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MICROBIOLOGY

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

Cláudia Balzan[1*] Rosangela Estel Ziech[2] Letícia Trevisan Gressler[3] Agueda Palmira Castagna de Vargas[1]

[1]Programa de Pós-graduação em Medicina Veterinária (PPGMV), Departamento de Medicina Veterinária Preventiva, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil. E-mail: cl.balzan@gmail.com. *Corresponding author.
[2]Médica Veterinária, Prefeitura Dois Vizinhos, Dois Vizinhos, PR, Brasil.
[3]Laboratório de Microbiologia e Doenças Infecciosas, Instituto Federal de Educação, Ciência e Tecnologia Farroupilha (IFFar), Frederico Westphalen, RS, Brasil.

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 failure and economic losses. In these cases, the bull is the asymptomatic carrier of the bacterium and the infected females can have infertility and even abortions. The techniques for the diagnosis of C. fetus are isolation in culture medium and identification by biochemical tests, immunofluorescence, immunoenzymatic assays and molecular techniques. Disease control is based on vaccination with bacterins. This review described the epidemiology, etiology, pathogenesis, and advances in the diagnosis and control of BGC.*
Key words: *Campylobacter fetus subsp. venerealis, infertility, venereal disease, beef cattle.*

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

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 por testes bioquímicos; imunofluorescência; ensaios imunoenzimáticos e técnicas moleculares. 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, doenças venéreas, bovinos de corte.*

INTRODUCTION

Bovine genital campylobacteriosis is caused by the microaerophilic bacterium *Campylobacter fetus* subsp. *venerealis*, a gram-negative rod. This 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 as in Brazil. The BGC was first diagnosed in Brazil in 1955 in an aborted fetus (RAMOS et al., 1983) and remains in the herds of this country to date. Also, BGC is on a list of notifiable diseases that are significant in international trade in animals or animal products, maintained by the World Organization for Animal Health (OIE, 2017). Published reviews have highlighted the prevalence, epidemiology, diagnosis and/or control of BGC (ALVES et al., 2011; BONDURANT, 2005; CORBEIL et al., 2003; HOFFER, 1981; MICHI et al., 2016; MSHELIA et al., 2007; SILVEIRA et al., 2018). Therefore, facing the woldwide impact of BGC, this review discussed the distribution, etiology, pathogenesis, *C. fetus* virulence factors, epidemiology, and advances in diagnosis and control of BGC, emphasizing its occurrence in Brazil.

Epidemiology and distribution

Recent data from the OIE (2019) indicated the presence of BGC in Argentina, Brazil, Colombia, Uruguay, Australia, New Zealand, Ireland, France,

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South Africa, Iran, and Nigeria between January and June 2018. In Nigeria, approximately 520,000 cattle are affected by BGC with a direct loss of 8.5 million dollars due to abortions and low fertility rates (MSHELIA et al., 2012).

In Brazil, BGC is among the most important causes of reproductive failure in beef and dairy farms which use natural breeding (VARGAS et al., 2002; LEAL et al., 2012; MIRANDA, 2005; OLIVEIRA et al., 2015; STYNEN et al., 2003). As shown in table 1, over the last 23 years, BGC has been reported in all regions of Brazil. There are specific prevalence studies, which usually involve a unique region of a state or diagnostic laboratory data. In Brazil, there are no official surveys, no estimates of losses caused by the disease, and no prediction of an eradication and control program. Moreover, BGC incidence remains understimated due to the absence of systematic diagnosis, which is associated with logistic issues, such as sample collection and shipment, as well as a limited number of laboratories qualified to perform BCG diagnosis (ALVES et al., 2011).

The main risk factor for the spread of BGC is 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 AI is a very common practice (STYNEN et al., 2003). Another risk factor is the use of semen without appropriate antimicrobials (BONDURANT, 2005).

Table 1 - Surveys reporting the prevalence of BGC in Brazil between 1995 and 2018.

