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Prevalence of *Campylobacter fetus* subsp. *venerealis* in Dairy Cows from Brejo Paraibano, Brazil*

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ABSTRACT

***Background*:** Bovine genital campylobacteriosis (BGC) results in an increase in the interval between calving, increase in age at first calving, increase in the number of doses of semen or services by conception, and reduction in the number of animals born and weaned. Due to the importance of cattle breeding in Brazil, to the impact of BGC on bovine reproductive health, and since campylobacteriosis has never been studied in this region of Brazil, epidemiological studies on *C. fetus* infection in bovine herds are essential. The objective of this study was to determine prevalence of *Campylobacter fetus* subsp. *venerealis* infection in dairy cows from the Brejo Paraibano region, northeastern Brazil.

***Materials, Methods & Results*:** A cross-sectional study was conducted to determine prevalence of animals infected by *C. fetus* subsp. *venerealis*. In order to compose the sample of the number of farms, a total of 30 farming establishments with milk cattle and expected prevalence of 1.8%, 95% confidence interval (CI) and statistical error of 5% were considered, which provided a minimum of 15 farms. Samples of cervico-vaginal mucus were collected from 273 dairy cows from 19 farms. Polymerase chain reaction was used for laboratory diagnosis using the oligonucleotides VENSF1 (5'CTTAGCAGTTT-GCGATATTGCCATT3') and VENS2 (5'GCTTTTGAGATAACAATAAGAGCTT3') for detection of a 142 base-pairs product. In order to confirm the results, positive samples were purified after amplification and bidirectional sequenced. A thematic map was prepared with prevalence distributions in the studied area. The prevalence of *C. fetus* subsp. *venerealis* infection in cows was 7.7% (confidence interval [CI] 95%, 4.8%-11.5%), and 31.6% (6/19) of the farms showed at least one positive animal. Of the six counties surveyed, all (100.0%) had positive animals, with a positive farm per county. Regarding age, it was observed that all positive animals were between two and 15 years old, with a mean age of 6.2 years.

***Discussion*:** This is the first report of *C. fetus* subsp. *venerealis* infection in dairy cows in this region of Brazil. In this microregion, 7.7% (21) were positive in the PCR. Considering only the samples of females, in Brazil a result close to that of the present study was obtained in the Federal District and Goiás, where a prevalence of 10.5% (27/258) was determined using direct immunofluorescence (DIF) in samples of uterine and vaginal swabs from animals slaughtered in slaughter houses. However, the prevalence observed in the present study was lower than that generally reported, including in other regions of the country. In Minas Gerais, a prevalence of 25.5% (40/157) was found using DIF in samples of cervical-vaginal mucus from cows from herds with reproductive problems. In the state of Rio Grande do Sul, 13.6% of samples from cows were PCR positive. The use of high sensitivity tests, such as PCR, which can detect a small number of microorganisms, is important in studies of this nature. The prevalence of farms with positive animals, associated with the detection of infection in cattle of all the counties surveyed, makes it possible to affirm that *C. fetus* subsp. *venerealis* infection is present in cattle in the Brejo Paraibano microregion. This study demonstrates the presence of *C. fetus* subsp. *venerealis* DNA in dairy cows in the surveyed region. It is recommended to adopt an artificial insemination program on the farms, as well as a vaccination program to stimulate immunity in order to reduce the occurrence of infection and possible reproductive problems.

Keywords: bovine, Brazil, campylobacteriosis, PCR.

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INTRODUCTION

Bovine genital campylobacteriosis (BGC) is a contagious disease caused by *Campylobacter fetus* subsp. *venerealis* [17]. Transmission of *C. fetus* subsp. *venerealis* occurs sexually and females become infected after copulation with infected bulls or vice versa [1].

Reproductive problems such as BGC result in an increase in the calving interval by frequent repetition of oestrus, increase in age at first calving as the heifer category is the most affected, increase in the number of doses of semen or services by conception, and reduction in the number of animals born and weaned [9,23].

BGC is more prevalent in developing countries, where natural mating in cattle is widely practiced [18]. In Brazil, some epidemiological studies were carried out in different regions, with different frequencies of infection being reported in cattle, ranging from 1.8% [21] to 52.3% [22].

Some factors may contribute to the introduction or dissemination of the agent, such as the high number of animals in the herd [21], acquisition of animals from markets [12], lack of herd prophylactic measures [8,12,13], and herds in which the cattle graze communally during the rainy season but may cover large distances in search of pasture and water during critical period of dry season [13].

