at 14 days after the revaccination with 0.5 mL of the *C. chauvoei* spore suspension intramuscularly containing 100 x LD_{50} of the MT reference strain and of the field strain SBP 07/09 in $CaCl_2$ (Inlab, Brazil) at the final concentration of 5%. After challenge, guinea pigs were observed for 72 hours and deaths during this period were recorded (FARIAS, 2011). According to the Brazilian legislation Portaria-SDA nº.49/97, the evidence is considered valid when at least 4/5 sentinel animals do not survive the challenge and the vaccine should protect at least 7/8 vaccinated guinea pigs to be considered efficient (BRASIL, 1997).

After 72 hours of observation, serum samples were obtained from all animals and stored at -80°C to be used in the enzyme-linked immunosorbent assay (ELISA) (FARIAS, 2011). The animal study was performed in accordance with the ethical and animal welfare

requirements of Ethics Committee on Animal Research of Universidade Federal de Santa Maria.

Enzyme-linked immunosorbent assay (ELISA)

The positive serum sample used to standardize the serological test was a pool of serum samples from all guinea pigs vaccinated and the negative serum sample was obtained from the pool of unvaccinated guinea pigs. The full bacterial antigens from the reference strain MT (received from LANAGRO – Rio Grande do Sul) and from the field strain (SBP 07/09) were standardized as recommended by Crichton et al. (1990) and considering the growing phase of higher immunogenicity of the *C. chauvoei* culture according to Mattar et al. (2002). The antigen was obtained from the *C. chauvoei* culture at the early stationary phase of growth (± 14h). The culture was centrifuged at 4000 rpm for 20 minutes and washed twice with phosphate buffered saline (PBS) (0.14 M NaCl in 20 mM sodium phosphate buffer, pH 7.2). The pellet was suspended in sodium phosphate buffered solution (pH 8.9) and the cells were disrupted by sonication (Ultronique QR, Eco-sonics, Brazil). The total protein concentration of each antigen was determined by the Total Protein Kit (Labtest, Brazil) being 2.65 μg mL^{-1} for MT strain and 3.8 μg ml^{-1} for SBP 07/09 strain. The antigen was stored at -20°C until further use.

The 96-well microplates (Nunc, Denmark) were coated with 1:100 of antigen diluted in carbonate-bicarbonate buffer pH 9.6 and incubated for 12 hours at 4°C. After the plate was washed three times with PBS plus 0.5% Tween 20 (PBS-T), incubated with blocking buffer (1% skim milk (w/v), 0.05% Tween 20 (v/v) in PBS) at 37°C for 1 hour, and washed once more. Then, 100μl of the test and control sample serum were diluted 1:50 in blocking buffer and applied to the microplate, following by incubation at 37°C for 90 minutes. The plates were washed three times with PBS-T. Then, a 1:5000 dilution of peroxidase-conjugated anti-guinea pig IgG produced in rabbit (Bethyl®) was added to the plate and incubated for 90 minutes at 37°C and were again washed three times with PBS-T. Immediately afterward, the plates were incubated at room temperature with o-phenylenediamine substrate solution (Sigma, USA). The reaction was interrupted after 15 minutes with 2N sulfuric acid and the absorbance at 450 nm filter was measured by a microplate reader.

Statistical analysis

The ELISA data were transformed by log (x+1) and submitted to analysis of variance

with repeated measures and residual were checked for normality and homoscedasticity, which are prerequisites for analysis of variance. The means were compared with the Tukey test at 5% probability in the Genes software (CRUZ, 2006).

RESULTS

The results obtained in the vaccine efficacy trial are presented in Table 2. None of the guinea pigs of groups A and B showed clinical signs and/or death after the challenge, demonstrating that the vaccines protected all vaccinated guinea pigs (vaccinated with either of the two vaccines) against the challenge direct with 100 times the LD_{50} of both challenge strains used. Vaccinated guinea pigs also did not present adverse reactions to vaccination or revaccination throughout the experimental period. In contrast, all the animals of the control groups had lesions characteristic for the disease, such as necrosis and increased muscle mass at the inoculation site and died between 24 and 48 hours after inoculation.

The four vaccinated and challenged groups seroconverted after immunization. The mean of OD by group are presented in Table 2. The control group remained seronegative. Guinea pigs inoculated with the B vaccine had higher OD rates when compared to guinea pigs inoculated with vaccine A. Significant differences by Tukey test ($P<0.05$) occurred between the OD mean of animals vaccinated with vaccine B and challenged with the reference strain (MT) and animals vaccinated with vaccine A and challenged with the field strain (SBP 07/09), regardless of the coating antigen used in the microplates. When the plates were observed according to the antigen used to coat, in the plates coated with MT strain antigen, the lowest OD means were those of the guinea pigs immunized with vaccine A and challenged with the SBP 07/09 strain. Meanwhile, there was no statistical difference between the OD means of the different strains used as challenge in each group of vaccinated guinea pigs. In the plate sensitized with the SBP 07/09 strain antigen the lowest mean OD were also those of the guinea pigs immunized with the A vaccine and challenged with the SBP 07/09 strain, however, in this case these averages were statistically different from the mean of the animals immunized with vaccine B (Table 2).

Whole genome sequencing of the two challenge strains MT and SBP 07/09 revealed solely 19 single nucleotide polymorphisms (SNP) (Table 3). Nine SNPs concerned intergenic loci that seem not to be attributed to regulatory RNA or promoter sequences. Three SNPs are located in open reading frames (ORF) of annotated proteins with known functions (ABC transporter related protein, ribose phosphate pyrophosphokinase, 50S ribosomal subunit

protein L1 and alkyl-hydro-peroxide reductase), were they induce minor amino acid changes, two SNPs result in silent mutations in hypothetical proteins without known functions and one SNP results in an amino acid change in a hypothetical, currently unassigned protein. There was full identity of the sequences of the *cctA* and *nanA* virulence genes between the two strains. The minimal inhibitory concentrations (MIC) of field strain SBP 07/09 was 4 μg ml^{-1} for metronidazole and 0.12 μg ml^{-1} for penicillin, whereas for the reference strain MT the MIC was larger than 16 μg ml^{-1} for metronidazole and 0.25 μg ml^{-1} for penicillin. These differences could not be explained by specific genetic differences found at the genome level. Both strains were sensitive to all the other antimicrobials tested.

DISCUSSION

The scientific evidences on the efficacy of vaccination against *C. chauvoei* to prevent diseases and lethality in cattle has been scantily presented in the literature (UZAL, 2012). Vaccination is still the main and most efficient preventive measure used to control blackleg worldwide. For example, in an outbreak of blackleg in a Norwegian cattle herd in which 72 housed animals died over a period of 12 days, immediate subsequent vaccination of the remaining animals efficiently stopped the outbreak (GROSETH et al., 2011).

The results obtained in this study demonstrated that the vaccines tested induced humoral response and were able to protect the immunized animals against challenge with the standard strain (MT) and the field strain (SBP 07/09). These results differ from those found by Santos (2003), who observed differences in the degree of protection in guinea pigs vaccinated and subsequently challenged with strain MT or a field strain (SP). According to this study that includes 22 commercial vaccine batches, 95% presented protective immunity in guinea pigs when challenged with strain MT. However, when challenged with the field strain SP, only 36% of the vaccines protected sufficiently in order to be approved (SANTOS, 2003). Araújo et al. (2010) also showed the difference between the serological responses of cattle when evaluated by ELISA based on the field strain SP compared to the reference strain MT. Lopes (2005), using the rapid agglutination test, found that bovine sera immunized with commercial multivalent vaccines showed an expressive immune response against the reference strain (MT) and a low response to the field strain. The antigenic complexity and lack of information regarding which strains are used in the commercial clostridial immunogens impose difficulties in the vaccines evaluation. Although it is not known which antigens composed the vaccines, it appears to be antigenically related with the clostridial

strains used in the challenge.

