UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

Cláudia Balzan

Campylobacter fetus: REVISÃO SISTEMÁTICA, DESENVOLVIMENTO E CARACTERIZAÇÃO ANTIGÊNICA DA PROTEÍNA SapA MUTANTE COM POTENCIAL IMUNOBIÓLOGICO

Santa Maria, RS
2019

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

Orientador: Profª. Drª. Agueda Castagna de Vargas

Santa Maria, RS
2019

Balzan, Cláudia
Campylobacter fetus: revisão sistemática, desenvolvimento e caracterização antigênica da proteína SapA mutante com potencial imunobiológico / Cláudia Balzan.- 2019.
86 p.; 30 cm

Orientador: Agueda Castagna de Vargas
Tese (doutorado) - Universidade Federal de Santa Maria, Centro de Ciências Rurais, Programa de Pós Graduação em Medicina Veterinária, RS, 2019

1. Campilobacteriose genital bovina 2. doenças venéreas 3. proteínas recombinantes 4. proteínas de superfície 5. fatores de virulência I. de Vargas, Agueda Castagna II. Título.

Sistema de geração automática de ficha catalográfica da UFSM. Dados fornecidos pelo autor(a). Sob supervisão da Direção da Divisão de Processos Técnicos da Biblioteca Central. Bibliotecária responsável Paula Schoenfeldt Patta CRB 10/1728.

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Aprovado em 08 de março de 2019:

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Santa Maria, RS
2019

AGRADECIMENTOS

À Universidade Federal de Santa Maria, pelo ensino disponibilizado e pelas oportunidades de aprendizado e convívio com professores excelentes.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROEX) pelo apoio financeiro no Brasil e no período de estágio sanduíche.

À professora e orientadora Agueda Castagna de Vargas, pelos momentos de aprendizado, de diversão e pela influência na minha formação.

À toda a minha família, principalmente meus pais, Daniel e Ivanete, por nunca medirem esforços para dar educação às filhas, sempre com muito amor, dedicação, incentivo e paciência. À minha irmã Caroline pela cumplicidade e amizade.

Aos pós-graduandos Gustavo Moreira, João Antônio Guizzo, Julia Pires Espindola e Bibiana Barasuol, bem como às professoras Drª Letícia Gressler e Drª Juliana Felipetto Cargnelutti pelo imenso auxílio durante a realização dos experimentos.

À equipe do Laboratório de Bacteriologia, tanto aos que já passaram pelo LABAC quanto os mais novos, por toda a paciência e apoio em toda a trajetória, bem como os vários momentos de aprendizados e diversão.

À equipe do Laboratório de Imunologia Aplicada e professor Fabricio Rochedo Conceição pelo enorme conhecimento adquirido, pelo auxílio nas análises e nos experimentos.

À equipe do Laboratório de Microbiologia e Imunologia Avançada e professor Rafael Frandoloso pela disponibilidade de estrutura para os testes laboratoriais e para a realização de experimentos.

Ao Anderson Luiz Zwirtes pelo incentivo, pelo carinho, pela paciência e pelo auxílio na finalização desta tese.

Às minhas amigas Gabriela, Giovana, Paula e Rosangela pelos momentos de diversão e por estarem ao meu lado neste período.

À todas as pessoas que de alguma forma ou de outra contribuíram para esta trajetória.

À todos o meu agradecimento.

RESUMO

Campylobacter fetus: REVISÃO SISTEMÁTICA, DESENVOLVIMENTO E CARACTERIZAÇÃO ANTIGÊNICA DA PROTEÍNA SapA MUTANTE COM POTENCIAL IMUNOBIÓLOGICO

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ORIENTADORA: Agueda Castagna de Vargas

A campilobacteriose genital bovina (CGB) é uma importante enfermidade reprodutiva de caráter venéreo, causada pela bactéria *Campylobacter fetus* subsp. *venerealis* e que acarreta perdas econômicas mundialmente significativas. Os principais fatores de virulência deste agente são relacionados com a adesão, motilidade, proteínas de superfície, produção de toxinas e sistemas de secreção e regulação. Esse patógeno possui proteínas de superfície (SapA), as quais são consideradas importantes na patogenia da CGB por apresentar variação antigênica e são responsáveis pela persistência da infecção no trato genital bovino. A colheita de amostras e o diagnóstico são bastante laborioso, sendo as amostras preconizadas para o diagnóstico são muco vaginal, esmegma prepucial e sêmen, placenta e fetos abortados. A prevenção e o controle desta enfermidade baseiam-se na vacinação, no uso de touros negativos para a enfermidade e implementação de programas de inseminação artificial. Com o intuito de simplificar as análises laboratoriais, este estudo objetivou a padronização dos processos de produção de uma proteína quimérica recombinante de *C. fetus* e avaliação de seu potencial como ferramenta para o diagnóstico e prevenção da CGB. Utilizando as sequências dos nove genes homólogos de *sap*A de *C. fetus* publicamente disponíveis, foram determinadas duas regiões para a contrução de um gene sintético, denominado *sapAN78*, com a possibilidade da clonagem e expressão do gene total e também das duas subunidades. O fragmento total e as subunidades foram clonados no plasmídeo pAE, expressos em *Escherichia coli* BL21(DE3) pLySs e purificados por cromatografia de afinidade ao níquel. A quimera recombinante de aproximadamente 60 kDa e as subunidades foram obtidas em quantidades significativas como corpos de inclusão, solubilizadas com ureia e detectadas por *Western blot* com anticorpo monoclonal anti-polihistidina e por anticorpos presentes no soro de bovinos positivos para CGB. A rSapAn78 foi utilizada para hiperimunização de coelhos, apresentando soroconversão desde a primeira imunização e os anticorpos produzidos ao final do protocolo de hiperimunização demonstraram-se ávidos ao ensaio com tiocianato de amônio. Estes anticorpos também reconheceram a rSapAn78 em testes de *Dot blot*, bem como a rSapAn78 e as proteínas nativas em cepas de *C. fetus* por *Western blot* e ELISA. Desta forma, demonstrou-se a imunogenicidade e antigenicidade da proteína quimérica contruída e seu potencial para aplicações em pesquisas futuras para o diagnóstico e prevenção da CGB.

Palavras-chave: *sap*A, proteína recombinante, imunogenicidade, antigenicidade.

