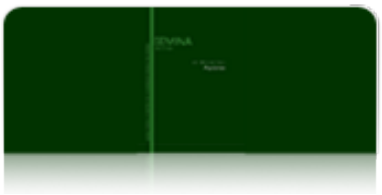

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# Occurrence of *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus* dna in bulls from Alagoas State, Brazil

# Ocorrência de dna de *Campylobacter fetus* subsp. *venerealis e Tritrichomonas foetus* em touros no Estado de Alagoas, Brasil

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## Abstract

The aim of the present study is to diagnose the occurrence of infections caused by *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus* in reproducer bulls from Alagoas State breeders, Brazil. The total of 162 preputial smegma samples were collected from nelore bulls from ten rural properties in the East, Agreste and Sertão mesoregions. The samples were subjected to the Polymerase Chain Reaction (PCR) technique in order to assess *C. fetus* subsp. *venerealis* and *Tritrichomonas foetus* DNA and cultivated in Modified Diamond Medium (DMM) for *Tritrichomonas foetus* isolation. Four point nine percent (4.9% - 8/162) of the evaluated bulls were infected with *C. fetus* subsp. *venerealis* and 3.0% (5/162) of the sample were infected with *T. foetus,* which was not isolated in any of the assessed animals. Based on our results, there was *C. fetus* subsp. *venerealis* and *T. foetus* DNA in bulls from Alagoas State, Brazil. Accordingly, it is necessary performing laboratory examinations in animals living in properties breeding animals for reproduction purpose in order to monitor and control such infections.
**Key words**: Campylobacteriosis. PCR. Bulls. Trichomoniasis.

## Resumo

Objetivou-se com esta pesquisa diagnosticar a ocorrência das infecções por *Campylobacter fetus* subsp. *venerealis e Tritrichomonas foetus* em touros, provenientes de propriedades localizadas no estado de Alagoas, Brasil. Foram coletadas 162 amostras de esmegma prepucial de touros da raça nelore, procedentes de dez propriedades rurais das mesorregiões Leste, Agreste e Sertão. Para a pesquisa de DNA de *C. fetus* subsp. *venerealis* e *Tritrichomonas foetus* as amostras foram submetidas à técnica Reação em Cadeia da Polimerase (PCR) e para isolamento de *Tritrichomonas foetus* foram cultivadas em Meio Diamond Modificado (DMM). Observou-se a ocorrência da infecção por *C. fetus* subsp. *venerealis* em 4,9% (8/162) dos touros analisados e 3,0% (5/162) para *T. foetus*. Não foi isolado *T. foetus* em nenhuma das amostras analisadas. Os resultados deste estudo confirmam a ocorrência de DNA de *C. fetus* subsp. *venerealis* e *T. foetus* em touros no estado de Alagoas, Brasil. Desta forma, constata-se a necessidade da realização de exames laboratoriais em propriedades de bovinos destinadas

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à reprodução para um monitoramento e controle dessas infecções.
**Palavras-chave:** Campilobacterioses. PCR. Touros. Tricomonose.

## Introduction

Bovine reproductive diseases influence herd's reproduction efficiency and, consequently, lead to significant economic losses. Moreover, lower reproduction success can be related to the presence of specific pathogens that in most cases are not diagnosed (BELLOWS et al., 2002).

Bovine genital campylobacteriosis and trichomoniasis are caused by *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus,* respectively. The infection caused by classical agents mainly happens at coitus through natural service (BONDURANT, 2005). These diseases have been mainly reported in extensive cattle production system due to the adopted reproduction management practices, which are often based on natural bred (MOLINA et al., 2013).

The infected bull becomes a reservoir and does not present the clinical signs of both diseases, fact that allows agents to long insidiously in herds. The difficulty in identifying the infected bulls, along with inappropriate management practices, makes intra-herd pathogen outbreak easy (MADOROBA et al., 2011).

Reproductive losses resulting from the herein addressed diseases are represented by infected animals' discharge and replacement (MADOROBA et al., 2011). Moreover, lack of diagnosis and monitoring are some of the factors contributing to the limited knowledge about the adverse effects caused by these diseases (MICHI et al., 2016).