Clostridium chauvoei whole antigen ELISA of guinea pig serum vaccinated for the potency test revealed an indirect measure of serum antibodies. This technique is traditionally used to evaluate the efficacy of vaccines containing the *C. chauvoei* antigen (CRICHTON et al., 1990; HAMAOKA et al., 1990). Many authors claim that immunity against this microorganism is stimulated primarily by cellular proteins of the agent and the response can be measured by detecting antibodies that are responsible for protection (CHANDLER, 1975; MATTAR et al., 1999; MICALIZZI; GUZMAN, 1997; STEVENSON; STONGER, 1980). Therefore, the ELISA standardized to measure the humoral immune response induced after vaccination and challenge with both strains used in this study allowed the comparison between the vaccines and the immunogenicity of the strains. Guinea pigs inoculated with vaccine B had higher antibody levels detected when compared to guinea pigs inoculated with vaccine A, suggesting the superiority of vaccine B in inducing humoral response when compared with vaccine A. The main difference occurred between the group B challenged with the MT strain relative to the group A challenged with the field strain. When comparing the titers of antibodies in the animals of group A and B, it can be observed that in the guinea pigs that were challenged with MT the antibodies levels were higher than those detected in the guinea pigs challenged with the field strain. However, no statistical difference was detected between the animals immunized with the B vaccine and challenged with the field strain and the animals immunized with the A vaccine and challenged with the MT. These results agree with those of a study on the humoral response with the field strain in some cases was also lower than the MT strain (ARAUJO et al., 2010). Although there are differences in response to both strains and vaccines, the strains used for challenge showed the same results in the ELISA test and there was no difference in depending on the vaccine protection.

Studies were published regarding the differences in the level of immunogenicity and the virulence of *C. chauvoei* strains (CHANDLER; GULASEKHARAM, 1974; KIJIMA-TANAKA et al., 1998; MATTAR et al., 1999; NAZ et al., 2005). Furthermore, the existence of non-flagellate mutants isolates with significantly lower virulence and immunogenicity were proposed by Kijima-Tanaka et al. (1994) and Tamura et al. (1995) and the variation of immunogenic proteins of cellular antigens associated with the growth phase of *C. chauvoei* culture were alleged by Mattar et al. (2002). The causes of vaccine failures were assumed to be due to antigenic differences between the strains used in the composition of the vaccines (DUTRA et al., 2011). In our study of guinea pigs vaccinated with vaccine A or vaccine B

and subsequently challenged with either of the two strains did not result in clinical disease and death and revealed the same degree of protection. In addition, genomic analysis of the field strain SBP 07/09 demonstrated nearly full genomic identity with the MT strain and 100% identical virulence genes *cctA* and *nanA*. This high homology between the two strains used in the challenge agrees with most recent data on a genomic analysis of twenty *C. chauvoei* strains collected worldwide over the last 60 years that revealed to be fully conserved, showing that the species *C. chauvoei* has reached a dead end in evolution (RYCHENER et al., 2017).

The antimicrobial profile of clostridia generally shows sensitivity to penicillin, tylosin, metronidazole, florfenicol and vancomycin (SALVARANI et al., 2012; SILVA et al., 2014). In this study, the MIC values showed differences between the reference strain (MT) where they were superior to those of the field strain (SBP 07/09).

Polyvalent vaccines are largely used, particularly in beef cattle, showing advantages such as time savings, allowing the immunization of populations. However, these vaccines often contain too many pathogens, some even without evidence of occurrence in the region where the vaccine is being used. In addition, vaccine indications are not always the same for all agents (age of first vaccination, number of booster doses, time elapsed between the first immunization and booster, route of administration) (GASPAR; SANTOS, 2014). With regard to important *C. chauvoei* antigens, there is concern that this pathogen produces little toxin under *in vitro* growth conditions. Together with other antigens in mixed bacterins this leads to a dilution, and hence could reduce protection against blackleg (LOPES, 1977). In contrast, in a study assessing the serological response of bovines to combined vaccine containing foot and mouth disease virus, rabies virus, *Pasteurella multocida* and *C. chauvoei* antigens compared to individual component vaccines there was no significant variation in the serological response elicited by individual component vaccines and combined vaccine containing all four antigens after 21 and 90 days post vaccination (SRINIVASAN et al., 2001). Hanna et al. (2014) also found no significant difference in *C. chauvoei* and *C. septicum* antibody titers between bivalent Clostridial and *Pasteurella* combined vaccines in sheep. In this study, although both vaccines protected guinea pigs against challenge, OD averages were higher in animals immunized with vaccine B composed by nine different antigens.

Despite the increasing demand to reduce *in vivo* studies, to evaluate some responses the animal model still remains the most robust. Buys et al. (2014) developed for use in vaccine potency testing an indirect cytometric bead immunoassay and the results were

compared with those obtained using an indirect ELISA and the *in vivo* mouse neutralisation test. The *in vitro* alternatives tested by Buys et al. (2014) did not have satisfactory results to allow the substitution of animal studies. The challenge in immunized guinea pigs is currently the method legally required for release of ready for trade vaccines. The current legislation in several countries has a standardization for vaccines containing *C. chauvoei* that require this efficacy test of all vaccine batches to be carried out by the manufacturer and by official agencies (BRASIL, 1997; BRITISH PHARMACOPEIA, 1998; USDA, 2003). However, we expect that novel knowledge on the potential major protective antigens such as CctA, NanA and NagH (hyaluronidase), will allow replacing the animal test by *in vitro* methods (RYCHENER et al., 2017).

While new knowledge clearly shows that, no antigenic variations between *C. chauvoei* strains from worldwide locations could be found (RYCHENER et al., 2017), different expression levels of the toxins could be responsible for variations of the clinical signs of the disease found. Furthermore, efficacy of vaccines could be strongly affected by the differential expression of toxins as valuable protective antigens depending on minor differences of composition of growth medium or fermentation processes. Controlling these factors will further lead to an improvement of the quality of blackleg vaccines. It has, however, to be noted that most of the informal and formal reports of cases of blackleg in Brazil are related to unvaccinated herds or to management failure, such as non-application of the booster vaccination.

CONCLUSION

The two clostridial vaccines tested induced humoral response and were able to protect the vaccinated guinea pigs against the challenge with the field strain (SBP 07/09) and the reference strain (MT). The similar performance observed between the strains in challenge *in vivo* and ELISA correlates with the homology found in their full genome sequences.

The results of this study together with the consolidated knowledge on this subject reiterate the importance of vaccination as the main preventive measure, especially in herds located in areas known to be endemic. Furthermore, it is important to mention the importance of effective vaccine management, correct route of administration, dose and booster.

Conflicts of interest: Authors declare no conflict of interests.

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Table 1. Primer sequences for PCR.