Study	Year	State	Technique	Sample from	Cattle	% positive animals
PELLEGRIN et al. (2002)	1995-1996	MS	DFAT	B	Beef	51.65% (171/327)
STYNEN et al. (2003)	1998	MG	DFAT	C	Dairy	25.5% (40/157)
MIRANDA (2005)	2000	BA, GO, MA, MT, MS, MG, PA, PR, RS, RO, SP, TO	DFAT	B	Beef	19.7% (224/1191)
ROCHA et al. (2009)	2009	RJ	DFAT/I	B	Beef/Dairy	IFD 35.9% (14/39) I 10.3% (4/39)
LEAL (2012)	2009	DF	DFAT	B/C	n/i	11.1% (44/398)
ZIECH et al. (2014)	1999-2010	RS	PCR	B/C/F	Beef/Dairy	10.9% (89/816)
OLIVEIRA et al. (2015)	2013	PE	PCR	C	Dairy	1.8% (7/383)
BOTELHO et al. (2018)	2013	MG	PCR	B	Beef	17.5% (35/200)
NASCIMENTO et al. (2018)	2016	AL	PCR	B	Beef	4.9% (8/162)
FILHO et al., 2018	2016	PB	PCR	C	Dairy	7.7% (21/273)
BALZAN, unpublished data	2011-2018	RS	I/PCR	B/C/F	Beef/Dairy	8% (21/261)

MS: Mato Grosso do Sul; MG: Minas Gerais; BA: Bahia; DF: Distrito Federal; GO: Goiás; MA: Maranhão; MT: Mato Grosso; PA: Pará; PR: Paraná; RS: Rio Grande do Sul; RO: Rondonia; SP: São Paulo; TO: Tocantins; RJ: Rio de Janeiro; PE: Pernambuco; AL: Alagoas; PB: Paraíba. DFAT: Direct Fluorescence Antibody Test; I: Isolation; PCR: Polymerase Chain Reaction (HUM et al., 1997).
B: Bulls; C: Cows; F: fetuses; n/i: no information.

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

Etiology and pathogenesis

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

Microaerophilic bacteria comprised the genus *Campylobacter*, which generally require oxygen concentrations between 3 and 15% and carbon dioxide concentrations from 3 to 5%. Only a few species are aerotolerant and can grow in the presence of oxygen (HOLT et al., 1994). *C. fetus* as a pathogen in cattle and sheep and is subdivided into two subspecies, *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, by habitat characteristics, transmission, clinical manifestations, and laboratory phenotypic identification (VÉRON & CHATELAIN, 1973). These subspecies are phenotypically differentiated according to HOLT et al. (1994), with tests of tolerance to 1% glycine and production of hydrogen sulphide (H_2S), with *C. fetus* subsp. *fetus* returning positive results in both assays, and *C. fetus* subsp. *venerealis* as negative. *C. fetus* subsp. *venerealis* still presents as a biovar, classified by the ability to produce H_2S but not 1% glycine tolerant, and is referred to as *C. fetus* subsp. *venerealis* biovar intermedius (VÉRON & CHATELAIN, 1973).

C. fetus subsp. *fetus* is commensal in the gastrointestinal tract and occasionally causes abortion in sheep and cattle (FLORENT, 1959 apud SKIRROW, 1994); whereas, *C. fetus* subsp. *venerealis* is present exclusively in the genital tract of cattle resulting in infertility and abortions, the bacterium being transmitted exclusively by the venereal route (HUM & QUINN., 1994).

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 microorganism persists in the epithelial crypts of the prepuce. Transmission to females occurs during natural mating with infected bulls, or by AI with contaminated semen, when the bacterium is distributed through the mucosa of the vagina, cervix, uterus, and uterine tubes (BONDURANT, 2005). Approximately one third of infected cows become carriers (QUINN et al., 2011), and other males may be contaminated by mating with 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 the animal remains as an asymptomatic carrier (VAN BERGEN, 2005a). Bulls older than 4 years of age are more likely to be carriers because they have deeper crypts where microorganisms find favorable conditions and safe harbor (SAMUELSON & WINTER, 1966). Infection can persist for months to years in older bulls (BIER et al., 1977a).