Although bovine genital campylobacteriosis and trichomoniasis are Compulsory Notification Diseases (CND), there is no specific regulation about the need of diagnostic tests applied for bull trading purposes (MICHI et al., 2016). Such lack of regulation may favor the shortage of information about the real epidemiologic situation of such diseases (SWAI et al., 2005).

The aim of the present study was to diagnose infection caused by *C. fetus* subsp. *venerealis* and *T. foetus* in producer bulls from breeder farms located in Alagoas State, Brazil.

## Material and Methods

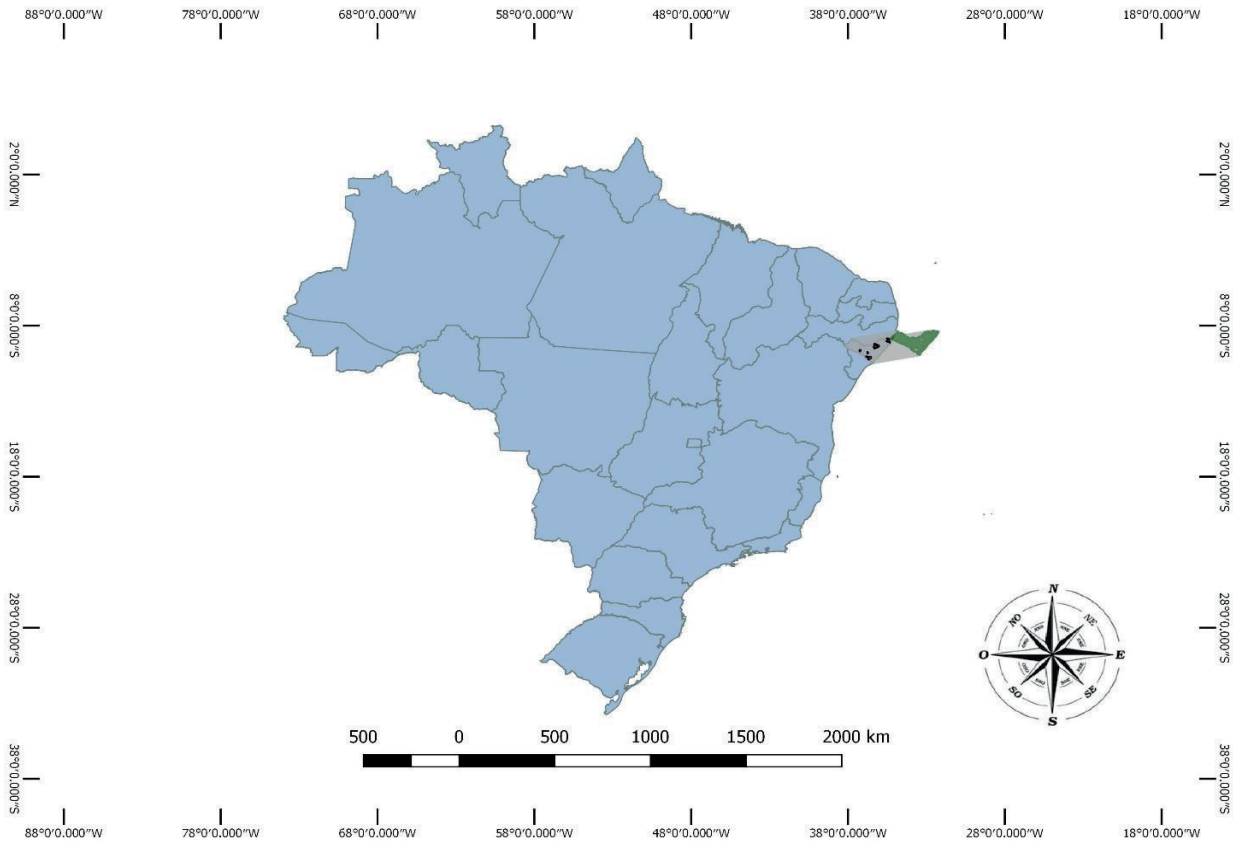
*Study site*

The research was conducted in ten counties located in Alagoas State mesoregions: East Alagoas (7) - Capela, Chã Preta, Igreja Nova, Jacuípe, Pindoba, Porto Calvo and Viçosa; Agreste (2) - Lagoa da Canoa and Olho d'Água Grande; Sertão (1): Jacaré dos Homens (Figure 1).

