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Prevalence of *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis* among bulls slaughtered in the state of Minas Gerais, Brazil

Prevalência de *Tritrichomonas foetus, Campylobacter fetus* subsp. *venerealis* e de *Campylobacter fetus* subsp. *fetus* em touros abatidos no Estado de Minas Gerais, Brasil

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Abstract

Bovine genital campylobacteriosis (BGC) and bovine genital trichomonosis (BGT) are globally widespread, predominantly sexually transmitted diseases that mainly affect herds in which natural mating takes place. Bulls serve as good epidemiological indicators for studying the causative agents of these diseases in cattle herds. The objective of the present work was to determine the prevalence of BGC and BGT among bulls sent to slaughterhouses in southern Minas Gerais, Brazil. Samples of preputial smegma from 200 sexually mature, non-castrated bulls of several ancestries were collected in 2013 from four regional slaughterhouses. The polymerase chain reaction (PCR) was employed to detect *Tritrichomonas foetus*, *Campylobacter fetus* subsp. *fetus*, and *C. fetus* subsp. *venerealis*. Isolation also was used aiming to diagnosis of BGT. All smegma samples were negative in culture for *T. foetus*. Molecular tests revealed 8% (16/200) of animals to be positive for *T. foetus*, 17.5% (35/200) for *Campylobacter. fetus* subsp. *fetus*, and 13.5% (27/200) for *C. fetus* subsp. *venerealis*. The results of the present study indicate that *T. foetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp. *fetus* are present among bulls slaughtered in southern Minas Gerais, and that BGC and BGT occur in this region. These diseases therefore need to be considered during differential diagnosis of reproductive diseases affecting cattle herds in which natural mating is employed.

Key words: Campylobacteriosis. Bovine diseases. Diagnosis. PCR. Reproductive diseases. Trichomonosis.

Resumo

A Campilobacteriose Genital Bovina (CGB) e a Tricomonose Genital Bovina (TGB) são doenças de transmissão predominantemente venérea, de ampla disseminação mundial, acometendo principalmente

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rebanhos submetidos à monta natural. Touros constituem bons indicadores epidemiológicos para a pesquisa destes agentes nos rebanhos**.** O objetivo deste estudo foi determinar a prevalência da CGB e da TGB em touros encaminhados a abatedouros da região sul de Minas Gerais, Brasil. Amostras de esmegma prepucial de 200 touros (machos não castrados em idade compatível com a monta) de diversas genealogias foram coletadas em quatro abatedouros da região, no ano de 2013. A reação em cadeia da polimerase (PCR) foi utilizada para detecção dos patógenos *Tritrichomonas foetus, Campylobacter fetus* subsp. *fetus* e *Campylobacter fetus* subsp. *venerealis.* O isolamento também foi utilizado para o diagnóstico da TGB. Todas as amostras analisadas apresentaram resultado negativo na cultura para isolamento de *T. foetus.* Por meio dos testes moleculares, verificaram-se prevalências de 8% (16/200) de animais positivos para *T. foetus;* 17,5% (35/200) para *C. fetus* subsp. *fetus* e de 13,5% (27/200) para *C. fetus* subsp. *venerealis*. Os dados do presente estudo apontaram a presença de *T. foetus, C. fetus* subsp. *venerealis* e *C. fetus* subsp. *fetus* em touros abatidos na região sul de Minas Gerais, evidenciando a ocorrência da TGB e da CGB nesta região e a necessidade de inclusão destas enfermidades no diagnóstico diferencial das doenças da reprodução em rebanhos bovinos submetidos à monta natural.
Palavras-chave: Campilobacteriose. Diagnóstico. Doenças de bovinos. Doenças reprodutivas. Tricomonose. PCR.

Introduction

Brazil has the world's largest population of commercial cattle, estimated at 212.8 million animals, and cattle farming is of great socioeconomic relevance in this country, enjoying a privileged position in Brazilian agribusiness and contributing to the settling of rural areas. Among the states of the Southeast Region, Minas Gerais (MG) stands out as having largest cattle population, representing 11% of the total in Brazil (IBGE, 2015), and being the leading milk producer, having produced approximately 6.5 billion liters in 2016 (IBGE, 2017).