Due to the importance of cattle breeding in Brazil, to the impact of BGC on bovine reproductive health, and since campylobacteriosis has never been studied in the Brejo Paraibano microregion of Brazil, epidemiological studies on *C. fetus* infection in bovine herds are essential. Thus, this study aimed to determine the prevalence of *C. fetus* subsp. *venerealis* infection in cattle from the Brejo Paraibano microregion, Brazil.

MATERIALS AND METHODS

Study area

A cross-sectional study was conducted to determine prevalence of animals infected by *C. fetus* subsp. *venerealis*. The Brejo Paraibano microregion is part of the Agreste mesoregion of the state of Paraíba, and consists of eight counties: Alagoa Grande, Alagoa Nova, Areia, Bananeiras, Borborema, Matinhas, Pilões, and Serraria [7].

Sampling

In order to compose the sample of this study, the prevalence was estimated by means of a two-stage sampling design, primarily directed to detect farms with positive animals for *C. fetus* subsp. *venerealis* infection. In the first stage, the number of farms (primary sampling units) to be sampled was calculated using Win Episcope version 2.0 software[1].

The selection of the primary sampling units was based on the register of rural properties of cattle of the Secretariat of State for the Development of Agriculture and Fishery (SEDAP), being considered the farms with more than 50 bovines, since these properties are part of the cattle production chain. In order to compose the sample of the number of farms, a total of 30 farming establishments with milk cattle [6] and expected prevalence of 1.8% [21], 95% confidence interval (CI) and statistical error of 5% were considered, which provided a minimum of 15 farms.

Farms were selected randomly. The selected farm that for some reason could not be visited was replaced by another nearby with the same production characteristics. The selected farm that at the time of the visit had less than the 50 cattle previously estimated was also sampled. In total, 19 properties were sampled.

In relation to the farms sampled by county, the numbers were as follows: Areia (7); Serraria (1); Alagoa Grande (3); Bananeiras (6); Pilões (1); and Alagoa Nova (1).

The number of cows in each farm (secondary unit) was calculated using Win Episcope version 2.0 software, using the values of expected prevalence, statistical error, and CIs mentioned above. Thus, 273 samples of cervico-vaginal mucus were collected from adult dairy cows, non-pregnant, from July 2015 to May 2016.

Sampling of cows by county was as follows: Areia (n = 90), Serraria (n = 13), Alagoa Grande (n = 42), Bananeiras (n = 54), Pilões (n = 53), and Alagoa Nova (n = 21).

Collection of biological material

It was not possible to identify the stage of the estrous cycle due to the lack of record in zootechnical records by the owners. In order to collect the biological material, the cows were restrained and then a cleaning of the vulvar region was carried out with water and 70% alcohol, and later drying with paper towels. For collection of cervicovaginal mucus, the vulvae lips were separated and then a uterine lavage pipette coupled to a 20 mL syringe was introduced into the vagina to aspirate the mucus. Samples were transferred and

stored in tubes containing 5 mL of phosphate buffered saline (PBS, pH 7.2) and transported immediately to the laboratory for due processing [15].

Laboratory processing of samples

In the laboratory, samples were transferred to polypropylene tubes and submitted for DNA extraction using the "Wizard® SV Genomic DNA Purification System"[2] commercial kit, following the manufacturer's instructions.

After DNA extraction, the amplification reactions of the genomic material to *C. fetus* subsp. *venerealis* were performed using the oligonucleotides VENSF1 (5'CTTAGCAGTTTGCGATATTGCCATT3') and VENS2 (5'GCTTTTGAGATAACAATAAGAGCTT3'), as described by Hum *et al*. [5]. In addition, positive and negative controls were used in all reactions. As a negative control, ultra-pure water was used. As a positive control, a sample isolated from bovine in the state of Pernambuco was used. The thermal profile used was: initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 20 s; annealing at 50°C for 20 s and extension at 72°C for 2 min; and final extension at 72°C for 10 min. The PCR product was analyzed by electrophoresis in agarose gel at 1.5%, stained with Blue Green[3], visualized in a transilluminator with ultraviolet light, and photodocumented to detect product with 142 base pairs (bp). Amplicon sizes were determined in comparison to a 100 bp marker.

Sequencing

In order to confirm the results, positive samples were purified after amplification and bidirectional sequenced using the BigDyeTerminator v3.1 Cycle-Sequencing kit[4], according to the manufacturer's instructions. Sequencing was performed by capillary electrophoretic separation in an ABI 3500 Genetic Analyzer sequencer[4]. Data were collected using Data Collection Software[4] and underwent quality inspection through SequencingAnalysis Software[4]. After sequencing, the contigs were submitted to BLAST in the NCBI GenBank database (www.ncbi.nlm.nih.gov/BLAST/) in order to investigate correspondence in species identification.