*Sampling*

Ten rural properties focused on bovine reproduction were selected through non-probabilistic convenience. The number of farms was limited since only few of them in Alagoas State are focused on breeding bovine reproducers of high genetic standard. The total of 1.264.053 bovines was taken into account to compose the sampling study (IBGE, 2016). The expected prevalence was 1.8% for *C. fetus* subsp. *venerealis* (OLIVEIRA et al., 2015) and 6.6% for *T. foetus* (OLIVEIRA et al., 2016), at confidence interval 95% and statistic error 5%. These parameters set the minimum sample of 28 bulls for bovine genital campylobacteriosis analysis and of 95 for trichomoniasis bovine evaluations. We herein collected 162 preputial smegma samples from nelore bulls in the age group 24 months or older, at sexual rest of 7-15 days.

**Figure 1.** Study site, counties holding bovine breeders focused on reproduction that compose East, *Agreste* and *Sertão* Alagoas.




*Biological material collection*

Preputial ostium haircut and sanitation with water and 70% alcohol were performed after animal restraining; water and alcohol excess were dried with the aid of paper towel. Next, a specific sterilized scraper was used for smegma collection; it was introduced in the preputial ostium to smear the preputial smegma. The biological material was transferred to flacon tubes containing 4mL of PBS (pH 7.2) after smearing. Subsequently, 1ml of sample was transferred to Diamond Modified Medium (DMM) for *T. foetus* isolation purposes. The samples were stored at room temperature and taken to the laboratory for proper processing.

*DNA extraction*

Smegma samples stored in DMM were subjected to DNA extraction in commercial "Wizard® SV Genomic DNA Purification System" (Promega®) kit, according to the manufacturer's protocol. The extracted DNA was analyzed and quantified in 1.5% agarose gel and molecular weight marker 100pb, stained with *Blue Green* (LGCbio), visualized under ultra-violet light, and photo-documented in order to have its quality checked.

*Molecular diagnostic tests*

The amplification reactions of *Campylobacter fetus* subsp. *venerealis* genomic material was carried out with oligonucleotides VenSF

(5′CTTAGCAGTTTGCGATATTGCCATT3′) and VenSR (5′GCTTTTGAGATAACAATAAGA GCTT3′), after sample DNA extraction. The reactions were amplified to 142pb, according to the protocol set by Hum et al. (1997).

Oligonucleotides TFR3 (5'CGGGTCTTCCTA TATGAGACAGAACC3') and TFR4 (5'CCTGC CGTTGGATCAGTTTCGTTAA3') were used in *T. foetus*. The reactions were amplified to 310pb, according to the protocol set by Felleisen (1997).

Reference DNA samples were used as the positive control of each agent and ultra-pure water was the negative control. The amplified material was detected through electrophoresis in 1.5% agarose gel stained with *Blue Green* (LGCbio), visualized under ultra-violet light and photo-documented.

*T. foetus isolation*

The DMM samples were incubated in microbiological stove at 37°C for 72 hours to isolate *T. foetus*. The samples were examined in dark-field microscope every 24 hours. Positive cultivation confirmation was based on the visualization of at least one living flagellated trophozoite presenting characteristic abrupt and undulating moves on each slide (DUFERNEZ et al., 2007).

## Results

The occurrence rate of infection caused by *C. fetus* subsp. *Venerealis* was 4.9% (8/162) and of 3.0% (5/162) for *T. foetus* when the PCR method was adopted. Isolation did not show *T. foetus* in any of the assessed samples.

Results of infections caused by *C. fetus* subsp. *venerealis* and *T. foetus* in bulls distributed by property are shown in Table 1. Co-infections were not observed in the analyzed animals; however, of the five propertys presenting positive animals, two (40.4%) had animals positive for *Campylobacter fetus* subsp. *fetus* or *Tritrichomonas foetus*.