Sanitary problems are a limiting factor in the livestock industry, increasing production costs and reducing the competitiveness of the goods produced. Of these problems, reproductive diseases have an important effect owing to their insidious and disseminated nature, and eventually compromise the reproductive performance of the affected animals (FASSIO et al., 2006). The protozoan *Tritrichomonas foetus* and the bacterium *Campylobacter fetus* subsp. *venerealis* are among the main causative agents of reproductive diseases in cattle submitted to mate naturally. These microorganisms cause bovine genital trichomonosis (BGT) and bovine genital campylobacteriosis (BGC), respectively. The bacterium *Campylobacter fetus* subsp. *fetus* is the causative agent of sporadic abortus in cows (ALVES et al., 2011). Despite the fact that they are globally widespread, studies concerning these diseases remain scarce in Brazil, where management practices predominantly based on natural mating certainly favor their occurrence (ALVES et al., 2011; MICHI et al., 2016).

BGC and BGT are responsible for considerable economic losses in the livestock industry, mainly due to the temporary infertility of infected females and resulting prolongation of the interval between pregnancies (BONDURANT, 2005; ALVES et al., 2011), indirect expenses due to professionals' fees, and costs related to the diagnosis, treatment, and disposal of infected animals (PELLEGRIN, 2002). Furthermore, BGC and BGT feature in the list of notifiable diseases maintained by the World Organization for Animal Health (OIE), which include transmissible diseases of socioeconomic importance and public health concern and therefore relevant to the international trade of animals and animal products (OIE, 2012).

The economic impact of these diseases with respect to Brazilian herds has been little investigated, and the studies that are available are scarce and obsolete (STYNEN et al., 2003; ROCHA et al., 2009; LEAL et al., 2012). This probably results from the lack of effective diagnostic routine methods,

along with problems associated with shipping and laboratory analyses of clinical samples, and the small number of laboratories with the technical capacity to diagnose these diseases (PELLEGRIN; LEITE, 2003).

The majority of studies concerning BGC and BGT carried out in Brazil have used traditional diagnostic methods, such as isolation (LEITE et al., 1997; ROCHA et al., 2009), mucus agglutination (CASTRO et al., 1971), and direct immunofluorescence (STYNEN et al., 2003; LEAL et al., 2012). However, these tests have low sensitivity and specificity (MCMILLEN et al., 2006), constituting a serious obstacle to understanding the real importance of these diseases in Brazilian herds.

Despite advances in molecular biology, the use of molecular tests in the diagnosis of BGC and BGT in Brazil remains rather rare. Indeed, no previous studies using such tests to detect BGT are to be found in the Brazilian researches. Concerning BGC, only a single report exists in which these diagnostic tests were applied to Brazilian herds, and only a small number of such studies have been performed in other countries (MADOROBA et al., 2011; HAMALI et al., 2011). In general, molecular tests offer the advantages of high sensitivity and specificity without the need to preserve the feasibility of pathogen in question. In the case of BGC and BGT, these benefits mean that such tests likely generate more reliable estimates of pathogen prevalence than conventional diagnostic methods, mainly the isolation, due to the low viability of the causative agents, especially when transport and enrichment media (TEM) are not utilized (BONDURANT, 2005).

Considering the importance of BGC and BGT in cattle submitted to natural mating and the scarcity of recent data concerning these diseases among Brazilian herds, the objective of the present work was to determine their prevalence among bulls sent to slaughterhouses in southern Minas Gerais during 2013.

Material and Methods

All procedures carried out in this study complied with regulatory standards for biosafety and ethics, having been approved by the Ethics Committee on Animal Experimentation (CEUA) of the Federal University of Lavras, process nr. 048/13.