After coitus, an ascending infection is established in females, and in 12 to 14 days the microorganism is distributed from the vagina/cervix to the uterine horns (BONDURANT, 2005; CORBEIL, 1999; YAEGER & HOLLERY, 2007). Then, development of endometritis and salpingitis occur during progestational phase of the estrous cycle, when both the amount and activity of neutrophils decline (VAN BERGEN, 2005a; 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 uterine invasion, in a period of 3 to 6 months, the cow can remain infertile and a source of infection (VAN BERGEN, 2005a); and subsequently, acquired immunity is developed.

The predominant immunity in *C. fetus* infected animals is local (CORBEIL et al., 1974), with reported differences between males and females. In females, antibody production in the vagina and uterus begins 40 to 60 days after infection (CORBEIL et al., 1974).

In preputial secretions of healthy bulls, IgG predominates in abundance, followed by IgA (BIER et al., 1977b), and in bulls infected with *C. fetus* subsp. *venerealis*, the serum antibody response is undetectable (VASQUEZ et al., 1983). 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; IgGs are produced in the uterus and these opsonize the pathogenic organisms, facilitating phagocytosis by neutrophils and mononuclear cells (CORBEIL, 1999; table 2). As BGC is caused by an extracellular pathogen, it would be expected to elicit predominantly a humoral immune response (CORBEIL et al.,

Table 2 - 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

1974), likely causing short duration of immunity to infectious agent (BONDURANT, 2005).

The presence of carrier cows is due to the evasion of local immune responses, 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 microorganisms, and by variation of surface antigens of the microorganism against local immune responses. In bulls, infections are persistent and result in asymptomatic carrier animals, indicating that immunity during inflammation does not eliminate mucosal microorganisms (COBO et al., 2011). Nevertheless, according to TIZARD (2015), *C. fetus* infections are associated with the presence of several mononuclear cell types, 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, whereas the uterus is free of infection (BONDURANT, 2005). This allows for the return of the cow's fertility in many cases, and may last up to 2 years in the absence of antigenic stimulation (CORBEIL et al., 1974). In bulls, persistence of the bacteria in the lower genital tract is also attributed to evasion of immune responses (VARGAS et al., 2002; CORBEIL, 1975).

Genes and virulence factors

Complete genomic sequencing of *C. fetus* is relatively recent. In 2006, the first complete and closed genome of *C. fetus* was made available by The Institute for Genomic Research (TIGR, USA, 2006), and this was the 82-40 strain isolated from an individual in the United States and later identified as *C. fetus* subsp. *fetus*, with a 1.77 Mpb genome and approximately 1,820 predicted genes. The first sequenced genome of *C. fetus* subsp. *venerealis* was published by MOOLHUIJZEN et al. (2009) as a not assembled genome consisting of multiple contigs of the Azul-94 strain, which was isolated from an aborted bovine fetus in Argentina.

Since then, genomic studies of *C. fetus* have expanded. This can be verified by queries on databases of DNA sequences, such as GenBank®, where currently there are 74 genomes of *C. fetus* published, including 11 complete genomes (IRAOLA et al., 2017; KIENESBERGER et al., 2014; VAN DER GRAAF-VAN BLOOIS et al., 2014; WANG et al., 2015). Whole genome sequencing can be used to differentiate the mammal-associated *C. fetus* strains based on their core genomes (VAN DER GRAAF-VAN BLOOIS et al., 2014) and provides data on strains prevalent worldwide. Thus, genomic research in *C. fetus* is very important in providing new information about the species and its subspecies, to enable scientific advances regarding their origins and hosts, and for the correct identification of strains.