ABSTRACT

Campylobacter fetus: SYSTEMATIC REVIEW, DEVELOPMENT AND ANTIGENIC CHARACTERIZATION OF SapA MUTANT PROTEIN WITH IMMUNOBIOLOGICAL POTENTIAL

AUTHOR: Cláudia Balzan
ADVISOR: Agueda Castagna de Vargas

Bovine genital campylobacteriosis (BGC) is an important reproductive disease of the venereal nature, caused by the bacterium Campylobacter fetus subsp. venerealis and entails worldwide economic losses. The main virulence factors of this agent are related to adhesion, motility, surface proteins, production of toxins and secretion and regulation systems. This pathogen has surface proteins (SapA), which are considered important in the pathogenesis of CGB due to antigenic variation and are responsible for the persistence of infection in the bovine genital tract. Sampling and diagnosis are laborious, and the recommended samples for diagnosis are muco vaginal, preputial smegma and semen, placenta and aborted fetuses. The prevention and control of this disease are based on vaccination, the use of bulls negative for the disease and the implementation of artificial insemination programs. In order to simplify the laboratory procedures, this study aimed to standardize the production of a chimeric protein of *C. fetus* and evaluate its potential as a tool for the diagnosis and prevention of BGC. Using nine *sap*A sequences of *C. fetus* gene publicly available, two regions were determined for the construction of a synthetic gene, called *sapAN78*, with the possibility of cloning and expression of the whole gene and also of the two subunits. The whole fragment and subunits were cloned into plasmid pAE, expressed by Escherichia coli BL21 (DE3) pLySs and purified by nickel affinity chromatography. Recombinant chimera of approximately 60 kDa and subunits were obtained in significant amounts as inclusion bodies, solubilized with urea and detected by Western blot with anti-polyhistidine monoclonal antibody and by antibodies present in BGC positive bovine serum. rSapAn78 was used for rabbit hyperimmunization, presenting seroconversion from the first immunization and the antibodies produced at the end of the hyperimmunization protocol were avid by the ammonium thiocyanate assay. These antibodies also recognized the rSapAn78 in Dot blot as well as rSapAn78 and native proteins in *C. fetus* strains by Western blot and ELISA. In this way, the immunogenicity and antigenicity of the constructed chimeric protein and its potential for applications in future research for the diagnosis and prevention of BGC were demonstrated.

Keywords: *sap*A, recombinant protein, immunogenicity, antigenicity.

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Manuscrito 1

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1 INTRODUÇÃO

O Brasil possui o maior rebanho comercial de bovinos a nível mundial com 221 milhões de cabeças (ABIEC, 2018) e produziu 35,1 bilhões de litros de leite em 2017 (ROCHA & CARVALHO, 2018). Apesar dos números positivos, elevados índices produtivos nem sempre são alcançados tendo em vista falhas nutricionais, de manejo e reprodutivas. Segundo Bellows, Ott e Bellows (2002), as doenças reprodutivas resultam em perdas econômicas na criação de bovinos, principalmente causadas pela diminuição da produção e retardo na reprodução. Dentre as enfermidades reprodutivas está a campilobacteriose genital bovina (CGB), a qual é responsável por perdas econômicas com descarte e necessidade de reposição de animais subférteis (fêmeas repetidoras de cio e touros contaminados), custo de sêmen, queda na produção de bezerros e maior intervalo entre partos (BELLOWS, OTT & BELLOWS, 2002; BONDURANT, 2005). A enfermidade é de distribuição mundial, com incidência elevada nos países em desenvolvimento, onde a reprodução dos animais por monta natural é amplamente praticada (MSHELIA et al., 2010).

Em rebanhos de corte, quando não há controle dos índices zootécnicos, as perdas reprodutivas com a CGB podem ficar despercebidas inicialmente (BONDURANT, 2005). Já em bovinos de leite, Roberts (1986 apud AKHTAR et al., 1993) afirma que a primeira indicação de doenças venéreas no rebanho é o aumento de intervalos entre partos-concepção ou do repasse com touro até a prenhez, além da produção de leite reduzir 7% em vacas positivas para *C. fetus* quando comparadas com vacas negativas (AKHTAR et al., 1993).

A CGB é uma doença infecciosa de transmissão sexual, causada pela bactéria *Campylobacter fetus* subespécie *venerealis*, apresenta distribuição mundial e pertence à lista de doenças de notificação obrigatória ao comércio internacional de animais ou produtos de origem animal da Organização Mundial da Saúde Animal (OIE, 2015).

Conforme a OIE, as técnicas para o diagnóstico de *C. fetus*, são o seu isolamento em meio de cultura e identificação por testes bioquímicos; imunofluorescência; ensaios imunoenzimáticos e identificação molecular (OIE, 2018). As amostras preconizadas para estes diagnósticos são muco vaginal, esmegma prepucial e sêmen, placenta e fetos abortados (OIE, 2008). Devido às dificuldades na coleta desse material e no armazenamento correto, assim como a baixa

disponibilidade de laboratórios capazes de realizar o diagnóstico recomendado para *C. fetus*, poucas amostras são encaminhadas para diagnóstico e a prevalência da CGB é subestimada (HUM et al., 1997; BONDURANT, 2005; VAN BERGEN et al. 2005ª; ALVES et al., 2011). As amostras devem chegar ao laboratório para processamento no mesmo dia da colheita, entretanto essa eficiência no transporte das amostras é um desafio que possuímos no Brasil visto que algumas fazendas são distantes dos laboratórios. Além disso, o diagnóstico convencional da CGB normalmente é laborioso e demorado e a ineficiência do transporte das amostras pode inviabilizar o isolamento de *C. fetus*.

Uma particularidade da CGB é a persistência de *C. fetus* no aparelho genital de bovinos infectados por longos períodos e esta é associada às falhas nos mecanismos de defesa e estas se devem principalmente pela variação nas proteínas de superfície do micro-organismo (CORBEIL et al., 1975; FAGAN & FAIRWEATHER, 2014). Essas proteínas de superfície (Surface Array Proteins - Sap) compõem externamente o envelope celular do *C. fetus* com importante papel na patogênese da CGB (MCCOY et al., 1975; WINTER & CAVENEY, 1978; FAGAN & FAIRWEATHER, 2014).

Devido às dificuldades envolvidas no diagnóstico convencional da CGB, métodos alternativos para diagnóstico, baseados em amostras de mais fácil colheita, como amostras de soro, vêm sendo estudados nos últimos anos. ZHAO e colaboradores (2010) desenvolveram um ensaio imunoenzimático indireto com proteína recombinante, obtendo bons resultados. Esta técnica é simples, de rápida execução e de baixo custo, bem como pode ser utilizada em larga escala (CROWTHER, 2001).

A disponibilidade de métodos sorológicos para detecção da CGB pode facilitar o diagnóstico convencional da enfermidade e com base no conhecimento das características da proteína SapA, o presente estudo objetiva a produção, caracterização e verificação do potencial de uma nova proteína recombinante tendo em vista o desenvolvimento de teste diagnósticos e a prevenção da CGB.