Gene	Direction	Amplicon (bp)	5' to 3' sequence	Reference
CctA	Forward	1120	TGCTTGCTTTAGCAACAACAACT	JQ728486*
CctA	Reverse		GGATGCGTCAACAATTTCTCA	Strain JF3703
NanA	Forward	1100	TCTTTGGCATACACCGTGGG	FM213082*
NanA	Reverse		CATCCCAAGTTACCCCACCA	Strain JF4135

*GeneBank Database acess number

Table 2. Challenge result with the reference strain of *Clostridium chauvoei* MT (Manguinhos-Teixeira) and field strain SBP 07/09 (Identification of the Laboratory of Bacteriology) in a guinea pig of polyvalent clostridial vaccines (A and B) and the mean values ± standard deviation of the humoral response of guinea pigs expressed in optical density (OD) in the ELISA test. *Antigens used in plate sensitization.

Groups		**Animal study**	**Serological response**	
	Challenge strains	*Protected guinea pigs/ Challenged guinea pigs*	*Mean OD*	
			*MT**	*SBP 07/09**
Vaccine A	*MT*	8/8	$0,277 \pm 0,014^{ab}$	$0,318 \pm 0,009^{B}$
	SBP 07/09	8/8	$0,182 \pm 0,050^{b}$	$0,255 \pm 0,019^{C}$
Vaccine B	*MT*	8/8	$0,367 \pm 0,015^{a}$	$0,405 \pm 0,006^{A}$
	SBP 07/09	8/8	$0,291 \pm 0,034^{ab}$	$0,355 \pm 0,066^{AB}$
Control	*MT*	0/5	$0,048 \pm 0,002^{cD}$	
	SBP 07/09	0/5	$0,049 \pm 0,001^{cD}$	

Means followed by different letters; lower case in the column of MT antigen (standard error: 20.38%) and upper case in the columm of SBP 07/09 antigen (standard error: 7.99%) are significantly different by Tukey test $P<0.05$. Adapted from Farias (2011).

Table 3. Single nucleotide polymorphisms (SNP) found between *Clostridium chauvoei* strain MT and SBP 07/09.

SNP nr	Genetic locus affected	Effect on amino acid sequence of ORF
1	Hypothetical protein	S298 > R298
2	ABC transporter related protein	G35 > R35
3	Ribose phosphate pyrophosphokinase	Y315 > D315
4	50S Ribosomal subunite protein L1	Y315 > D315
5	tRNA pseudouridine synthetase	silent
6	Hypothetical protein	silent
7	Intergenic SNP	silent [1)]
8	Intergenic SNP	silent [1)]
9	Tranpososase IS*240*	silent
10	Intergenic SNP	silent [1)]
11	Intergenic SNP	silent [1)]
12	Intergenic SNP	silent [1)]
13	Intergenic SNP	silent [1)]
14	Intergenic SNP	silent [1)]
15	Intergenic SNP	silent [1)]
16	α-mannosidase	silent
17	Intergenic SNP	silent [1)]
18	Hypothetical protein	silent
19	alkylhydroxyperoxide reductase	A80 > T80

[1)] no potential regulatory RNA affected.

4 MANUSCRITO 3

Molecular characterization of virulence genes *cctA*, *nanA*, and *fliC* in *Clostridium chauvoei*

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(Manuscrito a ser submetido para publicação)

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Abstract

Clostridium chauvoei toxin A (*cctA*), neuraminidase (*nanA*) and flagellin (*fliC*) proteins contribute to pathogenicity of *Clostridium chauvoei*, causative agent of blackleg in cattle. The aim of this study was to analyze the genetic variability of *cctA*, *nanA*, and *fliC* genes in *C. chauvoei* isolates from different regions and sampling dates in Brazil. The presence of these genes was verified by PCR amplification, and the partial gene sequencing from 17 strains was performed. Alignments of the PCR amplification of genes combined with bioinformatics analysis were used in an attempt to study the variability across *C. chauvoei* isolates. The similarity of partial sequences of *cctA* and *nanA* genes was 100%. The sequencing of the *fliC* revealed three different paralog alleles of flagellin, and two strains showed to be polymorphic causing amino acid alterations. Strains of *C. chauvoei* isolated in Brazil are highly conserved in relation to the virulence factors evaluated in this study.

Key-words: Blackleg, *Clostridium chauvoei* toxin A, neuraminidase, flagellin.

Introduction

Blackleg is a universal disease of cattle. It is an acute, endogenous/soil borne infection caused by *Clostridium chauvoei*, a Gram positive, motile, histotoxic, sporulating anaerobic bacterial species [1]. Current information indicates that toxins, DNAse, hyaluronidase, hemolysin, sialidase and flagellar protein seem to make a major contribution to pathogenicity of *C. chauvoei* [2]. At present, the only well characterized virulence factors of *C. chauvoei* are sialidase [3–6], *Clostridium chauvoei* toxin A (CctA) [7] and flagellin [8–10].

The β-barrel protein porin leukocidine CctA has been shown to constitute the main virulence factor of *C. chauvoei*. It is likely that *C. chauvoei* is not able to cause disease without the presence of *cctA* gene, associated to CctA. However until now, the generation of a knockout mutant of *cctA* was not reported. Frey et al. [7] demonstrated that vaccination of guinea pigs with CctA, in the form of a fusion protein, protected animals against virulent *C. chauvoei* spores.

Neuraminidases or sialidases have been found in viruses, bacteria, protozoa, fungi and metazoans [18]. They are suggested to be implicated in pathogenesis of blackleg. They are enzymes that cleave N-acetylneuraminic acid from carbohydrate polymers, such as mucin, glycoproteins, gangliosides and other sialoglycoconjugates, located on many mammalian cell membranes [6]. Vilei et al. [6] showed that antibodies produced against recombinant sialidase

NanA of *C. chauvoei* neutralized the activity of the infectious agent to degrade sialic acid indicating NanA to be a good candidate as an antigen for design of novel vaccines against blackleg.

Flagella of *C. chauvoei* drew particular attention in characterization of this pathogen. Flagella are not directly involved in pathogenesis of blackleg [11]. However, they are considered to contribute to the infectious process, since they provide mobility to bacteria and enable them to reach the target tissue where the pathogen causes injury and disease. Moreover, flagellar antigens were studied as potential candidate for vaccines [10,12,13]. Flagella also showed to be important for immune response in other pathogenic bacteria such as *Salmonella enterica* [14], *Vibrio cholerae* [15] and *Pseudomonas aeruginosa* [16]. In *C. chauvoei*, twenty flagellar biosynthesis genes were characterized [2]. Flagellin is an immuno-dominant surface protein of the *C. chauvoei*, which may have a protective role as well as a potential to be used in diagnostics [17].

Full genome sequences of twenty strains of *C. chauvoei*, isolated from four different continents, over a period of sixty-four years, revealed that *C. chauvoei* genome is highly conserved in contrast to other *Clostridium* species [19]. The aim of the study was to investigate genetic diversity of CctA (*cctA*), neuraminidase (*nanA*) and flagellin (*fliC*) across strains from different origins and periods of isolation in Brazil.

Materials and Methods

Bacterial source, growth conditions and characterization

The seventeen *C. chauvoei* isolates used in this study there were origin from Brazilian blackleg cases between 2002 and 2016 (Table 1). In addition, we included the Brazilian official reference strain (MT) and the *C. chauvoei* type strain ATCC 10092 from the American Type Culture Collection (ATCC). All strains were stocked in Reinforced Clostridial Medium (Oxoid™). The isolates were confirmed by polymerase chain reaction (PCR) for the *fliC* gene according to protocol proposed by Sasaki [20], and further analysis was performed by 16S rRNA gene sequencing.

