

Bovine Genital Campylobacteriosis in Pantanal, State of Mato Grosso do Sul, Brazil

A.O. Pellegrin^{1,2} A.P. Lage^{2*} J.R.B. Sereno¹
E. Ravaglia¹ M.S. Costa¹ R.C. Leite²

Key words

Cattle – Bull – *Campylobacter fetus* subsp. *venerealis* – Fluoroimmunoassay technique – Epidemiology – Pantanal – Brazil.

Summary

Prevalence of bovine genital campylobacteriosis was estimated in bulls of Pantanal, Mato Grosso do Sul, Brazil. Preputial washings of 327 bulls from 19 farms were submitted to the direct fluorescent antibody test. One hundred seventy bulls (52.3%) from 17 farms (89.5%) were positive. There was no difference in the number of positive bulls when considering the various age groups. There was a significant difference in farms with areas larger than 15,000 ha ($\chi^2 = 6.40$; $df = 1$; $P = 0.01$) with an estimated relative risk of 1.33 ($1.07 < RR < 1.66$) compared to farms smaller than or equal to 15,000 ha. These results indicated that bovine genital campylobacteriosis was widespread in the studied region, probably as a result of management practices.

INTRODUCTION

With a herd of approximately 3.8 million animals, beef cattle breeding has been the most important economic activity for almost two centuries in Pantanal, the largest flooded plain in South America (5), which covers a 139,558 km² area (31). In Pantanal, Nelore bulls (purebred and zebu crossbred) are either animals culled from the breeding regions located in the margins of the plain or animals selected among the young males of the herd (28). Andrological examinations, libido tests and venereal disease diagnosis are rarely performed. Brucellosis is the only reproduction-related disease actually diagnosed. In Pantanal, the bull to cow ratio varies from 1:10 to 1:17, which renders difficult the acquisition of high quality bulls because of the huge number of bulls to be acquired each year (28). Due to the lack of infrastructure and skilled labor, artificial insemination has a limited use in Pantanal, stressing the importance of careful bull selection (27).

Bovine genital campylobacteriosis (BGC) is a venereal disease caused by *Campylobacter fetus* subsp. *venerealis* and characterized mainly by infertility and embryonic death, as evidenced by estrus repeated at irregular intervals, and by abortion (9). Bulls are asymptomatic carriers; the agent is found in the preputial mucosa and is transmitted to almost every susceptible female by mating or by contaminated semen (11). The occurrence of BGC both in bulls and in cows has already been reported in several Brazilian regions, with 8.0 to 72.3% infection rates (14). There is, however, very little data on the situation in Pantanal (25). The objectives of the present study were to estimate BGC prevalence in Pantanal region, Mato Grosso do Sul State, Brazil, and to evaluate the effects of animal age and farm size on the rates obtained.

MATERIALS AND METHODS

Sampling

Cluster sampling was used to assess BGC prevalence in Pantanal region, based on an estimated 56% prevalence (25), 95% confidence interval and 11% error (3). This option was chosen because there is no general register of animals and random sampling would have required visiting a large number of farms, covering large distances, thus increasing the cost of the survey. An average of 20 animals were sampled per cluster. An intraclass correlation coefficient of 0.20 was used to calculate the design

1. Empresa Brasileira de Pesquisa Agropecuária, Centro de Pesquisa Agropecuária do Pantanal, Rua 21 de Setembro 188, 79320-900 Corumbá, MS, Brazil
Fax : +55 67 231 10 11; e-mail: aiesca@cpap.embrapa.br

2. Laboratório de Bacteriologia Aplicada, Núcleo de Pesquisa em Saúde Animal, Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Caixa Postal 567, 30123-970 Belo Horizonte, MG, Brazil
Fax: +55 31 34 99 20 80; e-mail: alage@vet.ufmg.br

* Author for correspondence

effect (23). The sample size was calculated using the correction for cluster sampling according to Cochran (8). The number of clusters to be sampled was calculated according to Otte and Gumm (23). This resulted in a sample size of 380 animals from 19 farms. The farms selected were randomly chosen from a list of those registered in EMBRAPA-Pantanal.