**Table 1.** Occurrence of infections caused by *Campylobacter fetus* subsp. *venerealis* and *Tritrichomonas foetus* in bulls distributed by rural property focused on bovine reproduction in Alagoas State, Brazil.

| PROPERTY | REGION | N. OF SAMPLES | *C. fetus* subsp. *venerealis* | *T. foetus* |
|---|---|---|---|---|
| **A** | **EAST** | 17 | 5.9% (1/17) | 5.9% (1/17) |
| **B** | **EAST** | 18 | 27.8% (5/18) | 16.7% (3/18) |
| **C** | **SERTÃO** | 2 | - | - |
| **D** | **EAST** | 15 | - | - |
| **E** | **AGRESTE** | 4 | - | - |
| **F** | **AGRESTE** | 6 | - | - |
| **G** | **EAST** | 28 | - | - |
| **H** | **EAST** | 21 | 4.8% (1/21) | - |
| **I** | **EAST** | 33 | 3.0% (1/33) | |
| **J** | **EAST** | 18 | - | 5.6% (1/18) |

## Discussion

The present study is pioneer in reporting infection caused by *C. fetus* subsp. *venerealis* and *T. foetus* in bulls from Alagoas State, Brazil. The herein recorded results are similar to records from other Brazilian states (LEAL et al., 2012; OLIVEIRA et al., 2015), as well as from foreign countries, where there is prevalence lower than 10% (SWAI et al., 2005; MADOROBA et al., 2011; MOLINA et al., 2013). Higher infection prevalence has been reported; however, it is worth highlighting that variations in outcomes may result from sampling design, animal's age, sanitation and reproductive management, and from the adopted diagnostic

method. Madoroba et al. (2011) state that other variables, such as herd size and geographic area, can influence results in different researches.

Despite the low *C. fetus* subsp. *venerealis* (4.9%) and *T. foetus* (3.0%) occurrence in the present study, the importance of including the diagnosis of these agents in bulls from breeders focused on reproduction is noteworthy. These animals are reservoirs and can spread the agents through natural bred or through artificial insemination with contaminated semen (EAGLESOME; GARCIA, 1997; CAMPERO; COBO, 2006). Infections caused by these pathogens are enzootic, mainly in beef cattle bred in extensive cattle production systems, wherein sanitation control is poor and animals reproduce through natural bred, clean-up bull and artificial insemination with contaminated semen (BONDURANT, 2005).

Since bulls are asymptomatic, they should not stay with the herds, because the newly acquired animals in it must be infection-free. That is the reason why regulation tests must be conducted before the breeding season. Brazil does not have specific Bovine Genital Campylobacteriosis and Trichomoniasis control and eradication programs; however, it is recommended to replace the infected animals by younger bulls with health certificate (TRUYERS et al., 2014; YINZHU et al., 2014). With regard to Campylobacteriosis, it is possible associating antibiotics and vaccination (BONDURANT, 2005), but antibiotic therapy presents limited efficacy (MICHI et al., 2016).

Reproduction management in the visited breeders associates artificial insemination and natural bred with clean-up bull in females who did not have pregnancy confirmation 30 days after artificial insemination. It is known that the natural bred with infected animals and the use of clean-up bull in positive herds are risk factors for these sexually transmitted diseases (PELLEGRIN et al., 1999). The use of contaminated semen in association with natural bred are risk factors that enable *C. fetus* subsp. *venerealis* and *T. foetus* outbreak maintenance in bovine herds; consequently, these cows can be infected and infect other bulls in the herd (HANCOCK et al., 2015).

Collections showed that breeders who had positive animals recorded bull:female relation 1:30, on average, thus evidencing the possible outbreak potential of these pathogens in females. Such result can considerably influence herd reproduction indices. It is essential identifying infected bulls due to epidemiological aspects, since these animals can have contact with a large number of females throughout the reproduction period, fact that can facilitate agent transmission (HUM et al., 2009; MSHELIA et al., 2010). According to the review carried out by Bondurant (2005), *T. foetus* transmission rate through natural bred from infected bulls to susceptible females varies from 30% to 70%. The study conducted by Waldner et al. (2017) showed that herds presenting at least one infected bull had their pregnancy rates 2.35 times lower than herds without contaminated bulls.