The present study was performed in the southeast region of the state of Minas Gerais, Brazil, using samples of preputial smegma collected from bulls slaughtered in four slaughterhouses in three municipalities: Perdões (1), Boa Esperança (1), and Campo Belo (2). In keeping with the Guidelines for Animal Transport (GTA), the bulls were mainly originated from herds located in municipalities of southern Minas Gerais.

Non-castrated bulls in the slaughter line were selected for the study, with animals raised under confinement or less than 2 years old being excluded in order to increase the chances of sampling sexually active bulls. Convenience sampling was used to select 200 bulls (non-castrated males of mating age) of various genealogies from slaughterhouses in southern Minas Gerais during December 2013. Given the capacity of the slaughterhouses and availability of animals meeting these criteria, 200 samples of preputial smegma were collected in total, consisting of 116, 20, and 64 samples from the slaughterhouses in Campo Belo, Perdões, and Boa Esperança, respectively.

Samples were collected immediately after the animals had been stunned and slaughtered. The penis, including the prepuce, was sectioned using a knife and cleaned with single-use paper towels when excessive dirt was present. The penis was exposed on a tray using a rat-tooth forceps, and the entire penis and preputial regions were scraped with a spatula to retrieve the smegma. All tools, including trays, forceps, spatulas, and gloves, were changed after each sample to avoid cross-contamination. The smegma samples were stored in properly labeled sterile flasks containing 5 mL sterile saline solution, which were then placed in thermal boxes

at room temperature and promptly transported to the laboratory.

The methodology described by Pellegrin and Leite (2003) was used to isolate *T. foetus*. For this propose, a 200-µL aliquot of each freshly collected smegma sample was spread on Lactopep^{T} medium within 12 h after collection. The cultures were then placed in an incubator at 37 °C and analyzed by dark-field microscopy at 100× and 400× magnification every day between the 1st and 7th days of incubation. The presence of at least one protozoan exhibiting the morphology and motility typical of *T. foetus* was sufficient to categorize the sample as positive for this organism. Samples of *Trichomonas vaginalis* strain FMV-1 and *T. foetus* strain K were used as references to control the culture medium quality. After spreading a portion of each sample on Lactopep medium, the remaining smegma material was frozen at -20 °C for later use in molecular tests.

Simplex and multiplex PCR for the detection of the target disease agents (*T. foetus*, *C. fetus* subsp. *fetus*, and *C. fetus* subsp. *venerealis*) were carried out using DNA extracted from preputial smegma as a template. Prior to DNA extraction, the samples were treated in a concentrator (model 5301; Eppendorf, Hamburg, Germany) in order to increase the sensitivity of the molecular tests. The samples were concentrated by reducing their volume (in 0.85% saline solution at pH 7.2) from 1.5 mL to 150 µL.

DNA was extracted using a commercial kit (DNeasy® Blood & Tissue Kit; QIAGEN, Venlo, The Netherlands) following the manufacturer's instructions. The DNA samples were subsequently stored at -20 °C until their use in PCR assays, which were performed in a PT100 thermocycler (Applied Biosystems, Carlsbad, USA).

The ITS1 region of rDNA was amplified for the detection of *T. foetus* using the primers described by Felleisen et al. (1998), which generate a 347-bp amplicon (Table 1). Each PCR comprised a solution containing 3 µL 10× *Taq* polymerase buffer, 2.5 µL 50 mM $MgCl_2$, 1.0 mM dNTPs, 2 U *Taq* DNA polymerase, 10 µM each primer, 10 µL template DNA, and ultrapure water up to a final volume of 30 µL. Cycling conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 66 °C for 30 s, and 72 °C for 90 s; and a final extension at 72 °C for 15 min. DNA samples from *Trichomonas vaginalis* strain FMV-1 and *T. foetus* strain K, both isolated in the context of a clinical case, were used as positive controls in the molecular tests.

Table 1. Primers used in PCR for detection of *Tritrichomonas foetus* and in the multiplex PCR for detection of *Campylobacter fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* in samples of preputial smegma from bulls.