Georeferencing

A thematic map was prepared with prevalence distributions in the studied area. Each property was georeferenced to define and visualize its location in physical space, i.e., identifying the farm properties on the map of the Brejo Paraibano microregion. The location of the properties was obtained with the aid of satellite tracking equipment (GPS-Global Positioning System), configured to provide the positions in the coordinate latitude/longitude system in the SAD-69 (South American Datum, 1969) system, which is the coordinate system of the cartographic base in the Brejo Paraibano microregion. In order to map the occurrence, the georeferenced data were processed in TerraView 3.1.3 software [2].

RESULTS

The prevalence of positive cows was 7.7% (21/273) (CI 95%, 4.8%-11.5%). Of the cattle herds, 31.6% (6/19) had at least one positive animal. Prevalence in positive herds ranged from 3.2% to 40.0%. Of the six counties surveyed, all (100.0%) had positive animals, with a positive farm per county (Table 1 and Figure 1). Regarding age, it was observed that all positive animals were between two and 15 years old, with a mean age of 6.2 years.

In the genetic sequencing, the samples presented had between 100% and 99% similarity with *C. fetus* subsp. *venerealis*.

DISCUSSION

This is the first report of *C. fetus* subsp. *venerealis* infection in cattle in the state of Paraíba, northeastern region of Brazil. In this microregion, 7.7% (21) were positive in the PCR. Researches in several parts of the world have revealed prevalence's ranging from 0.0% [15] to 15.0% [3]. Information regarding prevalence and outbreaks of the disease is not regularly reported by many countries [19], and this may be due to the lack or inefficiency of surveillance and monitoring programs on these sites. The difficulty of diagnosing BGC is well reported; this may be related to deficient infrastructure, lack of proper isolation media, and the sensitive nature of the bacteria. This difficulty has been the major limitation of the control of this disease in developing countries [18].

Considering only the samples of females, the prevalence observed in the present study was lower than that generally reported, including other regions of the country.

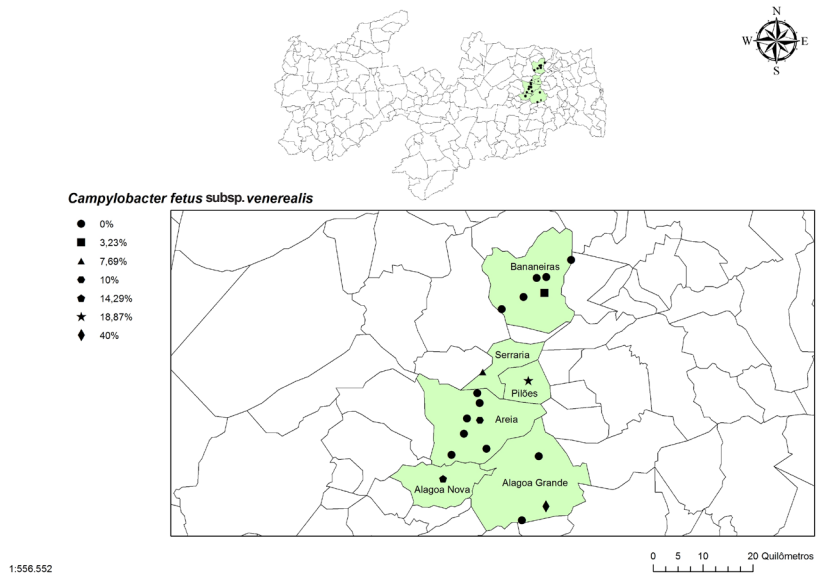

Figure 1. Distribution and prevalence of *Campylobacter fetus* subsp. *venerealis* infection in Brejo Paraibano microregion, Paraiba, Brazil. Upper map: Paraiba state; Bottom map: surveyed counties.

Table 1. Prevalence of dairy cows positive for *Campylobacter fetus* subsp. *venerealis* in Brejo Paraibano microregion, Brazil.

County	Nº samples	Positive samples	%	Nº farms	Positive farms	%
Alagoa Grande	42	4	9.5	3	1	33.3
Farm A	4	0	0			
Farm B	28	0	0			
Farm C	10	4	40			
Alagoa Nova	21	3	14.3	1	1	100.0
Farm D	21	3	14.3			
Areia	90	2	2.2	7	1	14.3
Farm E	6	0	0			
Farm F	15	0	0			
Farm G	25	0	0			
Farm H	20	2	10.0			
Farm I	8	0	0			
Farm J	8	0	0			
Farm K	8	0	0			
Bananeiras	54	1	1.8	6	1	16.7
Farm L	2	0	0			
Farm M	3	0	0			
Farm N	5	0	0			
Farm O	8	0	0			
Farm P	31	1	3.2			
Farm Q	5	0	0			
Pilões	53	10	18.9	1	1	100.0
Farm R	53	10	18.9			
Serraria	13	1	7.7	1	1	100.0
Farm S	13	1	7.7			
Total	273	21	7.7	19	6	31.6