Immunoblot analysis of CctA

Clostridium chauvoei strains were grown anaerobically in Brain Heart Infusion (BHI) broth (Oxoid Microbiology products, Thermo Scientific, Waltham, MA, USA) supplemented with 0.05% L-cysteine (Sigma-Aldrich chemical, St. Louis, MO, USA) at 37°C for up to 72

hours to mid exponential growth phase. 1 mL aliquots of each culture were centrifuged at 10,000 x g for 30 min at 4°C, the supernatant and bacterial pellet were separated carefully. Supernatants (5 µl aliquots) from each *C. chauvoei* strain were mixed 1:1 (v/v) with SDS-PAGE loading buffer (65.8 mM Tris-HCl, pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue) containing 5% β-mercapto-ethanol. The samples were separated by SDS-PAGE using 12% acrylamide gels. The proteins were electro-transferred onto polyvinylidene diflouride (PVDF) membranes (Millipore, IPVH10100) in transfer buffer (25mM Tris, 192mM Glycine and 20% (v/v) methanol). The membranes were blocked for 30 min using TBST (20mM Tris, 137mM NaCl and 0.1% Tween-20 detergent, pH 7.6) with 5% skimmed-milk powder and then probed overnight at 4°C with rabbit anti-CctA antiserum [7] diluted 1:1000 in TBST and for 2 h at room temperature with phosphatase labelled goat Anti-Rabbit (KPL 4751-1516) diluted 1:5000 in TBST. The signals were detected by incubating the blot in fresh alkaline phosphatase substrate buffer (100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$) containing BCIP (Roche 11585029001) and NBT (Roche 10760994001) staining solutions.

DNA extraction, PCR amplification, sequencing and nucleotide analysis

Bacterial DNA was extracted according to the protocol proposed by Takeuchi et al. [21]. Pairs of primers were designed in the Primer-BLAST (NCBI, USA), targeting the specific region of the leukocidin toxin gene of *cctA* and the neuraminidase gene *nanA* of *C. chauvoei*. For the amplification of the *fliC* gene the primers were adapted from Usharani et al. [17] (Table 2).

DNA amplification by PCR was performed in a reaction volume of 25 µl consisting of 5 µL buffer 10X + mM magnesium chloride, 20 mM each dNTP (Ludwig Biotec, Brazil), 1.25 U Taq DNA polymerase (Promega, USA), 10 mmol each primer, ultrapure distilled water qsp (Invitrogen, USA), and 1 µL DNA. PCR was carried out in Veriti 384-well Thermal Cycler (Applied Biosystems, USA). Cycles were as follows: initial denaturation of 94ºC for 5 minutes, followed by 35 cycles of 95°C for 50s, 61°C for 50s (*cctA*); 62°C for 50s (*nanA*); 68°C for 50s (*fliC*), and 72ºC for 7 minutes. Final extension was carried out at 72ºC for 4 min.

PCR products were sequenced by Sanger (ACTGene Molecular Analysis LTDA, Biotechnology Center, UFRGS, Porto Alegre, RS, Brazil). Additionally, were used flagellin reads of eight strains (Table 1) that were to sequencing by Illumina HiSeq (Fasteris SA, Plan-les-Ouates, Switzerland), according to the protocol by Rychener et al. [19].

The DNA sequences was analyzed by Geneious software version 7.1.5 [22].

Results

All strains amplified specific fragments of *cctA*, *nanA* and *fliC* genes. The identity of nucleotide sequences of the seventeen Brazilian *C. chauvoei* strains and the reference strain MT and Type strain ATCC 10092 was 100% for *cctA* gene. Minor differences were found comparing to strain JF3703 a strain isolated in 1956 in New Zealand (99.62% identity). Also for the *nanA* gene the sequences, were 100% identical among the Brazilian strains as well as compared to the reference MT and type ATCC 10092 strains. Again minor differences were found when comparing the *nanA* gene sequences to the old New Zealand strain [19], JF3703 which revealed 96.50% identical nucleotides.

Immunoblot analysis of culture supernatants from eight Brazilian *C. chauvoei* strains reveal that CctA is expressed and secreted by all of these strains (Figure 1) at relatively equal amount. No reactions with anti-CctA serum were seen on immunoblots containing the culture pellet material (not shown).

Sequencing of PCR products for the *fliC* gene showed triple peaks in some nucleotides in all the seventeen *C. chauvoei* strains used in this study. The sequencing of the eight strains by Illumina HiSeq revealed three different paralog alleles of flagellin (*fliC1*, *fliC2* and *fliC3*). Two of the eight strains evaluated, 2828/2004 and SB 52/2011, presented single nucleotide polymorphisms (SNPs) in the *flic3* allele with amino acid alteration (Figure 2).

Discussion

CctA is the major virulence factor of *C. chauvoei*, no genetic variability was found for the 17 *C. chauvoei* strains, Frey et al. [7] reported that the strain JF3703/1956 showed a slightly different allele of *cctA*, which however does not affect the amino acid sequence [19]. CctA belongs to the leucocidin superfamily of bacterial toxins and was not found in other clostridia [7]. The high degree of identity between strains linked to the fact that this toxin is specific to *C. chauvoei.* It suggests that CctA gene is a good candidate to identify *C. chauvoei* by PCR in clinical veterinary microbiology laboratories. Immunoblot analysis of supernatants and sedimented bacteria from cultures of *C. chauvoei* indicate that all strains tested secrete most or all of the CctA toxin that is synthetised.

Sialidases are considered as virulence factors in several pathogenic organisms. The sialidase activity of the NanA protein of *C. chauvoei* contains a CBM40 module that

specifically binds sialic acid residues as was reported for sialidase NanJ of *C. perfringens* [4,5,23,24]. The region amplified by the *nanA* primers used in this study includes mainly the CBM40 domain. All the strains showed a conserved DNA sequence revealing an identity of 100%, except for strain JF3703 where the observed differences are located in this domain [19]. Sialidase activity of *C. perfringens* type A was detected by Llanco et al. [25], the *nanH, nanI* and *nanJ* genes were investigated with four different patterns indicating heterogeneity of the microorganism. However, *C. chauvoei* unlike a bacterial enteric pathogen is highly specialized. The conserved gene sequences might reflect the restricted environment for survival of this pathogen and its adaptation to few hosts (cattle, sheep and goats) [2,19].

The multiplex PCR described by Sasaki et al. [20] has been widely used for identification and differentiation between of *C. chauvoei*, *C. haemolyticum*, *C. novyi* types A and B, and *C. septicum*. The primers for *C. chauvoei* encode a conserved initial region of the flagellin gene *fliC*. Sasaki et al. (2002) demonstrated that these clostridia have at least two copies of the flagellin gene (*fliC*) arranged in tandem (FliA and FliB). The deduced N and C terminal amino acid sequences of the FliCs are well conserved but central amino acid sequence are not. *C. difficile* strains also exhibit conservation in the N and C termini, while the central region is more diverse [26]. Rychener et al. [19] using full genome Illumina HiSeq sequencing, observed three putative genes *fliC1*, *fliC2* and *fliC3*, encoding three different paralog genes. Thomas et al. [27] by single molecule, real-time (SMRT DNA) sequencing also observed different copy number variations.