Microorganisms	Sequence of primers	PCR products	References
Tritrichomonas foetus	TFR3-5'CGGGTCTTCCTATATGAGACAGAACC-3' TFR4-5'CCTGCCGTTGGATCAGTTTCGTTAA-3'	347 pb	Felleisen et al. (1998)
Campylobacter fetus subsp. *fetus*	MG3F-5'GGTAGCCGCAGCTGCTAAGAT-3' MG4R-5'TAGCTACAATAACGACAACT-3'	750 pb	Hum et al. (1997)
Campylobacter fetus subsp. *venerealis*	VenSF-5'CTTAGCAGTTTGCGATATTGCCATT-3' VenSR-5'GCTTTTGAGATAACAATAAGAGCTT-3'	142 pb	

Multiplex PCR was carried out to identify *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* using the primers described by Hum et al. (1997) (Table 1). Each reaction comprised a solution containing 3 µL 10× *Taq* polymerase buffer, 2.5 µL 50 mM $MgCl_2$, 1.0 mM dNTPs, 1.5 U *Taq* DNA polymerase, 10 µM each primer, 10 µL template DNA, and ultrapure water up to a final volume of 30 µL. The following cycling conditions were employed: 95 °C for 5 min; 35 cycles of 95 °C for

30 s, 52 °C for 30 s, and 72 °C for 2 min; and a final extension at 72 °C for 7 min. For these molecular tests, *C. fetus* subsp. *fetus* ATCC 27374^T and *C. fetus* subsp. *venerealis* ATCC 19438^T were used as positive controls, while *C. jejuni* NCTC 10351 was used as the negative control.

The PCR products were subjected to electrophoresis on a 1.5% agarose gel for 2 h at 60 V, after which, the gels were stained with GelRed (Biotium, Fremont, USA) or 0.5 µg/mL ethidium bromide (Amresco, Solon, USA). Images of the gels were captured using a transilluminator (L-Pix Chemi Photo Digitizer; Loccus Biotecnologia, Cotia, Brazil) for subsequent analysis.

Results and Discussion

The use of bulls for the present study was based on the fact that they are regarded as an ideal sampling source due to their role as disseminators of BGC and BGT in breeding stock (ALVES et al., 2011), making them good epidemiological indicators of these venereal diseases (BONDURANT, 2005). Furthermore, since there are typically fewer bulls than cows in a cattle farm, the costs of diagnostic tests are lower (LAGE; LEITE, 2000; PELLEGRIN, 2002; BONDURANT, 2005).

Culture of smegma samples on Lactopep medium in order to isolate *T. foetus* failed to detect the presence of this parasite, even for samples with a positive PCR result. This may have been caused by the low sensitivity of the isolation method, which requires three consecutive negative results before an animal can be deemed free of infection by the agent in question (PELLEGRIN; LEITE, 2003). In addition, in the present work, it was not possible to observe a 45-day period of sexual rest before sample collection for BGT diagnosis by isolation, since the animals were sampled at the slaughterhouse. This may also explain the negative results of the culture tests for isolation of the *T. foetus*. According to Pellegrin and Leite (2003), the sensitivity of isolation is low when the animals to be sampled do not undergo this sexual rest period.

The absence of growth in the cultures for *T. foetus* isolation is consistent with studies performed in the states of Pernambuco (PAZ JÚNIOR et al., 2010) and Distrito Federal (LEAL et al., 2012), which also failed to detect this organism using the traditional isolation technique. Based on our results, the use of culture as the single diagnostic method for BGT may compromise the quality of the epidemiological data generated and the efficiency of disease control programs.

Regarding our molecular data, of the 200 samples analyzed, 8% (16/200) were positive for *T. foetus* (Figure 1), 17.5% (35/200) for *C. fetus* subsp. *fetus*, and 13.5% (27/200) for *C. fetus* subsp. *venerealis* (Figure 2). Four samples tested positive for both *T. foetus* and *C. fetus* subsp. *venerealis*, indicating the co-existence of both disease agents in certain animals. The occurrence of mixed infections with agents of venereal diseases may affect the reproductive performance of the animals and herds concerned, requiring the use of more onerous control measures and consequently causing greater economic losses.