It is difficult to accurately compare the results of this research with previously published studies because the gender of the animals among the studies may vary. Also, since males generally remain chronically infected and females can clear the infection after a period of sexual rest, the prevalence directed to bulls and herds with a history of reproductive problems may be higher. The differences between the performed studies in prevalence may also be due to other factors such as type of farming (extensive, semi-intensive, and intensive), time the samples were collected, use of artificial insemination, sample transport time to laboratory, differentiation between subspecies of *C. fetus*, and sexual rest before collection [16].

In some studies, the authors do not use an experimental design to estimate prevalence, only doing surveys specifically on problem farms and on animals with reproductive problems, as well as using different diagnostic techniques that vary in sensitivity and specificity [10,24]. In this study, the farms and the animals sampled were randomly selected, regardless of whether or not they presented characteristic clinical signs of the disease. This provided the general prevalence in the studied region, which was not only taken from herds with reproductive problems, and may also explain the lower prevalence found compared to other studies, since animals with infertility problems are more likely to be *C. fetus* subsp. *venerealis* positive.

The methods of collection, pregnancy, and the phase of the estrous cycle can also influence the quality of the collected material. The cows in this study were not pregnant, but it was not possible to identify the stage of the estrous cycle due to the lack of record in zootechnical records by the owners. The number of microorganisms in the vaginal mucus and the amount of secretion obtained are higher during estrus, which may decrease the sensitivity of diagnostic tests for carrier females, since not all collected females are in this condition [24,25], which probably occurred in this study, and may have influenced the detection of the agent DNA, and consequently the prevalence may be higher than that found. Therefore, it is recommended to collect from females in the estrus period and to use estrus synchronization when the objective is to obtain representative sampling of the herd [25], since a larger number of cows will be in heat at the same time during a visit to the farm. However, the use of drugs for synchronization only for collection, when the rural producer does not yet use this tool as a reproductive strategy, increases costs for the owner in the case of routine diagnosis or for the researcher when performing epidemiological surveys like this one.

Therefore, the use of high sensitivity tests, such as PCR, which can detect a small number of microorganisms, is important in studies of this nature. In the work of Groff *et al*. [4], the PCR technique was more sensitive and specific for the detection of *C. fetus* when compared with traditional isolation methods. The results of McMillen *et al*. [14] confirmed the sensitivity and specificity of the PCR assay by detecting an additional 40 bulls that were not detected by culture. The PCR is also faster and can be used as an effective alternative for genital campylobacteriosis diagnosis in cattle [4].

In general, epidemiological surveys should take into consideration the factors that may influence prevalence [11].

C. fetus subsp. *venerealis* was detected in six (31.6%) of the cattle herds sampled. The prevalence of farms with positive animals, associated with the detection of infection in cattle of all the counties surveyed, makes it possible to affirm that *C. fetus* subsp. *venerealis* infection is present in cattle in the Brejo Paraibano microregion.

Because of the damages that *C. fetus* subsp. *venerealis* infection can cause, the knowledge of distribution of this agent is important and the differences between regions should be considered. The prevalence rates found are, in general, randomly scattered in the study area. The spatial analysis of Molina *et al*. [16] showed that those herds in the center-south of La Pampa province (Argentina) were more likely to contain bulls infected with *C. fetus* than herds located in other areas of the province.

The vast spread of infectious agents constitutes a constant challenge for those involved with animal health, particularly in relation to control. Spatial epidemiology is useful for the visualization of areas at risk for infection and serves to rapidly alert of impending problems that need immediate action [20].

It is probable that the introduction of infected animals and lack of knowledge on the part of dairy farmers could have been responsible for the introduction and maintenance of the agent in the herds [21]. Considering the number of positive herds, BGC should

be considered in investigations of infertility problems in dairy cows from herds in this region.

Although the introduction of infected cows into breeding herds may contribute to the spread of BGC, non-virgin bulls involved in commercial exchanges are the major cause of spread [16]. Because of its epidemiological characteristics, mainly because it is a venereal transmission disease, BGC should be understood as a disease of the herd [23].

CONCLUSION

This study demonstrates the presence of *C. fetus* subsp. *venerealis* DNA in dairy cows in the surveyed region. Considering the percentage of farms with positive animals, an artificial insemination program should be adopted on the properties, as well as a vaccination program to stimulate immunity with the aim of reducing the prevalence of positive animals.

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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