The primer here used for amplification of *fliC* was chosen because Usharani et al. [17] reported that central region of flagellin gene is unique for *C. chauvoei*. However, in the sequencing of the PCR product of the strains evaluated in the study it has been observed that this primer anneals in a region of the flagellin that amplifies the three alleles, that was not reported by Usharani et al. [17]. For this reason, eight strains were submitted to sequencing by Illumina HiSeq. The sequences deposited on GenBank by Usharani et al. [17] are identical to the FliC sequence of Kojima et al. [10] and FliA sequence of Sazaki et al. [20].

In this study, the Illumina HiSeq sequencing revealed three different paralog alleles of flagellin too (*fliC1*, *fliC2* and *fliC3*). Six out of the eight *C. chauvoei* strains contain the same three alleles of strain JF 4335 that was used as a reference for the genomic DNA sequence [19] and of strains 12S0467 and ATCC 10092 [27]. Strain 2828 isolated in 2004 in Sao Paulo state showed a different allele of *fliC3* with one SNP affecting the third nucleotide (729 position) of the codon altering the 243 amino acid, there was a substitution of one Aspartic acid for a

Glutamic Acid (Figure 2). Strain SB 52 isolates in 2011 in Rio Grande do Sul state showed two SNPs, one affecting the third nucleotide (813 position) of the codon but not altering the amino acid (GGT_{Gly} instead of GGC_{Gly}) and other affecting the first (820 position) and third nucleotide (822 position) of the codon altering the 274 amino acid, there was a substitution of one Isoleucine for a Valine (Figure 2), both in *fliC3* allele. The central variable region of *fliC* is not easily resolved by sequencing techniques employing short reads, such as reversible terminator-based sequencing (Illumina). However, it was verified that there was no assembly error because the sequences obtained by Thomas et al. [27] by SMRT DNA sequencing demonstrated 100% identity with most of those obtained by the Illumina sequencing.

Flagella activity is characterized by motility and flagella reversibly is inherent in the wild-type strain and not just a peculiarity of the original variants [9]. Polymorphisms in flagellin genes might alter protein and influence flagella activity that could be related to virulence [10,28]. A poor protective immunity induced by the recombinant flagellin in mice was observed, suggesting that a conformation-dependent epitope may play an important role in the development of immunity against blackleg [10]. Alternatively antibodies against flagellin alone might simply not be sufficient to induce protective immunity as neutralizing antibodies against the main toxin is requested.

A study of Tayastere and collaborators [26] indicated that non-flagellated *C. difficile* serotypes retain transcription of *fliC* genes but the protein products have remained undetected. In *C. difficile* the flagella may be important for colonization and adherence, but not sufficient to induce protective immunity [11]. As the pathogenesis of blackleg by *C. chauvoei* infection and colitis caused by *C. difficile* infections are very different, the true involvement of the flagellum in the pathogenesis of blackleg, as well as how the transcription of the copies of the *fliC* is regulated in *C. chauvoei* needs to be unraveled to elucidate their role in pathogenesis.

Conclusion

Overall, the virulence genes *cctA* and *nanA* showed to be highly conserved among all Brazilian strains studied as well as comparing them with the Brazilian reference strain MT and the type strain ATCC 10092. Due to the conservation of CctA and NanA, these soluble antigens are good candidates as antigens for design and quality control of *C. chauvoei* vaccines. Despite of this, three different *fliC* alleles were detected among the strains studied. They are related to different serotypes of *C. chauvoei*. However, their role in infection and in inducing protective immunity remains to be elucidated further.

Conflicts of interest: none.

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Table 1. Brazilian *Clostridium chauvoei* isolates from blackleg cases during the period 2002 to 2016 used in this study. *Sequencing by Illumina HiSeq and Immunoblot analysis of CctA. SP: Sao Paulo, RS: Rio Grande do Sul.

	Strain designation/Year	City (geographical coordinates)	State
1	2655/2003	Araçatuba (21º12'S/ 50º25'W)	SP
2	2828/2004*	Luiziania (21º40'S/ 50º19'W)	SP
3	2974/2005*	Piracicaba (22º43'S/ 47º38'W)	SP
4	NF	Pelotas (31º46'S/ 51º20'W)	RS
5	SBP 07/2009*	Sao Martinho da Serra (29º32'S/ 53º51'W)	RS
6	SB 52/2011*	Sao Pedro do Sul (29º37'S/ 54º10'W)	RS
7	SB 65/2011	Santa Maria (29º41'S/ 53º48'W)	RS
8	SB 97/2011*	Jaguari (29º29'S/ 54º41'W)	RS
9	SB 105/2011	Jaguari (29º29'S/ 54º41'W)	RS
10	3270/2002*	Canguçu (31º23'S/ 52º40'W)	RS
11	SB 87/2013 286*	Toropi (29º28'S/ 54º13'W)	RS
12	SB 87/2013 285	Toropi (29º28'S/ 54º13'W)	RS
13	SB 131/2013	Sao Vicente do Sul (29º41'S/54º40'W)	RS
14	SB 46/2014	Jaguari (29º29'S/ 54º41'W)	RS
15	SBP 17/2015	Jaguari (29º29'S/ 54º41'W)	RS
16	SBP 43/2015*	Jaguari (29º29'S/ 54º41'W)	RS
17	SBP 59/2016	Ijui (28º23'S/ 53º54'W)	RS

Table 2. Forward (F) and reverse (R) primers used to gene sequencing of *Clostridium chauvoei* strains (n=17).

Primer	Sequence 5' – 3'	Bp	Temperature	Reference
cctA-F	TGCTTGCTTTAGCAACAACAACT	1120	61º	JQ728486*
cctA-R	GGATGCGTCAACAATTTCTCA			Strain JF3703/1956
nanA-F	TCTTTGGCATACACCGTGGG	1100	62º	FM213082*
nanA-R	CATCCCAAGTTACCCCACCA			Strain JF4135/2004
fliC-F	AGCTAACGATACAAACGTAG	708	58º	Adapted from
fliC-R	GCTGAATTTATAGTCTTTATGC			(USHARANI et al., 2015)

*GeneBank Database acess number

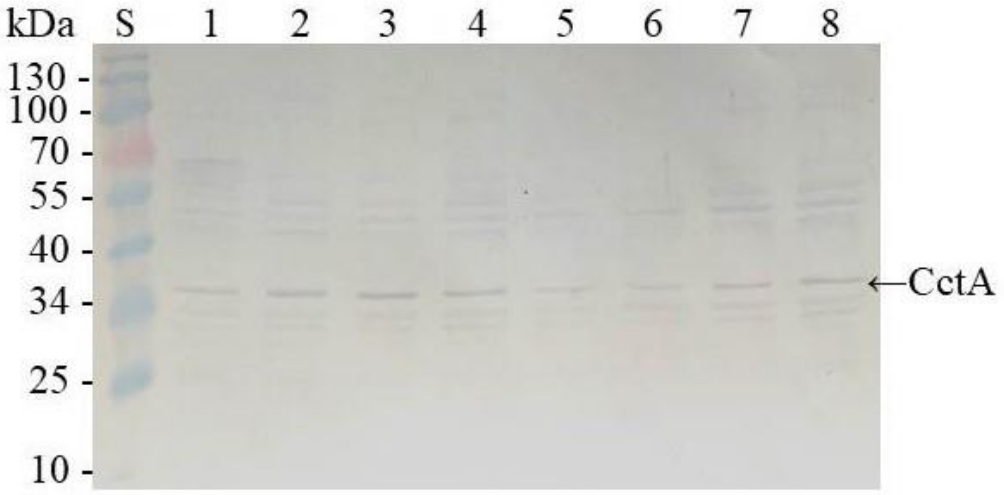

Figure 1. Expression of CctA analyzed on immunoblots of culture supernatants from eight Brazilian *Clostridium chauvoei* strains. Note the minor bands are background band due to the rabbit serum that were also present in pre-immunization serum. S: molecular mass standard as indicated on the left; 1 - 8: Brazilian *C. chauvoei* strains; 1: 2828/2004; 2: 2974/2004; 3: SBP 07/2009; 4: SB 52/2011; 5: SB 97/2011; 6: 3270/2002; 7: SB 87/2013 286; 8: SBP 43/2015.