A presente tese é composta por um manuscrito de revisão, intitulado “Bovine genital campylobacteriosis: main features and perspectives to diagnosis and control”, e outro manuscrito intitulado “Development and characterizaton of a recombinant protein with potential for Bovine Genital Campylobacteriosis diagnostic and prevention”.

2 MANUSCRITO 1

Bovine genital campylobacteriosis: main features and perspectives to diagnosis and control

Campilobacteriose genital bovina: principais características e perspectivas para o diagnóstico e controle

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(Artigo de revisão submetido para o periódico Ciência Rural)

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Bovine Genital Campylobacteriosis: epidemiology, diagnosis and control

Campilobacteriose Genital Bovina: epidemiologia, diagnóstico e controle

Cláudia Balzan[I*] Agueda Palmira Castagna de Vargas[I]

-REVISÃO BIBLIOGRÁFICA-

ABSTRACT

Bovine genital campylobacteriosis (BGC) is a venereal disease caused by ***Campylobacter fetus*** subsp. ***venerealis***. In countries with large cattle herds, such as Brazil, where the use of natural breeding as a reproductive strategy is a common practice, BGC is considered an important cause of reproductive failures and economic losses. In these cases, the bull is the asymptomatic carrier of the bacterium and the infected females can present infertility and even abortions. The techniques for the diagnosis of ***C. fetus*** are isolation in culture medium and identification; immunofluorescence; immunoenzymatic assays and molecular identification. Disease control is based on vaccination. In this sense, this review consists of an approach on epidemiology, etiology, pathogenesis, advances in the diagnosis and control of BGC.

Key words: ***Campylobacter fetus*** subsp. ***venerealis***, infertility, venereal disease, beef cattle.

RESUMO

A campilobacteriose genital bovina (CGB) é uma importante enfermidade de caráter venéreo, causada por ***Campylobacter fetus*** subsp. ***venerealis***. Em países com grandes rebanhos bovinos, como o Brasil, onde o uso da monta natural como estratégia reprodutiva é uma prática corrente, a CGB é considerada uma importante causa de falhas reprodutivas e perdas econômicas. Nestes casos, o touro é o portador assintomático da bactéria e as fêmeas infectadas podem apresentar infertilidade e até mesmo abortos. As técnicas para o diagnóstico de ***C. fetus***

são o isolamento em meio de cultura e identificação; imunofluorescência; ensaios imunoenzimáticos e identificação molecular. O controle da doença é baseado em vacinação. Neste sentido, esta revisão consiste em uma abordagem sobre a epidemiologia, a etiologia, a patogenia, os avanços no diagnóstico e controle da CGB.

Palavras-chave: ***Campylobacter fetus*** subsp. ***venerealis***, infertilidade, bovinos de corte, doenças venéreas.

INTRODUCTION

Bovine Genital Campylobacteriosis is caused by ***Campylobacter fetus*** subsp. ***venerealis,*** a gram negative and microaerophilic rod. BGC is a venereal disease with worldwide distribution and high incidence in developing countries where natural breeding is widely used for bovine reproduction (MSHELIA et al., 2010), such is in Brazil. BGC as firstly diagnosed in Brazil in 1955 from an aborted fetus (RAMOS et al., 1983) and remains without effective prevention measures so far. The disease also belongs to the list of notifiable diseases to international trade in animals or animal products of the World Organization for Animal Health (OIE, 2017). Published reviews highlited the prevalence, the epidemiology, the diagnosis of BGC and/or the control of BGC (ALVES et al., 2011; BONDURANT, 2005; CORBEIL et al., 2003; HOFFER, 1981; MICHI et al., 2016; MSHELIA et al., 2007; MURRAY, 2007; SILVEIRA et al., 2018). Therefore, facing the woldwide impact of BGC this review article will discuss the distribution, etiology, virulence factors of ***C. fetus***, epidemiology, pathogenesis, the advances in diagnosis and control of BGC emphatizing its occurrence in Brazil.

Epidemiology and distribution

Recent data from the OIE (2019) show the presence of BGC in Argentina, Brazil, Colombia, Uruguay, Australia, New Zealand, Ireland, France, South Africa, Iran and Nigeria between January and June 2018 (Figure 1). In the latter, approximately 520,000 cattle are

affected by BGC with a direct loss of 8.5 million dollars due abortions and low fertility rates (MSHELIA et al., 2012).

In Brazil, BGC is among the most important cause of reproductive failures in beef and dairy farms who use natural breeding (VARGAS et al., 2002; LEAL et al., 2012; MIRANDA, 2005; OLIVEIRA et al., 2015; STYNEN et al., 2003, ZIECH et al., 2014; Figure 2). However, BGC incidence remains understimated due to the absence of systematic diagnosis, which is associated to logistic issues, such as the samples collection and shipment as well as the reduced number of qualified laboratories to perform the diagnosis (ALVES et al., 2011).

The main risk factor for the high spread of BGC is the natural breeding (MSHELIA et al., 2012), especially when bulls older than 4 years and without sanitary control for BGC are employed (HOFFER, 1981; BONDURANT, 2005). Even in farms using artificial insemination (AI), the use of bulls after the AI is very common practice (STYNEN et al., 2003). Other risk factor is the use of semen without quality control (BONDURANT, 2005).

In farm which use extensive production of beef catle, the presence of bulls throughout the year contribute for easyly spread the BGC by the use of natural breeding (MIRANDA, 2005). In dairy cattle the use of AI normally is the breeding method, however many properties use bulls after AI. Bondurant (2005) sustains that the first indication in these cases of venereal disease in the herd is the increase in intervals between calving-conception or the increase in the interval between the coitus until pregnancy.

In clinical history are observed herds with low conception rates and in the recently exposed to the BGC the rates can be 50% lower than the expected rate (DEDIE et al., 1982 apud BONDURANT, 2005). The main groups at risk are newly introduced heifers and cows in the herd, in which clinical signs are most pronounced due to low levels of immunity (HOFFER, 1981).

Etiology

Micro-organisms of the genus ***Campylobacter*** are gram-negative curved rods with 0.2 to 0.5 μm diameter, mobile with polar flagella, oxidase positive and with variable catalase reactions (VAN BERGEN et al., 2005; QUINN et al., 2011).

Microaerophilic bacteria compose the genus ***Campylobacter***, which generally require oxygen concentrations between 3 and 15% and carbon dioxide concentrations of 3 to 5%. Only a few species are aerotolerant and can grow in the presence of oxygen (HOLT et al., 1994). ***C. fetus*** is studied as a pathogen in cattle and sheep and is subdivided into two subspecies, ***C. fetus*** subsp. ***fetus*** and ***C. fetus*** subsp. ***venerealis***, by the habitat characteristics, transmission, clinical manifestation and laboratory phenotypic identification (VÉRON & CHATELAIN, 1973). These subspecies are phenotypically differentiated, according HOLT et al. (1994), with tests of tolerance to 1% glycine and production of hydrogen sulphide (H_2S), with ***C. fetus*** subsp. ***fetus*** as positive in both assays and ***C. fetus*** subsp. ***venerealis*** as negative. ***C. fetus*** subsp. ***venerealis*** still presents a biovar, classified by biochemical characteristics, called ***C. fetus*** subsp. ***venerealis*** biovar intermedius (VÉRON & CHATELAIN, 1973).