Preputial washings

Preputial washings were collected in phosphate buffered saline (PBS, pH 7.4) and stored at 4°C until the test was performed (17). Collections were obtained after a minimum of 15 days sexual rest.

Direct Fluorescence Antibody Test

The direct fluorescence antibody test (DFAT) followed the methods of Mellick *et al.* (21) and Winter *et al.* (38). Preputial washings were centrifuged at 600 xg for 10 min to obtain a supernatant, which was then centrifuged at 13,000 xg for 30 min. The sediment was resuspended in 500 µl PBS (pH 7.4) and homogenized. Two 20 µl samples of this suspension were placed within the marks of an immunofluorescence slide. The slides were air-dried at room temperature and fixed in acetone at -20°C for 30 min. They were then covered with 20 µl of an anti-*Campylobacter fetus* subsp. *venerealis* fluorescein conjugate (29) at 1/128 dilution, prepared in rabbit with *C. fetus* subsp. *venerealis* NCTC 10354 (10). After incubation at 37°C for 30 min in a moisture chamber, the slides were washed three times with PBS (pH 7.4) during 10 min to remove excessive conjugate. Buffered glycerin (pH 9.2) was used to mount the slides with a coverslip. The positive control was a *C. fetus* subsp. *venerealis* NCTC 10354 cultured in BHI agar (Difco, USA) at 37°C for 48 h in a microaerophilic environment. An epifluorescence microscope (Carl Zeiss, Germany) with 40x and 100x objectives was used to examine the smears. Preputial washings were considered positive when fluorescent bacteria with typical *C. fetus* morphology ("S"-, comma- or gull wing-shaped spiral bacteria) were found.

Farms area and animals age

To study the effect of their area (Table I) on the frequency of bulls positive to *C. fetus* DFAT, the farms were divided into two categories: < 15,000 ha and ≥ 15,000 ha. The influence of age was studied in 90 animals from three farms that were grouped into two categories: six years of age or less, and older than six years of age, considering that in Pantanal most bulls are found in this older age group (25).

Statistical analysis

Prevalence, confidence interval (CI), design effect and intracluster correlation coefficient were calculated according to Bennett *et al.* (3). Real prevalence was estimated according to Martin *et al.* (18), adjusting the test prevalence obtained, with 88.9% specificity and 92.6% sensitivity, previously determined for DFAT (10). The frequency distribution of bulls positive to *C. fetus* DFAT was compared in the various categories (age and farm area) using the chi-square test, using α error equal to 0.05 (30), and relative risk (RR) (18).

■ RESULTS

Some samples were lost during transportation to the laboratory and in some farms it was not possible to sample 20 bulls per farm; therefore, only 327 preputial washings were processed. Preputial washings from 171 animals were positive to *C. fetus* DFAT (52.3%) with a 95% confidence interval of 42.6 to 62.0% (Table I). Seventeen out of 19 herds sampled

had positive bulls, prevalence ranging from 14-75%. Only two animals were tested in each of the negative herds. Real prevalence was estimated at 51.7%, within the calculated confidence interval. The design effect (D) value and intracluster correlation coefficient (ρ) were 3.20 and 0.14, respectively. A significant difference was found between the frequency of bulls positive to *C. fetus* DFAT in the two farm categories ($\chi^2 = 6.40$; df = 1; P = 0.011) (Table II). The relative risk was estimated at 1.33 (1.07 < RR < 1.66) for farms larger than 15,000 ha. When animals six years of age or older were compared with younger animals, there was no significant difference in prevalence ($\chi^2 = 1.24$, df = 1; P = 0.26) (Table III).