Current research on species of *Campylobacter* concentrates on total genomic analysis for the identification of virulence genes and characteristics that contribute to the pathogenicity differences among subspecies (FOUTS et al., 2005; MOOLHUIJZEN et al., 2009; ALI et al., 2012; VAN DER GRAAF-VAN BLOOIS et al., 2016; IRAOLA et al., 2017; GILBERT et al., 2018). In the *C. fetus* subespecies these virulence factors were clearly identified as classes of genes encoding proteins (MOOLHUIJZEN et al., 2009), such as surface polypeptides involved in bacterial adherence, motility, toxin production and resistance, and 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 shape of the cell and the corkscrew-like motility conferred by the flagella of *C. fetus* are needed 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*. 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, motility and adhesion capacity of host cells play fundamental roles in the diseases caused by bacteria belonging to the genus *Campylobacter*.

A genomic island encoding a type IV secretion system integrated into the genome of *C. fetus* subsp. *venerealis* was identified, and it was believed that this genomic island was a determinant for the tropism of this subspecies in the bovine genital tract (GORKIEWICZ et al., 2010). However, phylogenetic analyses between genomes of strains of the two subspecies of *C. fetus* showed that this coding region involving the type IV secretion system exists in both subspecies as an element acquired from different donors and contains *fic* (n = 4) and *virD4* (n = 10) genes (VAN DER GRAAF-VAN BLOOIS et al., 2016). In other research, SPRENGER et al. (2017) have demonstrated that *fic* genes predominate and are strongly conserved in *C. fetus* subsp. *venerealis*, and Fic proteins are related to changes from the normal to the static metabolic states, which assists in the maintenance of the bacterium for long periods in the host, even under stressful conditions.

Several bacteria, including *Escherichia coli* and *Shigella* spp., produce Cytolethal distending toxin (CDT). Among *Campylobacter* strains, the *cdt* gene cluster is distributed universally and is well conserved, particularly in *C. jejuni*, *C. coli*, and *C. fetus* (ASAKURA et al., 2007). In *C. jejuni,* CDT protein is recognized for causing damage to 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 a lipid fraction, denominated lipid A, and a polysaccharide fraction, with a central oligosaccharide and an "O" antigen (MORAN et al., 1996). In *C. fetus*, lipid A has low biological activity compared to other Enterobacteriaceae family members, and this is associated with persistent colonization of the host (BLASER & ENGBERG, 2008). Variation in O antigen is the basis of a serotyping scheme for *C. fetus*, and two main serotypes, denominated A and B, are recognized (VAN BERGEN et al., 2005a; DWORKIN et al., 1995a; PEREZ-PEREZ et al., 1986; TU et al., 2004). *C. fetus* subsp. *fetus* contains serotypes A and B, whereas *C. fetus* subsp. *venerealis* has only serotype A.

C. fetus has a protein structure 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, these SAPs are responsible for the resistance of this microorganism to bactericidal and phagocytic activities of the host (MCCOY et al., 1975; RAY et al., 2000). Their superficial location suggested that they may be an important mediator in interactions with the host (BLASER & GOTSCHLICH, 1990), and in persistence of the pathogen in the reproductive tract (CORBEIL et al., 1975).

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 and usually one of these being dominant (DUBREUIL et al., 1990; DWORKIN et al., 1995a; DWORKIN et al., 1995b; FUJIMOTO, 1991; PEI et al., 1988, VARGAS et al., 2002). During infection, extensive high frequency chromosomal rearrangements occurred in DNA, and this results in the modification of dominant epitopes during persistence of the bacteria in the genital tract (GARCIA et al., 1995; WANG et al., 1993). Thus, this evidence showed the importance of surface proteins for *C. fetus* subsp. *venerealis*; and consequently, for the maintenance of BGC infection. Because of this, such proteins are potential candidates for the development of serological diagnostic methods, and according to PEI et al. (1988), for use in vaccines.

Diagnosis of BGC

The subspecies of *C. fetus* showed different adaptations to host tissues. However, at the genetic level they are practically indistinguishable (MOOLHUIJZEN et al., 2009). Sequence alignment reveals that the genotypes of *C. fetus* subspecies are highly homologous, with 92.9% average identity (KIENESBERGER et al., 2014).