C. fetus subsp. ***fetus*** is commensal from gastrointestinal tract that cause abortion agent in sheep and cattle occasionally (FLORENT, 1959 apud SKIRROW, 1994), while ***C. fetus*** subsp. ***venerealis*** is found exclusively in the genital tract of cattle resulting in infertility and abortions, being transmitted exclusively by the venereal route (HUM et al., 1994).

Genes and virulence factors

The genomic sequencing of ***C. fetus*** is recent. In 2006, the first complete and closed genome of ***C. fetus*** was made available by The Institut for Genomic Research (TIGR, USA, 2006), the 82-40 strain isolated from human in the United States and later identified as ***C. fetus*** subsp. ***fetus***, with a 1.77 Mpb genome and approximately 1,820 predicted genes. Already the first sequenced genome of ***C. fetus*** subsp. ***venerealis*** was published by MOOLHUIJZEN et al.

(2009) as an unmounted genome and consisting of multiple contigs of the Azul-94 strain, that was isolated from a bovine aborted fetus in Argentina.

Since then, it has been observed the expansion of genomic studies in ***C. fetus***. This can be verified in queries databases of DNA sequences, such as GenBank, where currently there are 74 genomes of ***C. fetus*** published, being 11 complete genomes (GENBANK, 2019). Current research on species of ***Campylobacter*** concentrates on the total genomic analysis for the identification of virulence genes and characteristics that contribute to the pathogenicity differences of the subspecies (FOUTS et al., 2005; MOOLHUIJZEN et al., 2009; VAN DER GRAAF-VAN BLOOIS et al., 2016). In the ***C. fetus*** subespecies these virulence factors were clearly identified as classes of genes encoding proteins (MOOLHUIJZEN et al., 2009), such as surface proteins, involved in bacterial adherence, motility, toxin production and resistance, regulatory and secretion systems.

Linkage between bacterial pathogens and epithelial cells is a prerequisite for invasion of host cells and subsequent translocation to the deeper layers of the mucosa. The spiral form of the cell and the corkscrew motility conferred by the flagella of ***C. fetus*** are required to colonize and cross the mucus barrier that covers the vaginal epithelium (SPRENGER et al., 2012). In addition, the flagellum is an important adhesin of ***C. jejuni*** and may have a similar function in ***C. fetus***. The genomes of the two subspecies of ***C. fetus*** harbor homologs of adhesin PEB1, which is an outer membrane protein (OMP) and important in adherence to epithelial cells (SPRENGER et al., 2012). Therefore, the motility and adhesion capacity of host cells play a fundamental role in the diseases caused by bacteria of the genus ***Campylobacter***.

A genomic island encoding a type IV secretion system integrate the genome of ***C. fetus*** subsp. ***venerealis*** was identified and it was believed that this genomic island was determinant for the tropism of this subspecies to the bovine genital tract (GORKIEWICZ et al., 2010). However, phylogenetic analyzes between genomes of strains of the two subspecies of ***C. fetus***

that this coding region of the type IV secretion system exists in both subspecies of ***C. fetus*** an element acquired from different donors and contains *fic* (n= 4) e *virD4* (n= 10) genes (VAN DER GRAAF-VAN BLOOIS et al., 2016). In other research, SPRENGER et al. (2017) have proved that *fic* genes predominantly and strongly conserved in ***C. fetus*** subsp. ***venerealis*** and Fic proteins are related to the change from the normal to the static metabolic state, which assists in the maintenance of the bacterium for long periods in the host, even under stress conditions. ABRIL et al. (2007) reported the presence of a highly conserved original insertion sequence (ISCef1) in ***C. fetus*** subsp. ***venerealis*** and related to glycine tolerance. This result confirms the potential of the 1% glycine tolerance test in the phenotypic differentiation of ***C. fetus*** subspecies, besides contributing to the understanding of evolution and pathogenesis.

Cytolethal distending toxin (CDT) is produced by several bacteria, such as ***Escherichia coli*** and ***Shigella*** spp. Between ***Campylobacter*** strains the cluster of ***cdt*** gene was distributed universally and is well conserved, particularly in ***C. jejuni***, ***C. coli*** and ***C. fetus*** (ASAKURA et al. , 2007). In ***C. jejuni***, the CDT is recognized for causing damage on the DNA of the host and tissue necrosis (JOENS et al., 2011), presenting as a potential virulence factor also in ***C. fetus***.

The lipopolysaccharide (LPS) of ***C. fetus*** is typical of gram negative bacteria. It has the lipid fraction, denominated lipid A, and the polysaccharide fraction, with the central oligosaccharide and an antigen "O" (MORAN et al., 1996). In ***C. fetus***, lipid A has low biological activity compared to Enterobacteriaceae family bacteria and this is associated with the selection of micro-organisms with LPS of low biological activity and it can then persistently colonize the host (BLASER et al., 2008). The variation in the O antigen of LPS is the basis of the serotyping scheme of ***C. fetus*** and two main serotypes, denominated A and B, are recognized (VAN BERGEN et al., 2005; DWORKIN et al., 1995; PEREZ-PEREZ et al., 1986; TU et al., 2004) ***C. fetus*** subsp. ***fetus*** contains serotypes A and B and ***C. fetus*** subsp. ***venerealis*** has only the serotype A.

C. fetus has a structure of protein origin covering the outer membrane. MCCOY et al. (1975) were the first to describe the presence of a microcapsule called the S layer, formed by protein subunit arrays known as surface array proteins (SAPs) and recognized as important in the pathogenesis of BGC (MCCOY et al., 1975; FAGAN & FAIRWEATHER, 2014; WINTER et al., 1978). Because of this, they are potential candidates for the development of serological diagnostic methods and, according to PEI et al. (1988), for use in vaccines.

Surface array proteins

Surface proteins (S-layer) are some of the most abundant proteins in the bacterial cell (SLEYTR & BEVERIDGE, 1999) and externally form the cell envelope (VAN BERGEN, 2005). These are protein subunits, in regular arrangements that cover the LPS of the bacterium, making it inaccessible to host defenses (GARCIA et al., 1995). The Sap subunits are secreted in the absence of an N-terminal signal sequence and bind specifically to type A or B of the LPS of ***C. fetus*** (YANG et al., 1992; VAN BERGEN, 2005), being also a form of classification of the species.