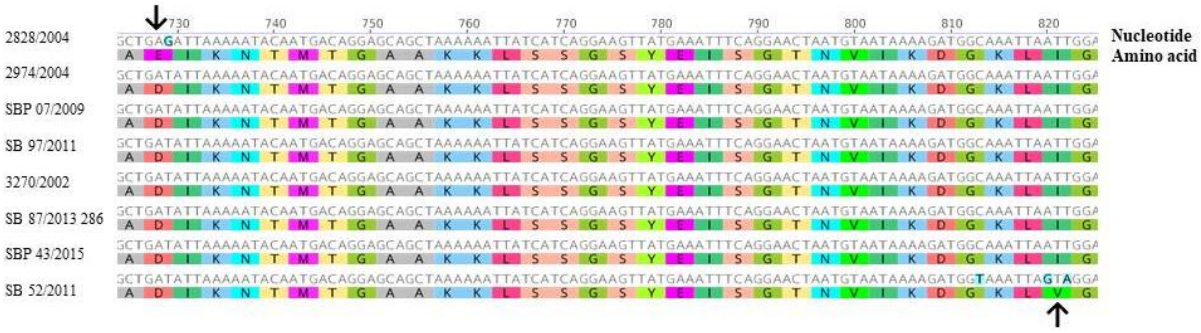

Figure 2. Nucleotide and amino acid sequences alignments of *fliC3* of eight *Clostridium chauvoei* strains by Geneious software. The arrows indicate the polymorphisms.

5 MANUSCRITO 4

Genomic comparison of Brazilian *Clostridium chauvoei* isolates from blackleg

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(Manuscrito a ser submetido para publicação)

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Abstract

Clostridium chauvoei is the etiological agent of blackleg. The disease has been reported worldwide, and although it can be prevented by vaccination, sporadic cases and occasional outbreaks still occur. Blackleg is an important disease in Brazil, the present study aimed to determine and analyze the complete genome sequences of six Brazilian *C. chauvoei* strains, a especial approach was given to strain that caused visceral clinical signs. The results of this study revealed that the genome of the Brazilian *C. chauvoei* strains is highly conserved.

1. Introduction

Clostridium chauvoei is a Gram-positive, anaerobic, and spore-forming rod found in the soil, feces, and the digestive tract of many animals (Quinn et al., 2011). The proposed pathogenesis of blackleg is that *C. chauvoei* spores are initially ingested from contaminated soil. The ingested spores or those produced after germinative cycles in the gut, are transported from the intestine or lesions in the oral cavity to muscles and tissues by macrophages across Peyer's patches (Jubb et al., 1991; Uzal, 2012). Reaching the tissues the spores remain dormant until specific conditions are generated, as anaerobiosis, resulting in their germination, multiplication and consequently production of the exotoxins (Uzal, 2012). Usually, the antimicrobial profile of clostridia presents sensitivity to penicillin, tylosin, metronidazole, florfenicol and vancomycin (Salvarani et al., 2012; Silva et al., 2014).

There are two different clinical manifestation of blackleg in cattle: a classical and visceral form, both forms are usually fatal and the treatment with antibiotics is discouraging (Assis et al., 2010; Casagrande et al., 2015; Uzal, 2012). In most cases of classical blackleg occur acute neutrophil necrotizing myositis that affects skeletal muscle. The visceral myonecrosis, which is rarely diagnosed but can affect the heart, the sublingual muscles and the diaphragm (Assis et al., 2010; Casagrande et al., 2015). The pathogenesis of cardiac lesions has not been successfully explained (Abreu et al., 2017).

The relative small genome of *C. chauvoei*, consisting of 2.8 million base-pairs (bp), reflects its adaptation to a restricted host range (cattle, sheep and goats), where *C. chauvoei* is able to replicate and to cause disease (Frey and Falquet, 2015). Full genome sequences of twenty strains of *C. chauvoei*, isolated from four different continents, over a period of sixty-four years, revealed that *C. chauvoei* genome is highly conserved across other *Clostridium* (Rychener et al., 2017).

Whole genome sequencing is a powerful tool for understanding bacterial intra-species diversity keeps unveiling its secrets as the power and speed of sequencing technologies increases (Binnewies et al., 2006; Tettelin et al., 2008). Until now, there is limited information regarding the genetic diversity of *C. chauvoei*, only the twenty strains sequenced by Rychener et al. (2017) and the comparative genome analysis of ATCC 10092 with a field strain performed by Thomas et al. (2017).

Considering the disease importance, in order to get insight into the molecular genetic evolution of *C. chauvoei*, the complete genome sequences of six Brazilian *C. chauvoei* isolates were unraveled. Particular focus was given to *C. chauvoei* strain that caused visceral clinical signs, intending through molecular tools to elucidate the different clinical manifestations of the disease.

2. Materials and Methods

2.1. Bacterial strains

The six bacterial strains used in this study there were origin from Brazilian blackleg cases from 2002 to 2015 and are listed in Table 1. All strains were stocked in Reinforced Clostridial Medium (Oxoid™). Previously to genome sequencing, the isolates were confirmed by polymerase chain reaction (PCR) for the *fliC* gene according to protocol proposed by Sasaki (2002), and further analysis was performed by 16S rRNA gene sequencing.

2.2. Whole genome sequencing assembly, annotation and consensus comparison

The full genomes of the six *C. chauvoei* strains were sequenced by Illumina® technology HiSeq 2500/3000 (150 bp paired-end reads) platforms performed by GATC-Biotech (Konstanz, Germany) according to the manufacturer's protocols. Genome coverage varied from 50 x to 1000 x. Genome assembly was performed using the Geneious® de novo assembly algorithm (Biomatters Ltd, L2, 18 Shortland Street, Auckland, 1010, New Zealand). Annotation of the whole genomes was made using Prokka (Seemann, 2014).

To get an overview of the genomes of the six *C. chauvoei* strains, the consensus sequences were extracted from the BAM files using SAMTools and visualized using the BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011).

2.3. Minimal inhibitory concentration

Susceptibility of the strains was determined in supplemented Brucella Broth using a commercially available 96-well broth microdilution plates, Sensititre™ ANO2B susceptibility plates for anaerobic organisms (Trek Diagnostics Systems Ltda, England). The minimal inhibitory concentration (MIC) tested was of ampicillin/subactam 0.5/0.25 – 16/8 µg/ml; amoxillin/clavulanic acid 0.5/0.25 – 16/8 µg/ml; cefotenan 4 – 64 µg/ml; penicillin 0.06 – 4 µg/ml; imipenem 0.12 – 8 µg/ml; meropenem 0.5 – 8 µg/ml; clindamycin 0.25 - 8 µg/ml; cefoxitin 1 – 32 µg/ml; metronidazole 0.5 – 16 µg/ml; chloramphenicol 2 – 64 µg/ml; ampicillin 0.5 – 16 µg/ml; piperacillin 4 – 128 µg/ml; tetracycline 0.25 – 8 µg/ml; mezlocillin 4 – 128 µg/ml; piperacillin/tazobactam 0.25/4 – 128/4 µg/ml. The procedure and interpretation of the results were performed according to the manufacturer. The quality control test was based on *Clostridium septicum* ATCC 8065.