With regard to the number of breeders with positive animals, 40% and 30% of them had at least one animal infected with *Campylobacter fetus* subsp. *venerealis* and *T. foetus*, respectively. One hundred percent (100%) of these breeders were located in East Alagoas State and traded reproducers with neighbor regions. However, based on the adopted sampling design, it is not possible stating that animals bred in the East region are more prone to infections caused by these agents, since the sanitation and reproductive managements adopted in all its properties were similar. The study carried out by Molina et al. (2013) in Argentina did not show homogeneous distribution of these diseases among different regions countrywide. They also indicated that the risk factors are similar for both infections.

It was observed that 100% of breeders have miscarriage records. Infection caused by *C. fetus* subsp. *venerealis* leads to reproductive issues such

as temporary infertility, heat repetition, embryonic death and miscarriage (JIMENEZ et al., 2011). Besides the aforementioned clinic signs, pyometra was observed in approximately 5% of cows with Trichomoniasis, due to the bacterial contamination that happens when the fetus is lost (BURNS et al., 2010).

The lack of infected-animal diagnosis enables agent maintenance, and consequences deriving from the infection mainly compromise cows' reproductive function (GONZÁLEZ-CARMONA et al., 2012; YAO et al., 2015). Thus, it is possible recording significant losses due to infertility and miscarriages, and it increases the interval between labors and calf production decrease (MARDONES et al., 2008).

Most positive samples in our study were from bulls in the mean age group 3.5 years. However, the age of animals positive for *C. fetus* subsp. *venereali*s and *T. fetus* in property B was 5 years or older. Young and old bulls present different ability to harbor *C. fetus* subsp. *venerealis* and *T. foetus*. The mucosa of bulls older than 5 years has a larger number of folds and deeper crypts, which can reduce oxygen supply and favor the susceptibility to infections caused by anaerobic microorganisms (CLARK et al., 1974). Overall, young bulls can produce less smegma in their penile mucosa, where folds and crypts are poorly developed. Such process impairs the establishment and persistence of microorganisms. In addition, one must take into account that young animals are more resistant to infection (EAGLESOME, GARCIA, 1997; MICHI et al., 2016).

Co-infections of both agents were not observed in the present study; however, two breeders (A and B) had *Campylobacter fetus* subsp. *venerealis* and *T. foetus* positive animals. Biological material collection in these two rural properties was performed before the bred season, and it may have favored a more precise diagnosis of both agents. The biological material collection time can influence infection-prevalence results, since the concentration of these agents in the preputial smegma tend to be weaker during the bred season (MOLINA et al., 2013).

It was not possible isolating *T. foetus* in any of the collected samples. These results are similar to those recorded by Rocha et al. (2009) and Mai et al. (2013), who did not isolate *T. foetus* in bull preputial-wash samples. The non-isolation of the agent may due to the parasite's low concentration in the analyzed samples, since 3.0% of the samples were positive in PCR. The PCR technique is the most sensitive in comparison to isolation, because it enables detecting the parasite's DNA even at low concentrations (MUTTO et al., 2006).

Although the parasite was not detected in the culture, it was possible visualizing mobile and round-shaped structures during the microscopic analysis. *T. foetus* morphological changes into the pseudocysts form have been reported (PEREIRA-NEVES et al., 2003; PEREIRA-NEVES; BENCHIMOL, 2009). Pereira-Neves et al. (2011) demonstrated that *T. foetus* pseudocysts are found in the fresh preputial smegma from bulls. These changes can be attributed to stress conditions such as room temperature variations and inhibitors of cultivation medium constituents (ONDRAK, 2010).

Regular bull-monitoring programs concerning venereal diseases become necessary, since they aim at reducing infection risks and, consequently, at improving bovine reproduction indices in order to increase production, profitability and sustainability, mainly when it influences the number of calves born per year. It is worth taking into account that this is one of the most important variables affecting the economic efficiency in bovine herds (COLLANTES-FERNÁNDEZ et al., 2014).

## Conclusion

The occurrence of infections caused by *C. fetus* subsp. *venerealis* and *T. foetus* in bulls from Alagoas

State, Brazil was confirmed. Accordingly, it is recommended to conduct laboratory examinations focused on sexually transmitted diseases in rural properties breeding reproducers in order to reduce the financial impacts caused by them.

## Ethical aspects

The research was approved by the Ethical Commission on Animal Use of Pernambuco Federal University (License n. 108/2015).

## Conflict of interest

The authors declare no conflict of interest.

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