Table I

Frequency of *Campylobacter fetus* DFAT-positive bulls, and area of the studied farms in Pantanal, Brazil

Herd	Area (ha)	DFAT		
		Num. of animals sampled	Positive	%
F1	6732	9	4	44.4
F2	20,000	19	9	47.3
F3	12,300	19	12	63.2
F4	14,600	7	1	14.3
F5	112,000	44	33	75.0
F6	9000	2	1	50.0
F7	36,700	2	0	0.0
F8	16,400	19	12	63.2
F9	13,781	2	0	0.0
F10	1800	9	3	33.0
F11	25,500	18	13	72.2
F12	21,000	31	11	35.5
F13	4400	39	20	51.3
F14	25,000	15	11	73.3
F15	14,300	19	10	52.6
F16	26,000	12	4	33.3
F17	29,422	20	13	65.0
F18	9600	21	3	14.3
F19	14,000	20	11	55.0
Total	412,535	327	171	52.3*

DFAT: direct fluorescence antibody test

* 95% CI (42.6–62.0%)

Table II

Frequency of *Campylobacter fetus* DFAT-positive bulls by area of the farm in Pantanal, Brazil

Area (ha)	DFAT	
	Negative	Positive
≤ 15,000	82	65
> 15,000	74	106
Total samples	327	171

DFAT: direct fluorescence antibody test

$\chi^2 = 6.40$; df = 1; P = 0.011; RR = 1.33 (1.07 < RR < 1.66)

Table III

Frequency of *Campylobacter fetus*-positive bulls by age group from three farms in Pantanal, Brazil

Age group (years)	DFAT (%)		Total
	Positive	Negative	
≤ 6	20	8	28
> 6	35	27	62
Total	55	35	90

DFAT: direct fluorescence antibody test

$\chi^2 = 1.24$; $df = 1$; $P = 0.26$

DISCUSSION

Results of the present study indicate that BGC was frequent in beef bulls used in the survey area. The 52.3% prevalence was higher than that reported in other Brazilian regions (12, 13, 14, 15). Although some reported results were similar, they were not comparable because some authors used non-probabilistic sampling (15) or only studied herds with a recent history of reproductive disorders (13).

Bovine genital campylobacteriosis is widely distributed in Argentina, where natural breeding is most common, similarly to the Brazilian system. The rate of *C. fetus*-positive herds in Argentina varies from 21 to 43% and the frequency of bulls infected by *C. fetus* subsp. *venerealis* varies from 15 to 23% (6, 34, 37).

The diagnosis sensitivity is directly related to the method, either isolation or DFAT, to the collection frequency and mainly to the way the material is packaged and transported from the farm to the laboratory (35). Repeated sample collection in the same animal decreases the chance of a false negative result (35). To obtain maximum sensitivity in DFAT the recommendation is to obtain three samples from the same animal at 15-day intervals, with sexual rest before and also between collections (14).

Bulls in this experiment were sampled only once, because the animals had no identification, management was difficult and returning to the farm would have increased the costs involved. Thus, the determined prevalence could have been higher if two or three samples had been collected from each animal (35).

Indeed, apparent prevalence (52.3%) was not overestimated. Having used a more flexible accuracy (11%) to estimate prevalence, the real prevalence (51.7%) was within calculated confidence interval (95% CI: 42.6–62%).

Prevalence studies can be based on the examination of a group of animals at a certain point in time or during a time interval. Both are valid expressions, providing information solely about the risk of an animal in that population to have the disease (32). In this experiment, sampling 19 farms estimated prevalence in the time period from April 1995 to November 1996. Samples were not obtained, however, in the period between December 1995 and March 1996, as it was not possible to reach the farms because the area was flooded.

There is no breeding season in most farms of Pantanal, and the bulls are kept with the cows the whole year round (36). Even so, there are parturition peaks suggesting the existence of a natural

breeding season in spring and early summer, October through January (1). This probably coincides with better pastures, allowing cows to gain weight and come into heat. Sample collections for the present study were obtained mainly during fall and winter months, the only period when all the farms can be reached by road. It is also the time when bulls have a natural sexual rest period.