In previous research concerning BGT carried out in Minas Gerais, Medeiros and Figueiredo (1971) determined this disease's prevalence to be 14.4%, whereas Leite et al. (1997) reported that only 5.9% of the suspicious samples sent for culture tested positive. A number of prior studies have also used PCR to diagnose BGT, revealing a prevalence of 32% in beef cattle in Spain (MENDOZA-IBARRA et al., 2012), 5.5% in beef cattle in Texas, USA (SZONYI et al., 2012), and 4.1% in African cattle (MADOROBA et al., 2011). However, there have been no reports of PCR being used as a diagnostic tool in the detection of BGT in Brazilian cattle.

Figure 1. Electrophoresis of PCR products on a 1.5% agarose gel to detect *Tritrichomonas foetus*. (1) Positive control (*T. foetus* strain K); (2) negative control (*Trichomonas vaginalis* strain FMV-1); (3) *T. foetus*-positive field sample; (4).100-bp DNA ladder.

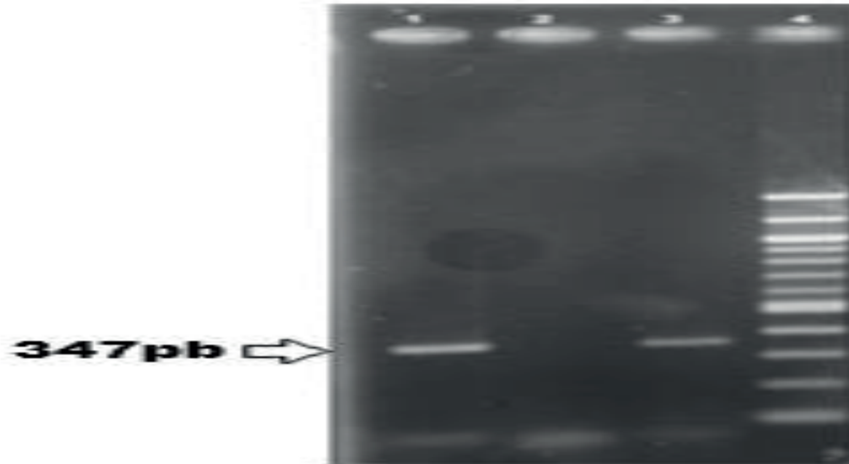
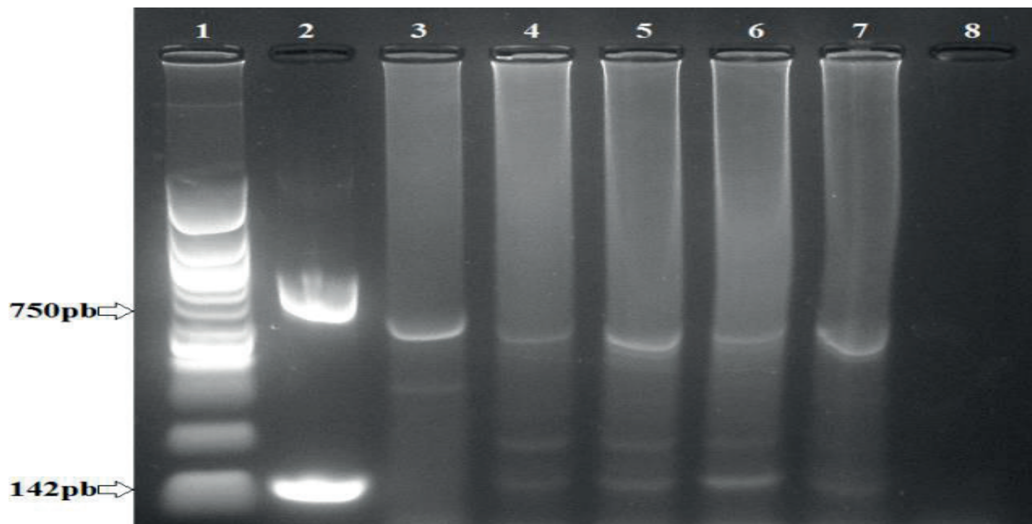