The materials for laboratory diagnosis of BGC from suspect animals include 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 be collected. The diagnosis of BGC is at the herd level, so it is not considered for isolated cases, but an epidemiological profile of the herd based on the history of clinical manifestations compatible with this disease and analysis of reproductive rates, to be confirmed with laboratory assays.

In females, cervicovaginal mucus can be collected with an insemination pipette or absorbent pad (OIE, 2017; STYNEN et al., 2003). Preputial samples from bulls can 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 to increase the sensitivity of the diagnosis (SKIRROW & BONDURANT, 1988). Moreover, the OIE (2017) indicated 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 container (temperature 4–10 °C) and protected from light.

Techniques for the diagnosis of *C. fetus* involve the isolation and identification of the infectious agent, immunofluorescence, immunoenzymatic tests, and molecular identification (OIE, 2017). The use of two combined techniques to obtain a result is preferred to give reliability to the result of the diagnosis, but several laboratories do not apply or do not perform the techniques correctly (VAN BERGEN et al., 2005a).

Isolation and identification of C. fetus

The isolation and identification of *C. fetus* by microbiological culture is considered the standard and confirmatory test for diagnosis of infection (BROOKS et al., 2004) and is a suitable assay for certifying individual animals prior to movement (OIE, 2017). However, the same depends on the sample quality, the way it is sent to the laboratory, and the viability of the microorganisms. *Campylobacter* species are microaerophilic and *C. fetus* grows fastidiously, requiring strictly atmospheric conditions for cultivation, including selective and enrichment media (QUINN et al., 2011).

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

However, researchers report doubts concerning 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 (a characteristic previously attributed only to *C. fetus* subsp. *fetus*). In terms of glycine tolerance, CHANG & OGG (1970) have shown that this characteristic can be influenced by transduction of this phenotypic characteristic by bacteriophages. In addition, antimicrobial resistance can be acquired through transduction or mutations (CHANG & OGG, 1970; SALAMA et al., 1992).

The procedures for isolation and identification of *C. fetus* are laborious and require special culture media and atmospheric conditions, restricting the number of laboratories able to carry out diagnosis of this microorganism. In addition, inconsistencies between phenotypic and genomic characteristics of *C. fetus* samples revealed in recent research (VAN DER GRAAF-VAN BLOOIS et al., 2014) have stimulated a critical evaluation of the clinical relevance of identification of *C. fetus* subspecies using phenotypic tests. Therefore, some techniques such as fluorescence antibody test (FAT) and polymerase chain reaction (PCR) are also used for the detection of BGC, combined with microbiological isolation (VAN BERGEN et al. , 2005a).

In Brazil, only one published research report used microbiological isolation in combination with another technique. The study of ROCHA et al. (2009) investigated the presence of *C. fetus* in samples from bulls on dairy and beef farms in the region of the Médio Paraíba, state of Rio de Janeiro and used microbiological isolation combined with direct immunofluorescence (DIF) testing. By DIF, ROCHA et al. (2009) confirmed the presence of *C. fetus* in 35.9% (14/30) of samples, and in the microbiological culture and biochemical tests, obtained 10.3% (4/30) positivity for *C. fetus* subsp. *venerealis* (Table 1).

For routine diagnostic activities of the Laboratory of Bacteriology of the Universidade Federal of Santa Maria, microbiological culture combined with PCR was established (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 (n = 147), cervical mucus (n = 108) and fetal abomasal content (n = 6) were analyzed. The presence of samples positive 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) had at least one *C. fetus* positive animal in 42.86% (9/21) of the municipalities analyzed (BALZAN, unpublished data).

Fluorescent Antibody Test (FAT)

Immunofluorescence tests can be applied for direct diagnosis of microorganism in samples, or to confirm the identification of microorganisms after isolation (VAN BERGEN et al., 2005a; OIE, 2017). FATs are generally used as a screening test for preputial samples (CIPOLLA et al., 2001).