The surface proteins of ***C. fetus***, also known as S-layer, are responsible for the resistance of this micro-organism to the bactericidal and phagocytic activity of the host (BLASER, 1993; MCCOY et al. 1975; RAY et al., 2000) and its superficial location suggests that they may be an important mediator in the interaction with the host (BLASER et al., 1990) and persistence of the pathogen in the reproductive tract (CORBEIL et al., 1975). Some bacterial species present genetic variation in Sap expression and the best example of this situation is ***C. fetus***, in which the S-layer variation is well characterized (FAGAN & FAIRWEATHER, 2014).

A particular aspect of the surface proteins in ***C. fetus*** is the fact that a single bacterium can produce up to three proteins, with molecular mass ranging from 97 to 149 kDa, being usually one of these dominant (DUBREUIL et al. , 1990; DWORKIN et al., 1995a; DWORKIN et al., 1995b; FUJIMOTO, 1991; PEI et al., 1988). The predicted proteins demonstrate high

levels of amino acid identity in the aminoterminal region amd present variation in the carboxyterminal domain, with the binding to LPS occurs in the conserved region (SPRENGER et al., 2017).

In recente studies (TU et al., 2003) determined the existence of eight complete homologous copies of the *sapA* gene and one partial homologous copy on the bacterial chromosome. The expression of the *sap* homologues occurs from a single promoter (TUMMURU & BLASER, 1992) and this promoter has reduced activity at 32°C when compared to 37°C, suggesting that Sap expression is more efficient at host temperature conditions (KIENESBERGER et al., 2010; SPRENGER et al., 2012).

During infection extensive chromosomal rearrangements of high frequency occur in DNA, involving inversion and recombination for the phenotypic change and expression of alternating *sap* homologues on the surface of the cell, which results in an antigenically distinct S-layer (BLASER, 1994; VARGAS et al., 2002; TU et al., 2004; TUMMURU & BLASER, 1993).

This is due to the modification in dominant epitopes during persistence of the bacterium in the genital tract (GARCIA et al., 1995; WANG et al., 1993). VARGAS et al. (2002) found variations in electrophoretic patterns of Sap after different samplings in ***C. fetus*** subsp. ***venerealis*** isolates from persistently infected cattle, however no changes were observed in the profile of surface proteins during in vitro multiple passages (VARGAS, unpublished data). Thus, is showed the importance of host and etiologic agent interaction for variation in surface proteins for ***C. fetus*** subsp. ***venerealis*** and consequently for the maintenance of the BGC.

Pathogenesis

The infection by ***C. fetus*** subsp. ***venerealis*** in bulls is not associated with clinical signs, changes in semen quality or genital abnormalities (BIER et al., 1977a) and the micro-organism is housed in the epithelial crypts of the prepuce. Transmission to the females occurs during

natural mating with infected bull or artificial insemination with contaminated semen, when the bacterium is distributed through the mucosa of the vagina, cervix, uterus and uterine horns (BONDURANT, 2005). About one-third of infected cows become carriers (QUINN et al., 2011) and other males may be contaminated by mating in sick or carrier cows (HOFFER, 1981).

C. fetus subsp. ***venerealis*** persistently colonizes the preputial cavity of the bull, preventing it from elaborating an effective immune response and thus this remains as asymptomatic carrier (VAN BERGEN, 2005). Bulls older than 4 years are more likely to be carriers because they have deeper crypts where micro-organisms find favorable conditions and harbor (SAMUELSON & WINTER, 1966). Infection can persist for months to years in old bulls (BIER et al., 1977a).

After coitus, an ascending infection is established in females and in 12 to 14 days the micro-organism is distributed from the vagina/cervix to the uterine horns (BONDURANT, 2005; CORBEIL, 1999; YAEGER & HOLLERY, 2007). Then it occurs the development of endometritis and salpingitis during the progestational phase of the estrous cycle, when both the amount and the activity of neutrophils decline (VAN BERGEN, 2005; QUINN et al., 2011). ***C. fetus*** subsp. ***venerealis*** does not interfere with the process of fertilization and initial embryonic development, however, endometritis prevents implantation of the embryo (HOFFER, 1981), followed by early embryonic death. After the uterine invasion, in a period of 3 to 6 months, the cow can remain infertile and source of infection (VAN BERGEN, 2005) and subsequently, acquired immunity is developed.

The predominant immunity in ***C. fetus*** infected animals is local (CORBEIL et al., 1974b), being reported differences between males and females in the immunological studies performed. In females, forty to sixty days after infection, the production of antibodies in the vagina and uterus begins (CORBEIL et al., 1974). In preputial secretions of healthy bulls predominates IgG followed by IgA (BIER et al., 1977b) and in bulls infected with ***C. fetus***

subsp. ***venerealis*** the serum antibody response was undetectable (VASQUEZ et al., 1983). The infiltration of lymphocytes and subepithelial plasma cells is similar in the prepuce and penis of infected and uninfected bulls (BIER et al., 1977b; SAMUELSON & WINTER, 1966). In the vagina IgA predominates, which may limit the spread of the infection; already in the uterus are produced IgGs and these opsonize the pathogenic organisms, facilitating phagocytosis by neutrophils and mononuclear cells (CORBEIL, 1999; Table 1). As BGC is caused by an extracellular pathogen it would be expected to find predominantly a humoral immune response from the host (CORBEIL et al., 1974), which would most likely be the cause of the short duration of immunity to the agent (BONDURANT, 2005).

The presence of carrier cows is due to the evasion of the local immune response, which CORBEIL (1999) attributes to factors such as the relative lack of spontaneous IgG response in the vagina, possible blockage of vaginal IgG effects by IgA binding in micro-organisms and by the variation of the surface antigens of the micro-organism against the local immune response. In bulls, infections are persistent and this is an asymptomatic carrier, indicating that immunity during inflammation does not eliminate mucosal micro-organisms (COBO et al., 2011). Still, according to TIZARD (2015), ***C. fetus*** infections are associated with the presence of several mononuclear cells, as well as late cutaneous reactions (type IV hypersensitivity), so that cell-mediated immunity also participates in resistance to infection. Once the prolonged vaginal carrier status has been determined for ***C. fetus*** subsp. ***venerealis***, the cow remains with the vaginal infection, but the uterus is free of infection (BONDURANT, 2005). This allows the return of the cow fertility in many cases, and may last up to 2 years in the absence of antigenic stimulation largest (CORBEIL et al., 1974). In bulls, persistence of the bacteria in the lower genital tract is also attributed to the evasion of the immune response (VARGAS et al., 2002; CORBEIL, 1975).