3. Results

3.1. Genome assembly and annotation

Genome assembly and circularization summary for the complete genomes are represented in Table 2. The final circular genomes ranging from 2,882,958 to 2,887,475 bp. All plasmids were identical with 3,941 bp.

The circular diagram shown in Figure 1 represents the consensus sequences of six *C. chauvoei* strains from Brazilian blackleg cases aligned against the reference sequence JF4335 isolated from a cattle that succumbed of blackleg in Switzerland in 2004 (Falquet et al., 2013).

3.2. Minimal inhibitory concentration

The minimal inhibitory concentrations (MIC) of *C. chauvoei* strains are showed in Table 3. The metronidazole showed a MIC_{50} of 4 µg/ml and a MIC_{90} of 8 µg/ml, whereas for penicillin 50% (3/6) of the strains were sensitive to all concentrations tested. All strains were sensitive to all the other antimicrobials tested.

4. Discussion

The total size of the genome of the Brazilian strains is more similar to the reference strain JF4335 from Switzerland (Falquet et al., 2013) than German strain 12S0467, described by Thomas et al. (2017). The chromosome sequence of *C. chauvoei* strain JF4335 is 2,887,451 bp with a GC content of 28.3% (Rychener et al., 2017). The cryptic plasmid (Frey

and Falquet, 2015) from Brazilian strains, however, showed 100% identity with the 12S0467 plasmid and 95% identity with the JF4335 plasmid.

The genomes of the six *C. chauvoei* Brazilian strains were compared, the circular diagram was developed and shown the high similarity between strains (Figure 1). *C. chauvoei* has been efficient in causing a highly fatal acute disease in a limited number of species. For this reason, this microorganism does not undergo pressure for mutations, unlike, for example, *C. difficile* where it is possible to epidemiological interpretation of genealogies. Didelot et al. (2012) revealed that the estimated date of the common ancestor of *C. difficile* could be as little as a few days before samples were taken. A completely different reality since a recent study involving whole genome analysis of 20 strains of *C. chauvoei* isolated across four continents over 64 years indicated high similarity (Rychener et al., 2017).

The most prevalent gaps were identified by Rychener et al. (2017) and represented mostly fragments of transposases or fragments of putative bacteriophage genes. The strain JF5842 isolated in 2015 was included in this study and revealed lack of IS256 transposase (Rychener et al., 2017). Interestingly, in strain JF5840, the only one who presented clinical signs in the myocardium, this transposase is also missing. Rychener et al. (2017) reported that none of observed gaps or islands contains any genes that would be known to have a vital function or to be involved in virulence or pathogenicity of the organism.

Transposable elements are major components of prokaryotic genomes and play a significant role in their evolution (Guérillot et al., 2014). IS256 transposase is a DNA-binding protein, its molecular function and lifestyle is very little known (Hennig and Ziebuhr, 2010). Multiple genomic IS256 copies may serve as crossover points for homologous recombination events and so play an important role in genome flexibility, adaptation, and evolution of staphylococcal and enterococcal genomes (Shankar et al., 2002; Ziebuhr et al., 2000). However, there are no studies on the role of IS256 in *C. chauvoei*.

The cattle that died whose material was sent to the laboratory had the criteria for presumptive diagnosis of blackleg, that include necrohemorrhagic emphysematous myositis, interstitial edema, hemorrhage, and gas bubbles between myofibers (Heckler et al., 2018). Only in one case myocardium was also affected (JF5840 strain). Usually, heart and skeletal muscle lesions occur in the same animals in cases of blackleg. It has been postulated that after skeletal muscle lesions, metastatic colonization of the heart and diaphragm occurs secondarily. However, the pathogenesis of cardiac lesions, in those rare cases in which only cardiac lesions are observed, has not been satisfactorily explained (Abreu et al., 2017). As

well as why clinical signs occur in the myocardium only in some cases and others are restricted to skeletal muscles. No genetic variability was found among JF5840 and the other *C. chauvoei* strains.

The strain JF5840 showed the highest MIC values, 8 µg/ml for metronidazole and 0.12µg/ml for penicillin. These differences could not be explained by specific genetic differences found at the genome level. The observed susceptibility reflects the fact that blackleg is not treated with antibiotics due to acute course of the disease. For this same reason it is difficult to compare the results with the MICs of other members of the genus *Clostridium*.

5. Conclusion

From the data of total sequencing of the genome it was not possible to observe some variation that suggests why in some cases clinical signs in the myocardium occur. The study reveals that the genome of the Brazilian *C. chauvoei* strains is highly conserved.

Conflict of interest statement

The authors confirm that they have no conflicts of interest in this work.

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Table 1. Description of Brazilian *Clostridium chauvoei* isolates analyzed in this work.

Strain Designation	Year of Isolation	Origin (geographical coordinates)	Source
JF5834	2004	Luziânia - SP (21º40'S/ 50º19'W)	skeletal muscle
JF5835	2005	Piracicaba - SP (22º43'S/ 47º38'W)	skeletal muscle
JF5837	2011	São Pedro do Sul - RS (29º37'S/ 54º10'W)	skeletal muscle
JF5839	2002	Canguçu - RS (31º23'S/ 52º40'W)	skeletal muscle
JF5840	2013	Toropi - RS (29º28'S/ 54º13'W)	skeletal muscle and heart
JF5842	2015	Jaguari - RS (29º29'S/ 54º41'W)	skeletal muscle

Table 2. Genome assembly and circularization summary (*Clostridium chauvoei* strains JF5834, JF5835, JF5837, JF5839, JF5840 and JF5842).

	JF5834	**JF5835**	**JF5837**	**JF5839**	**JF5840**	**JF5842**
Total genome size	2,884,627	2,887,475	2,882,982	2,883,492	2,884,200	2,882,958
Sequence coverage	135X	102X	111X	569X	133X	182X
Paired-ends reads	1,742,834	1,260,162	1,424,074	10,614,982	1,740,408	2,528,782
GC-content (%)	28.3	28.3	28.3	28.3	28.3	28.3
CDS	2,620	2,625	2,616	2,618	2,616	2,622
tRNA	87	87	86	87	87	87
rRNA	27	27	27	27	27	27

Table 3. The minimal inhibitory concentrations (MIC) of Brazilian *C. chauvoei* strains.