In a study by FIGUEIREDO et al. (2002), the direct FAT (DFAT) demonstrated good detection limits (100 CFU/mL) in preputial washes, with 92.59% sensitivity and 88.89% specificity. These results demonstrated the use of DFATs as an important support technique for the control of BGC. In Argentina, due to the implementation of a national health plan to control venereal diseases in cattle from 1983, there are approximately 30 laboratories that mainly use DFAT assays to diagnose BGC from bull 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 approximately 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 were false-positive results due to non-specific fluorescence and inability to differentiate *C. fetus* subspecies (SILVEIRA et al., 2018), with low availability of conjugate. In addition, technicians must be trained and experienced so that 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 (table 1) until 2009 and obtained high rates of positivity. Nowadays, 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) (WAGENAAR et al., 2001) and multilocus sequence typing (MLST) (VAN BERGEN et al., 2005c) methods were firstly recommended to differentiate the two subspecies, *fetus* and *venerealis*, but these tests are laborious, impractical for routine use and, according to VAN DER GRAAF-VAN BLOOIS et al. (2013) and IRAOLA et al. (2017), are not fully reliable for *C. fetus* subspecies differentiation.

A practical platform for BGC diagnosis is the PCR technique, since this assay is rapid, simple, and reliable. Several PCR assays have been developed to identify *C. fetus* (ABRIL et al., 2007; VAN BERGEN et al., 2005b; 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 those of 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 require the isolation of bacteria or culture of samples in medium for enrichment for DNA extraction.

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

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

To allow differentiation of *C. fetus* subspecies, MCGOLDRICK et al. (2013) adapted some PCR methods already published (CASADÉMONT et al., 1998; HUM et al., 1997) for quantitative PCR (qPCR), and reported a sensitivity of 98.7% and specificity of 99.8% for *C. fetus* subsp. *venerealis* identification, and this technique has been approved for routine subspecies characterization 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 *C. fetus*, but did not meet the objective of differentiating the subspecies of *C. fetus*.

Recently, papers published in Brazil (Table 1) used the conventional PCR technique described by HUM et al. (1997). Some of this research (FILHO et al., 2018; NASCIMENTO et al., 2018; OLIVEIRA et al., 2015) used only a pair of primers specific for *C. fetus* subsp. *venerealis* identification (VENS – *par*A gene), which is not recommended. As evaluated by VAN DER GRAAF-VAN BLOOIS et al. (2013), the technique of HUM et al. (1997) has 100% sensitivity and 100% specificity for the detection of *C. fetus*, and 58% sensitivity and

83% specificity for *C. fetus* subsp. *venerealis*, so it is reliable for species identification only.

Therefore, a critical evaluation of publications regarding available PCR techniques has been recommended for implementation of such techniques in a laboratory for the diagnosis of *Campylobacter fetus*.

Enzyme-linked immunosorbent assay (ELISA)

In the diagnosis of BGC, ELISA could be used for evaluating herd immunity, but is not acceptable for diagnosis of the infection in individual animals, and cannot differentiate between infections caused by the two subspecies of *C. fetus* that causes disease in ruminants (OIE, 2017). In Brazil, currently, reports on the use of ELISA to diagnose BGC are unavailable.

Previous studies to improve BGC diagnosis 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 & QUINN, 1994). These IgA antibodies were chosen because they persist for longer and their concentration remains constant in the genital tract for many months (HUM & QUINN, 1994). However, problems with sensitivity and specificity (false-positive/-negative) were reported by HUM & QUINN (1994) in an ELISA for IgA detection, in which the vaginal mucus of bovine females with suspicted *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 satisfactory detection limit (10^5 to 10^7 CFU/mL), making this technique using monoclonal antibodies viable only after 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 the recombinant proteins SapA-N and SapA-C. 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, as reported by WANG et al. (1993).

Differential diagnosis

Other agents transmitted by sexual contact cause reproductive problems in cattle herds and should be considered for a correct diagnosis. The diagnosis of reproductive disease is mostly performed with serum samples, but when herd history reveals infertility, samples such as 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 sample types are the same for 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 herds. In these cases, clinical signs and epidemiological characteristics should be taken into account, along with the results of laboratory tests (GIVENS, 2006).