Diagnosis of BGC

The subspecies of ***C. fetus*** show different adaptations to the host tissue, however, at the genetic level they are practically indistinguishable (MOOLHUIJZEN et al., 2009). One way to differentiate subspecies is to use 16S rRNA genes, since they are sensitive to small mutations, remain pockets for variation, and are useful evolutionary regulators to estimate the relationships between organisms (HANSSON et al., 2008; RASKIN et al., 2006). Sequence alignment revealed that the genotypes of ***C. fetus*** subspecies are highly synthetic, with 92.9% average identity (KIENESBERGER et al., 2014).

The materials for laboratory diagnosis of BGC from suspect animals are samples of prepucial smegma and semen in bulls and female specimens of cervicovaginal mucus (OIE, 2017). In addition, samples of aborted fetuses and placenta can also be collected. The diagnosis of BGC is at the herd level, so it is not considered isolated cases but an epidemiological profile of the herd based in the history of clinical manifestations compatible with this disease and analysis of reproductive rates, to be confirmed with laboratory assays.

In females, cervico-vaginal mucus can be collected with an insemination pipette or absorbent pad (OIE, 2017; STYNEN et al., 2003). Preputial samples from bulls could be obtained by scraping or washing with sterile saline (OIE, 2017). An important aspect to be observed is the maintenance of bulls in sexual rest for 7 to 15 days before harvesting the material and that three collections, with the same intervals and still with the animal in sexual rest, should be performed to increase the sensitivity of the diagnosis (SKIRROW & BONDURANT, 1988). Also, the OIE (2017) indicates the use of transport media to maintain viability in samples that will not be processed in the laboratory on the same day of collection. Enriched and selective transport media (Lander, Foley and Clark, for example) are suitable for transporting the samples and when these media are not available the collected material should be placed in a sterile recipient (temperatures 4-10°C) and protected from light.

The techniques for the diagnosis of ***C. fetus*** are the isolation and identification of the agent, immunofluorescence, immunoenzymatic tests and molecular identification (OIE, 2017). Isolation and identification of ***C. fetus***

The isolation and identification of ***C. fetus*** by microbiological culture is considered the standard and confirmatory test for the diagnosis of infection (BROOKS et al., 2004). However, the same depends on the quality of the sample, the way it is sent to the laboratory and the viability of the micro-organism. ***Campylobacter*** species are microaerophilic and ***C. fetus*** grows fastidiously, requiring strict atmospheric conditions for cultivation as well as selective and enrichment media (QUINN et al., 2011).

The discrimination of isolates in subspecies is based on colonial morphology, certain biochemical properties and antimicrobial susceptibility (QUINN et al., 2011). The gram staining, catalase test and oxidase activity are the tests most frequently used in diagnostic aboratories, followed by the motility test (VAN BERGEN et al., 2005). In addition, the differentiation between ***C. fetus*** subspecies is carried out mainly by the antimicrobial susceptibility test as cefalotin and nalidixic acid (HOLT et al., 1994) and by the evaluation of biochemical characteristics. Also, the growth at 25°C and 42°C and in the presence of 3.5% sodium chloride, H_2S production and the tolerance to 1% glycine (HOLT et al., 1994), the latter being the standard test for subspecies differentiation (VAN BERGEN et al., 2005).

However, several researchers report doubts about the differentiation by the phenotypic tests mentioned above (VAN DER GRAAF-VAN BLOOIS et al., 2014). First, ***C. fetus*** subsp. ***venerealis*** biovar intermedius is able to produce H_2S (characteristic previously attributed only to ***C. fetus*** subsp. ***fetus***). CHANG & OGG (1970) have shown that glycine resistance can be influenced by the transduction of this phenotypic characteristic by phage. In addition, the antimicrobial resistance can be acquired through transduction or mutations (CHANG & OGG, 1970; SALAMA et al., 1992).

The isolation and identification of ***C. fetus*** are difficult and require special culture media and atmosphere, restricting the number of laboratories that carry out the diagnosis of this micro-organism. In addition, the inconsistency between phenotypes and genomic characteristics of ***C. fetus*** samples revealed in recent research (VAN DER GRAAF-VAN BLOOIS et al., 2014) stimulate a critical evaluation of the clinical relevance of identification of ***C. fetus*** subspecies by phenotypic tests. Therefore, some techniques such as immunofluorescence test (IFT) and polymerase chain reaction (PCR) are also used for the detection of BGC combined with microbiological isolation (VAN BERGEN et al., 2005).

In Brazil, only one published research used microbiological isolation in combination with another technique. The study of ROCHA et al. (2009) investigated the presence of ***C. fetus*** in samples of bulls from dairy and beef farms in the region of the Middle Paraíba, state of Rio de Janeiro and used microbiological isolation combined with direct immunofluorescence test. By the direct immunofluorescence test, ROCHA et al. (2009) found the presence of 35.9% (14/30) of ***C. fetus*** and in the microbiological culture and biochemical tests obtained 10.3% (4/30) of positivity for ***C. fetus*** subsp. ***venerealis***.

In the diagnostic routine of the Laboratory of Bacteriology of the Universidade Federal of Santa Maria, we established the microbiological culture combined with PCR (HUM et al., 1997). From 2011 to 2018, 261 samples of beef and dairy cattle from 43 breeding farms in Rio Grande do Sul state were analyzed. Prepural aspirate (147), cervix mucus (108) and fetal abomasal content were analyzed (6). The presence of positive samples for ***C. fetus*** in this period was 2.72% (4/147) in bulls, 14.82% (16/108) in cows and 16.66% (1/6) in fetuses. For farms, 23.25% (10/43) of the farms had at least one positive animal in 42.86% (9/21) of the municipalities analyzed (BALZAN, unpublished data).

Fluorescence Antibody Test (FAT)

Immunofluorescence test can be applied for direct diagnosis of the micro-organism from samples or to confirm the identification of micro-organisms after isolation (VAN BERGEN et al., 2005; OIE, 2017). It is generally used as a screening test in preputial samples (CIPOLLA et al., 2001).

In the study of FIGUEIREDO et al. (2002), the direct fluorescence antibody test (DFAT) demonstrated good detection limit (100 CFU/mL) in preputial washes, with 92,59% of sensitivity and 88,89% of specificity. These results prove the use of DFAT as an important support technique for the diagnostic of BGC. In Argentina, due to the implementation of a national health plan to control venereal diseases in cattle from 1983, there are around 30 laboratories that mainly use direct fluorescence antibody test to diagnose BGC from bulls samples (CIPOLLA et al., 2001). This health plan was successful in controlling the disease, given the reduction in the percentage of BGC in herds of beef cattle from around 50% in 1983 to 15-18% in the period 1997-1999 (CIPOLLA et al., 2001).