The ρ value found in this study was close to that estimated by Otte and Gumm (23) for moderately contagious diseases, confirming that the 0.20 value is a good estimate to be used in calculating the sample size (number of clusters) when the true ρ is not known. On the other hand, McDermott and Schukken (20) indicate that the intraclass correlation coefficient can vary from 0.0017 to 0.46, although it is between 0.01 and 0.15 for most diseases, confirming what is suggested by Otte and Gumm (23) and was found in this study. In practice, BGC was homogeneously distributed in the population of the studied region, with a high rate of positive herds.

The intraclass correlation coefficient also influenced the calculation of the confidence interval (95%) that was more accurate (9.7%) than the confidence interval initially used to calculate the number of clusters. As a disease related to certain management practices, as natural breeding, bovine genital campylobacteriosis has a higher prevalence in farms where this practice is used. This correlation was found in the present study in all farms with natural breeding. Calculation of the design effect is based on sampling error, which is higher in cluster sampling (3). The design effect was influenced by the number of sampled clusters and by the irregular size of the samples in each. This resulted from material lost between collection and arrival at the laboratory and difficulties in gathering 20 bulls at the same time in some of the farms.

Problems related to sample transport are always a major obstacle in epidemiological studies in Pantanal that require sampling a large number of farms. Unpaved roads or even tracks are used to travel from a village to the farms. Thus, the time needed to reach the sampling site is increased, it is difficult to visit several farms in a short period of time and the costs of the sampling procedure are higher. As the region has no electricity, ice has to be transported from the laboratory to the farm in order to store the material under ideal temperature. As observed in this study, high values for the design effect have to be used in calculating the number of samples in studies that will be carried out in the region.

In only two of the farms infected bulls were not found. The samples obtained from these farms may not have been large enough to detect the disease. The high frequency of farms with infected animals shows once again that BGC is widespread in the region.

In Argentina, prevalence of bovine genital campylobacteriosis was thought to influence herd fertility (34). The authors report 65.2% incidence in farms with fertility problems and 35.2% in farms with unknown fertility, and consider that BGC is exerting a direct influence on reproduction rates. In the studied farms of Pantanal, however, fertility rates were not taken into consideration, as they were largely unknown because it was not a common practice to establish pregnancy diagnoses. In Pantanal, the birth rate is estimated by observing lactating cows or presence of calves. Perinatal mortality is also unknown as calves that were aborted, are weak or die at birth or fall prey to the wild carnivores of the region.

Bulls infected with *C. fetus* subsp. *venerealis* can markedly reduce herd fertility (22). A low calving rate was observed in the evaluation of a group of virgin heifers bred by infected bulls in the same region (24), results showing 59.3% calving rates, following pregnancy rates of 37 and 63% at 90 and 120 days, respectively.

The effect of the farm size on the occurrence of positive bulls may be related to the number of bulls being used and the number of subdivisions per farm. Cadavid Garcia (5) reports that farms with areas between 1000 and 3600 ha usually have a mean of four subdivisions, while farms with more than 14,400 ha have an average of 8.17 subdivisions, showing that while the farm size increases four times the number of subdivisions does not increase proportionally. In farms with areas between 1000 and 3600 ha, the average number of breeding cows and bulls was 325 and 30, respectively, and in farms with more than 14,400 ha this number was 3210 and 224. As multiple breeding is a common practice in Pantanal, the larger farms use almost twice as many bulls per subdivision than the smaller farms.

There is also a proportional increase in the number of older bulls (> 8 years of age); it is estimated that they represent 25% of the total number of bulls in the farm (25). This is probably also true for the other farms in the sample, although this information cannot be obtained in every farm because of the lack of individual animal identification. Due to social dominance, older bulls breed a larger number of cows (2), increasing the risk of infection by *C. fetus* (7, 26, 33).