Another microorganism very similar to *Campylobacter* in microbiological cultures is *Arcobacter* sp. (ETONSI, 2013). This species growth in aerobiosis and it is enough to undo the mistake, as well as the use of selective broth to enrichment can eliminate this bacterium. The *Arcobacter* sp.; although, isolated from bovine abortion cases (FERNÁNDEZ et al., 1995; NEILL et al., 1985), was also recovered from preputial bovine washes, and from vaginal swabs of cows without observable reproductive problems (KABEYA et al., 2004). Therefore, in situations of microbiological culture of samples from animals suspected of BGC infection, *Arcobacter* spp. should be included for differential diagnosis.

Prevention and control

The control of the disease in herds is carried out with the implementation of AI programs, avoiding the use of bulls (BONDURANT, 2005), and mainly with the introduction of vaccination in bulls and cows. The practice of discarding bulls bearing *C. fetus* subsp. *venerealis*, according to PELLEGRIN et al. (2002), as well as the implementation of a limited breding season (60 to 90 days), discarding of nonpregnant females at the end of the breeding season, and sexual rest for 3 to 4 cycles for recovery of females, are also measures recommended for BGC control.

Vaccination is 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 adjuvants, given as subcutaneous or intramuscular injections. There are reports of good efficacy when administered to females, inducing at least partial protection against experimental genital infection (COBO et al., 2004). In herds infected with *C. fetus* subsp. *venerealis*, reproductive age females should be vaccinated annually, approximately 30 days before the start of breeding (BONDURANT, 2005). In the case of first vaccination, heifers and cows should receive two doses, at approximately 60 and 30 days, respectively, before the beginning of the breeding season. Vaccination alone is often not considered sufficient to manage an outbreak of BGC, and in bulls it is necessary to test and cull positive animals (ERICKSON et al., 2017).

When comparing the efficacy of 10 commercial vaccines for the prevention of abortion following exposure to *C. fetus*, BRYNER et al. (1988) reported 0 to 89% in efficiency. These authors reported deficiencies in immune responses to 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 arise.

BGC positive cows and bulls can be treated. Cows are not usually treated, and after a few months, can eliminate the infection and return to estrus. Decisions regarding the treatment of bulls should be evaluated taking into consideration the age and value of the animal, since treatment incurs some costs and the animals will need to be managed for several days. There are reports of the use of topical and systemic antimicrobial treatments, with variable results. The tested antimicrobials included streptomycin, dimetridazole chlorhydrate, and oxytetracycline (CAMPERO et al., 1993; ERICKSON et al., 2017). ERICKSON et al. (2017) tested a combination of antimicrobial treatment with injectable oxytetracycline and vaccination with two doses of a monovalent oil-based *C. fetus* subsp. *venerealis* commercial vaccine in BGC-positive bulls. Two treatments with long-acting oxytetracycline at label doses and a commercial monovalent bacterin did not eliminate BGC in all study bulls. Therefore, this is not recommended as an effective management strategy. However, due to the antimicrobial resistance reported in the last decades and because of legislation in some countries, treatment must be carried out observing the legislation surrounding antimicrobial use in livestock production, and on the recommendation of a veterinarian.

CONCLUSIONS

BGC is a relevant disease because of its clinical presentation, which causes large economic losses. Published prevalence studies and a survey carried out involving diagnosis of the disease in LABAC/UFSM affirmed that the disease affects cattle herds in several regions of Brazil, and that the losses are underestimated. BGC diagnosis is laborious due to the culture characteristics of *Campylobacter* and the limitations of other techniques. In addition, there are no projections for disease control and eradication programs at either regional or national levels. Further research aimed to optimize the diagnosis of BGC using molecular techniques could facilitate the establishment of results regarding occurrence and; consequently, taking preventive measures at herd level. Finally, the use of combined diagnostic methods is essential to ensure reliable results.

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DECLARATION OF CONFLICTS OF INTERESTS

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

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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