The immunofluorescence test was not widely evaluated and the reported problems are false-positive results due to non-specific fluorescence, poor conjugate availability, and inability to differentiate ***C. fetus*** subspecies. In addition, technicians must be trained and experienced so that the performance of the test is not impaired by subjectivity (FIGUEIREDO et al., 2002).

In Brazil, DFAT was widely used in surveys of the occurrence of ***C. fetus*** in several regions. In Mato Grosso do Sul, region of Pantanal, positivity in beef cattle was estimated at 51.65% (171/327) by PELLEGRIN et al. (2002). In Minas Gerais, research in the Varginha region revealed 25.5% (40/157) of positivity in dairy herds (STYNEN et al., 2003). The occurrence in 12 Brazilian states with the highest herd of beef cattle in the year 2000 was estimated in 19.7% (224/1191) positive of the animals in 50.8% (61/120) of the breeding properties (MIRANDA, 2005). In Distrito Federal, the prevalence in cows and bulls was estimated in 11.1% (44/398) of the animals (LEAL et al., 2012). Now, the commercial

unavailability of conjugates makes the use of the technique restricted to certain research laboratories producing these inputs.

Molecular identification of ***C. fetus***

The amplified fragment length polymorphism (AFLP) methods (WAGENAAR et al., 2001) and multilocus sequence typing (MLST) (VAN BERGEN et al., 2005b) differentiate the two subspecies (***fetus*** and ***venerealis***) reliably, but these tests are cumbersome and impractical for routine use (VAN DER GRAAF-VAN BLOOIS et al., 2013).

A practical platform for the diagnosis is the PCR technique, since this assay is fast, simple and reliable. Several PCR assays have been developed to identify ***C. fetus*** (ABRIL et al., 2007; VAN BERGEN et al., 2005; HUM et al., 1997; MCMILLEN et al., 2006; TU et al., 2005; WANG et al., 2002)**.** The evaluation of these several PCRs (VAN DER GRAAF-VAN BLOOIS et al., 2013) showed that only HUM et al. (1997) and ABRIL et al. (2007) are valid for identification of ***C. fetus*** in terms of sensitivity and specificity, however both techniques need to isolate the bacteria or culture the sample in medium of enrichment for DNA extraction.

Also, multiplex PCR assays (HUM et al., 1997) and real-time PCR with specific probes (MCMILLEN et al., 2006), were able to identify ***C. fetus*** at the species level only. The OIE (2017) recommendation is that a PCR to identify ***C. fetus*** isolates reliably to subspecies level is not available and the researchers need to be careful and critical when publishing with results at the (unreliable) subspecies level.

CHABAN et al. (2012) used the primers of the conventional PCR of HUM et al. (1997) applied to a quantitative real-time PCR platform for direct processing of preputial samples, aiming at improving the original assay which had as factors the low analytical sensitivity. The test developed proved to be sensitive and low cost, however the processing of preputial samples for direct detection is laborious.

In order to determine the differentiation of the subspecies of ***C. fetus***, (MCGOLDRICK et al., 2013) adapted some methods of PCR already published (CASADÉMONT et al., 1998; HUM et al., 1997) for real-time PCR (qPCR) and approved two of these as complementary in the subspecies characterization routine when there are doubts in the OIE (2017) recommended trials.

VAN DER GRAAF-VAN BLOOIS et al. (2013) developed a qPCR assay with 100% sensitivity and 100% specificity for the detection of ***Campylobacter*** species, but did not meet the objective of differentiating the subspecies of ***C. fetus***. GUERRA et al. (2014) optimized a qPCR test to facilitate the access of field veterinarians to diagnostic tests for BGC, testing the influence of transport and prioritizing cost reduction.

Recently, papers published in Brazil used the conventional PCR technique described by HUM et al. (1997). ZIECH et al. (2014) tested samples from bulls, cows and fetuses from the state of Rio Grande do Sul between 1999 and 2010 by PCR. As a result, 10.9% of the samples (89/816) were ***C. fetus*** positive.

Enzyme-linked immunosorbent assay (ELISA)

Previous studies to improve the diagnosis of BGC have used ELISA to detect IgA in samples such as cervical mucus, preputial washings and contents of aborted fetuses (DEVENISH et al. , 2005; HEWSON et al., 1985; MSHELIA et al. , 2010; PELLEGRIN et al., 2011; HUM et al., 1994). These antibodies were chosen because they persist for longer and their concentration remains constant in the genital tract for many months (HUM et al, 1994). However, problems with sensitivity and specificity (false reactions) were reported by HUM et al. (1994) in an ELISA for IgA detection, in which the vaginal mucus of bovine females with suspicion of ***C. fetus*** infection was used.

Brooks et al. (2004) and Devenish et al. (2005) used monoclonal antibodies against ***C. fetus*** LPS antigen in capture ELISA, but did not obtain a good detection limit (10^5 to 10^7

CFU/mL), making this technique with monoclonal antibodies viable only with a period of enrichment of the sample for 4 to 5 days. Antigens used to sensitize plaques were whole bacteria or obtained by acid extraction from ***C. fetus*** cultures using glycine buffer. However, these samples are not easy to obtain and there are false-positive results. The main problems with ELISA techniques developed are limitations in sensitivity and / or specificity.

ZHAO et al. (2010) developed and evaluated a highly specific (94.3%) and sensitive (88.6%) indirect ELISA for the detection of IgG antibodies against ***C. fetus*** in bovine sera by validating as antigen recombinant proteins SapA-N and SapA-C coded. These researchers were successful in choosing the N-terminal region of SapA protein from a field strain, noting their immunodominance and the presence of multiple antigenic epitopes reported by (WANG et al., 1993).

Differential diagnosis

Other agents transmitted by sexual contact cause reproductive problems in the herds and should be considered for a correct diagnosis. The diagnosis of reproductive disease diagnosis is mostly performed from serum samples, but when herd history reveals infertility, preputial smegma, semen, fetal fluid, placenta and vaginal discharge can provide a definitive diagnosis (GIVENS, 2006).

The protozoan ***Tritrichomonas fetus***, responsible for bovine genital trichomoniasis, causes disease with aspects similar to BGC and should be investigated as a differential diagnosis (BONDURANT, 2005). Trichomoniasis is usually diagnosed using culture and/or PCR and the samples are the same for the BGC diagnosis (MICHI et al., 2016). In addition, BONDURANT (2005) and GIVENS (2018) cite ***Haemophilus somnus***, ***Ureaplasma*** and other ***Mycoplasmas*** and in some special conditions, ***Leptospira*** spp., ***Brucella abortus*** and viral diseases such as Bovine Viral Diarrhea (BVD) and Infectious Bovine Rhinotracheitis (IBR) causing infertility

and/or abortions in cattle herd. In these cases, clinical signs and epidemiological characteristics should be taken into account with the results of laboratory tests (GIVENS, 2006).