Mainly because of the increase in prepuce crypts, older bulls are more susceptible to infection by *C. fetus*, as the microaerophilic conditions needed by the agent to survive in the prepuce are optimized (7). Although a relationship between the animal age and *C. fetus* infection can be found in some cases (7, 26, 33), this has not always been observed (4, 19). This is probably because bulls are considered to be young when they are 2 to 3 years of age, since 50% of the bulls are introduced in Pantanal at 30 months of age. A bull 5 years of age or older has already participated in at least two breeding seasons (28) and is still considered as young. Data collected in three farms of this study did not indicate any significant difference in the infection rates of old and young bulls. This is probably because bulls start breeding later in Pantanal than in other regions, at the age of 3.5 to 4.0 years, when the prepuce crypts are already well developed, and they are still considered young.

The high prevalence in the studied herds suggests that bovine genital campylobacteriosis is an important disease contributing to low reproduction rates of Pantanal cattle herds. It emphasizes the need for a higher number of bulls in the herd, as several matings may be required before an infected cow becomes pregnant (16). As a result, profitability from cattle breeding in the area has decreased because of increases in production cost, interval between calvings and age at first birth.

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Résumé

Pellegrin A.O., Lage A.P., Sereno J.R.B., Ravaglia E., Costa M.S., Leite R.C. Campylobactériose genital bovina dans le Pantanal, Etat du Mato Grosso do Sul, Brésil

La prévalence de la campylobactériose genital bovina a été estimée chez des taureaux au Pantanal, Mato Grosso do Sul, Brésil. La recherche des anticorps par immunofluorescence directe a été effectuée avec les prélèvements des lavages des prépuces de 327 taureaux appartenant à 19 élevages. Ce test a révélé 170 taureaux (52,3 p. 100) positifs, provenant de 17 élevages (89,5 p. 100). Il n'y a pas eu de différence dans le nombre de taureaux positifs lorsque les différents groupes d'âge ont été considérés. En revanche, il y a eu une différence significative dans les élevages de plus de 15 000 ha ($\chi^2 = 6,40$; $df = 1$; $P = 0,01$), avec un risque relatif estimé de 1,33 ($1,07 < RR < 1,66$), par rapport aux élevages de 15 000 ha ou plus petits. Ces résultats ont indiqué que la campylobactériose genital bovina était répandue dans la région étudiée et était probablement liée au mode de gestion des troupeaux.

Mots-clés : Bovin – Taureau – *Campylobacter fetus* subsp. *venerealis* – Technique fluoroimmunologique – Epidémiologie – Pantanal – Brésil.

Resumen

Pellegrin A.O., Lage A.P., Sereno J.R.B., Ravaglia E., Costa M.S., Leite R.C. Campilobacteriosis genital bovina en Pantanal, estado de Mato Grosso del Sur, Brasil

La prevalencia de la campilobacteriosis genital bovina fue estimada en toros de Pantanal, estado de Mato Grosso del Sur, Brasil. Los lavados de prepucio de 327 toros de 19 fincas se sometieron al test de fluorescencia directa de anticuerpos. Ciento setenta toros (52,3%) de 17 fincas (89,5%) fueron positivos. No hubo diferencia en el número de toros positivos cuando se consideraron los diferentes grupos de edad. Hubo una diferencia significativa en fincas con más de 15 000 ha ($\chi^2 = 6,40$; $df = 1$; $P = 0,01$), con un riesgo relativo estimado de 1,33 ($1,07 < RR < 1,66$) comparado con fincas más pequeñas o iguales a 15 000 ha. Estos resultados indican que la campilobacteriosis genital bovina está altamente distribuida en la región estudiada, probablemente como resultado de las prácticas de manejo.

Palabras clave: Ganado bovino – Toro – *Campylobacter fetus* subsp. *venerealis* – Técnica de inmunofluorescencia – Epidemiología – Pantanal – Brasil.