Figure 2. Electrophoresis of multiplex PCR products on a 1.5% agarose gel for the detection of *Campylobacter fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus*. (1) DNA ladder (100 bp); (2) positive controls (*C. fetus* subsp. *fetus* ATCC 273742; *C. fetus* subsp. *venerealis* ATCC 19438); (3) positive sample for *C. fetus* subsp. *fetus*; positive samples for *C. fetus* subsp. *venerealis* (4-7); (8) negative control (*C. jejuni* NCTC 103518).



In the present work, 17.5% (35/200) and 13.5% (27/200) of the animals tested were positive for *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis*, respectively. Estimates of the frequency of BGC reported in previous investigations of cattle herds in Minas Gerais have been extremely variable, ranging from 0 to 46.9% (ALVES et al., 2011). In this context, it is worth mentioning the study carried out by Stynen et al. (2003) in south and southeast Minas Gerais, in which direct immunofluorescence was used to reveal that 25.5% of breeding females were positive for BGC. One of the disadvantages of this technique employing to diagnose BGC is that it does not allow for discrimination between

C. fetus subspecies (RUCKERBAUER et al., 1974). According to our results, this can lead to an overestimation of the prevalence of this disease, considering that 17.5% (35/200) of samples were positive for *C. fetus* subsp. *fetus* and only 13.5% were positive for *C. fetus* subsp. *venerealis*.

Studies in which PCR has been used as a tool for the diagnosis of BGC remain scarce. Nevertheless, Madoroba et al. (2011) did successfully use this technique to establish the prevalence of *C. fetus* to be 1.9% among cattle in Africa. Moreover, in a study performed in Iran, Hamali et al. (2011) employed PCR to determine that 3.9% of the samples that they collected from miscarried fetuses or placentas were positive for *C. fetus* subsp. *venerealis*. In Brazil, Ziech et al. (2014) used PCR to detect *C. fetus* at the species level. In theory, this might also result in an overestimation of the prevalence of BGC as direct immunofluorescence, because *C. fetus* subsp. *fetus* is commonly isolated from genital secretions of bulls and cows without reproductive implications. According to Blaser et al. (2008), *C. fetus* subsp. *fetus* is the causal agent of sporadic reproductive problems in cattle mostly in association with gastrointestinal infections.

Our study revealed the occurrence of BGC and BGT in bulls sent to slaughterhouses in south Minas Gerais. Considering the infrequency with which artificial insemination is used in Brazilian cattle farming, estimated to be 11.9% by ASBIA (2014), and the fact that BGC and BGT are transmitted predominantly by natural mating (BONDURANT, 2005), our results underline the need to include these diseases in the differential diagnosis of bovine reproductive diseases and to improve and expand the infrastructure for their diagnosis among cattle herds in Brazil.

Parallels between the data concerning the prevalence of BGC (13.5%) and BGT (8%) in the present work and those generated in previous studies should be drawn with care, due to differences between experimental designs and diagnostic techniques that may have distinct specificities and levels of sensitivity (BONDURANT, 2005). It is worthy of note that the use of molecular techniques such as PCR, which is quick and easy and enables the analysis of large numbers of samples (HO et al., 1994), may help overcome the limitations of traditional diagnostic tests for BGC and BGT, as evidenced by the results of culture and isolation tests for *T. foetus* in the current study.

Conclusions

The results of the present study revealed *T. foetus*, *C. fetus* subsp. *venerealis*, and *C. fetus* subsp. *fetus* infection of bulls sent to slaughterhouses in southern Minas Gerais, indicating the occurrence of BGC and BGT among cattle in this region. For the identification of animals infected by *T. foetus*, the use of molecular tests proved more effective than detection by isolation.

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