Another micro-organism morphologically very similar to ***Campylobacter*** in the microbiological culture is ***Arcobacter*** spp. (ETONSI, 2013), but this species growth in aerobiosis and it is enough to undo the mistake. The latter agent, although isolated from bovine abortion cases (FERNÁNDEZ et al., 1995; NEILL et al., 1985), was also recovered from preputial bovine washes and of vaginal swabs of cows without observable reproductive problems (KABEYA et al., 2004). Therefore, in situations of microbiological culture (without selective drugs) of samples of animals suspected of BGC should be included ***Arcobacter*** spp. as differential diagnosis.

Prevention and control

The control of the disease in the herds is carried out with the implantation of AI programs and avoiding the use of bulls (BONDURANT, 2005). The practice of discarding bulls bearing ***C. fetus*** subsp. ***venerealis***, according to Pellegrin et al. (2002), as well as the implantation of a limited breding season (60 to 90 days), discarding of empty females at the end of the mating season, and sexual rest for 3 to 4 cycles for females recovery are also measures recommended in the control of the BGC. Vaccination has been shown to be quite effective in preventing the recurrence of estrus and abortion caused by ***C. fetus*** subsp. ***venerealis***, according to BONDURANT (2005).

Commercial vaccines used for the control and prophylaxis of BGC are composed of bacterins with oil-based adjuvants and are subcutaneously or intramuscularly applied and there are reports of good efficacy when administered to females (GOMES, 2011), inducing at least partial protection against experimental genital infection (COBO et al., 2004).

When comparing the efficacy of 10 commercial vaccines for the prevention of abortion following exposure to ***C. fetus***, BRYNER et al. (1988) found a variation of 0 to 89% of

efficiency. These authors reported the deficiency in the production of immunity of some commercial vaccines, suggesting that the percentage of protection that a vaccine offers is directly proportional to the bacterial mass used and that immunogenicity of the samples used in the vaccine or type of adjuvant used may occur.

CONCLUSION

The BGC is a relevant disease because its clinical presentation and causes large economic losses. The published prevalence studies and the survey carried out from the diagnosis of the disease in LABAC /UFSM allow to affirm that the disease affects cattle herds in several regions of Brazil. The diagnosis is laborious due to the culture characteristics of ***Campylobacter*** and the limitations of other techniques. In addition, there is no projections for disease control and eradication programs neither at regional or national level. Further research aimed at optimizing the diagnosis from serum samples could facilitate the obtaining of results of occurrence and consequently the taking of preventive measures at herd level. Also, the use of combined diagnostic methods is essential to ensure reliable results.

ACKNOWLEDGEMENTS

The authors are thankful for the financial support received from the Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES/PROEX). C. Balzan received scholarship supported by CAPES Foundation, Ministry of Education of Brazil (grant number 88881.135832/2016-01/PDSE).

DECLARATION OF CONFLICTING INTERESTS

We have no conflict of interest to declare.

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Table 1 - Immunity to ***Campylobacter fetus*** subsp. ***venerealis***. Adapted from CORBEIL (1999).

	Ig class predominant		
	Uterus	Vagina	Clearance
Systemic immunization	IgG	IgG	Quick Uterus and vagina
Natural immunity (Local)	IgG	IgA	Slower - uterus then vagina

3 MANUSCRITO 2

DEVELOPMENT AND CHARACTERIZATION OF A RECOMBINANT PROTEIN WITH POTENTIAL FOR BOVINE GENITAL CAMPYLOBACTERIOSIS DIAGNOSTIC AND PREVENTION

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(Artigo a ser submetido para publicação no periódico Protein Expression and Purification)

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4 CONSIDERAÇÕES FINAIS

A Campilobacteriose genital bovina é uma enfermidade de caráter venéreo e amplamento distribuída em rebanhos que utilizam a monta natural. Apesar dos prejuízos econômicos ocasionados por esta enfermidade, ela permanece nos rebanhos com os touros portadores assintomáticos, uma vez que as fêmeas com problemas reprodutivos normalmente são descartadas sem efetivo diagnóstico. Devido à importância da CGB no Brasil e as várias falhas no diagnóstico, foi elaborado um artigo de revisão com o objetivo de discutir a situação da doença sob os seguintes aspectos: epidemiologia e distribuição geográfica, etiologia, patogenia, avanços no diagnóstico, diagnóstico diferencial e prevenção e controle.

A partir dos resultados obtidos no manuscrito 2 da presente tese pode-se concluir que através do processo de clonagem e expressão em *E.coli* foi possível obter uma proteína quimérica recombinante sob a forma de corpos de inclusão com aproximadamente 60kDa. A rSapAn78 foi purificada com êxito por cromatografia de afinidade ao metal, demonstrou-se imunogênica em coelhos e manteve as suas propriedades antigênicas quando testada frente a amostras de soro hiperimune produzido em coelhos e amostras de soro de bovinos positivos para CGB. Da mesma forma, o soro hiperimune produzido em coelhos reconheceu a proteína nativa em cepas de campo de *C. fetus* subsp. *venerealis.*

Devido à importância da CGB, da sua presença nos rebanhos bovinos brasileiros e de não haver um plano estratégico de controle e erradicação desta enfermidade em nosso país torna-se relevante a discussão levando em consideração as inúmeras pesquisas que trouxeram atualizações sobre a distribuição e diagnóstico. Somado à isso, a experiência do grupo do Laboratório de Bacteriologia da Universidade Federal de Santa Maria com o diagnóstico rotineiro desta enfermidade e em pesquisas relevantes já publicadas sustenta o potencial desta pesquisa para a produção futura de um teste diagnóstico baseado na proteína recombinante desenvolvida e também a possibilidade de uso na prevenção da CGB.

5 CONCLUSÕES

- Fundamentado nos dados apresentados nesta tese, conclui-se que a Campilobacteriose Genital Bovina continua disseminada no território brasileiro, é subdiagnosticada e apesar das tecnologias existentes no diagnóstico laboratorial, as análises para esta enfermidade ainda apresentam inúmeros obstáculos que vão desde a colheita da amostra até os procedimentos laboratoriais não padronizados.

- A proteína recombinante produzida e caracterizada neste estudo a partir de sequências da proteína de superfície A de *C. fetus* foi imunogênica em coelhos e sua capacidade de distinguir entre bovinos infectados com *C. fetus* e negativos foi comprovada.

- Dadas as falhas no diagnóstico da CGB, a necessidade de melhorias no processo de produção de vacinas e as características da proteína quimérica desenvolvida nesta pesquisa, a rSapAn78 desponta como um potencial antígeno para o diagnóstico e prevenção desta enfermidade.

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