Strain Designation	Metronidazole (µg/ml)	Penicillin (µg/ml)
JF5834	1	sensitive
JF5835	4	sensitive
JF5837	8	sensitive
JF5839	2	0.12
JF5840	8	0.12
JF5842	4	0.06

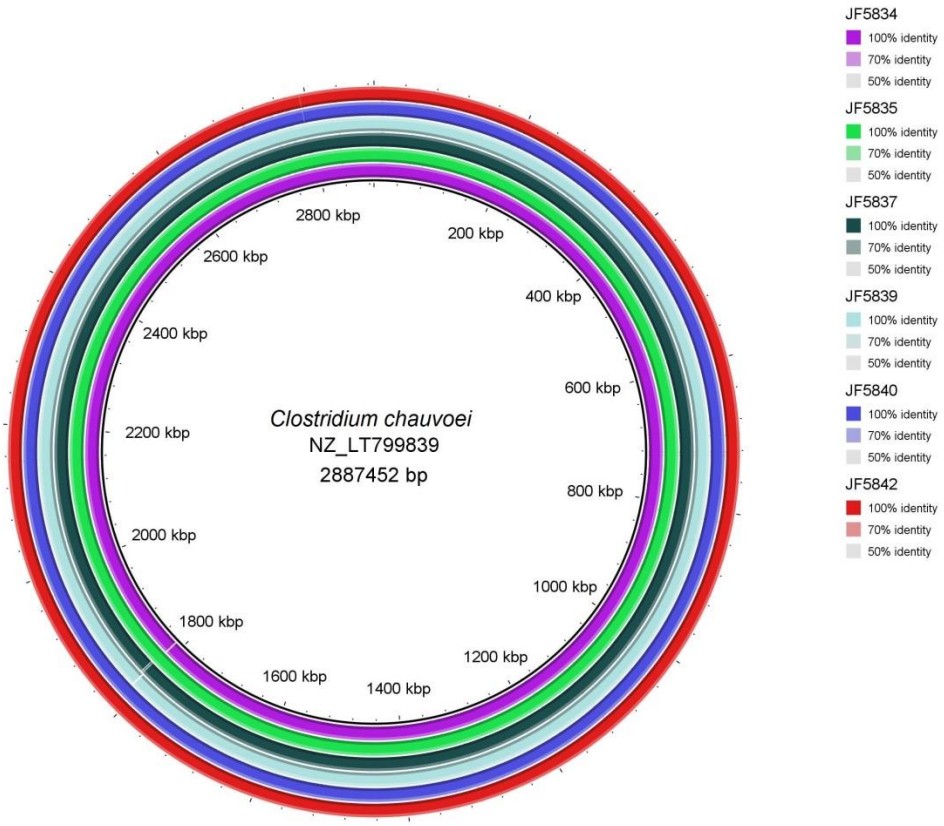

Figure 1. Consensus sequences of six Brazilian strains aligned against the reference sequence JF4335 (Reference number: NZ_LT799839) using BLAST Ring Image Generator.

6 CONSIDERAÇÕES FINAIS

Embora o carbúnculo sintomático seja uma das mais antigas doenças conhecidas que acometem os bovinos, ainda existem lacunas importantes no entendimento dessa doença. Os artigos sobre *C. chauvoei* são relativamente escassos e não havia uma atualização concisa sobre o assunto. Devido à importância do carbúnculo sintomático na produção pecuária e a evolução do conhecimento científico nos últimos anos, mais especificamente nos últimos cinco anos, foi publicado um artigo de revisão com o objetivo de resumir o conhecimento mais expressivo sobre a enfermidade de acordo com os tópicos: etiologia e fatores de virulência, epidemiologia e manifestação clínica e patológica, patogênese, diagnóstico, prevenção e áreas para desenvolvimento de pesquisas futuras.

A partir dos resultados do manuscrito 2 apresentado nesta tese, foi possível concluir que o desempenho idêntico das duas cepas de *C. chauvoei* após o desafio *in vivo* e a incapacidade de infectar os animais vacinados estão correlacionados com a homologia genética das cepas. Embora o insucesso das vacinações contra *C. chauvoei* tenha sido previamente relatado, os resultados deste estudo juntamente com a alta similaridade genética das cepas sugerem que as falhas vacinais não estão relacionadas à variabilidade antigênica, mas sim ao manejo vacinal inadequado.

No terceiro manuscrito, o sequenciamento parcial dos genes avaliados mostrou alta similaridade entre as cepas. Por esse motivo, os antígenos solúveis NanA e CctA são bons candidatos a antígenos vacinais. Além disso, três alelos foram detectados no gene *fliC*. No entanto, estudos adicionais deverão elucidar a sua real contribuição na infecção e na indução de imunidade protetiva. Talvez uma alta concentração dos antígenos solúveis nas vacinas seja suficiente para a adequada indução de imunidade protetiva contra *C. chauvoei*. E a produção desses antígenos seja a principal característica a ser observada na escolha das cepas que deverão compor as vacinas. Ou ainda, em futuras pesquisas poderão ser construídos micro-organismos recombinantes com base nos estudos desenvolvidos por Vilei et al. (2011) e Frey et al. (2012) para a produção em massa de NanA e CctA, respectivamente.

No quarto manuscrito foram comparados seis genomas completos de cepas brasileiras. Apenas uma cepa foi isolada de um bovino que apresentou sinais clínicos na musculatura esquelética e no miocárdio. Uma comparação do genoma desta cepa foi realizada, buscando mutações que pudessem estar associadas à diferença na manifestação clínica. Porém, com base na comparação genômica não foram observadas diferenças, as seis

cepas se mostraram altamente conservadas, tal como foi observado no sequenciamento parcial dos genes e na comparação das cepas utilizadas no desafio vacinal. Ressaltando que *C. chauvoei* é um micro-organismo que no decorrer da sua evolução obteve sucesso em causar uma enfermidade aguda, altamente fatal, limitada a algumas espécies. Estas características, associadas à similaridade genética são um indicativo de que neste momento evolutivo *C. chauvoei* não está sendo desafiado a desenvolver mutações.

Uma das seis cepas avaliadas no quarto manuscrito também foi incluída no artigo de Rychener et al. (2017), no qual participamos como colaboradores. Este artigo apresentou os primeiros resultados de sequenciamento do genoma completo de *C. chauvoei* e comparou cepas de 4 continentes isoladas de 1951 a 2015. O estudo revelou que o genoma da espécie *C. chauvoei* é altamente homogêneo e que a similaridade entre as cepas é alta.

Dessa forma, foi possível reunir nesta tese e consolidar por meio de artigos científicos o conhecimento acerca de *C. chauvoei* que durante anos foi construído pelo grupo de pesquisa do Laboratório de Bacteriologia do Departamento de Medicina Veterinária Preventiva da Universidade Federal de Santa Maria.

7 CONCLUSÕES

- Com base nos estudos apresentados nesta tese conclui-se que as falhas vacinais possivelmente decorrem de erros de manejo, visto que a similaridade genética das cepas sugere que não ocorrem variações antigênicas.
- A partir do que foi postulado por estudos anteriores e pela conservação dos genes de virulência da neuraminidade (*nanA*) e da toxina A de *C. chauvoei* (*cctA*) observada no sequenciamento parcial é sugerido que a adição destas toxinas às vacinas possa beneficiar a indução de imunidade protetiva.
- O sequenciamento do genoma de *Clostridium chauvoei* isolados de casos de carbúnculo sintomático no Brasil revelou alta similaridade.
- Ainda existem lacunas na compreensão da enfermidade como, por exemplo, por que os esporos resistem aos mecanismos microbicidas de macrófagos bovinos e haveria alguma forma de induzir imunidade protetiva contra os esporos?
- Além disso, por que em alguns casos ocorre manifestação clínica visceral visto que no sequenciamento do genoma não foram observadas mutações que pudessem estar associadas. Pode haver relação com variações no indivíduo hospedeiro?

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