Bovine campylobacteriosis in bulls: insights in the conventional and molecular diagnosis

Juan A. García (garcia.juanagustin@inta.gob.ar)
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

Pablo Farace
Instituto Nacional de Tecnología Agropecuaria, Consejo Nacional de Investigaciones Científicas y Técnicas (IABIMO INTA-CONICET)

Andrea K. Gioffré
Instituto Nacional de Tecnología Agropecuaria, Consejo Nacional de Investigaciones Científicas y Técnicas (IABIMO INTA-CONICET)

Claudia Morsella
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

María A. Méndez
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

Joaquín Acuña
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

Juan F. Aller
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

Marcelo Signorini
Instituto de Investigación de la Cadena Láctea (INTA – CONICET)

Fernando A. Paolicchi
Instituto de Innovación para la Producción Agropecuaria y el Desarrollo Sostenible (IPADS INTA-CONICET)

Research Article

Keywords: venereal disease, bovine campylobacteriosis, ruminants, diagnosis

Posted Date: May 23rd, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1670751/v1

Abstract

Campylobacter fetus is a gram-negative motile bacterium, with two subspecies relevant for cattle health: *C. fetus* subsp. *venerealis* (Cfv) and *C. fetus* subsp*. fetus* (Cff). Both subspecies are associated with reproductive losses in cattle. Herein, the *C. fetus* infection of three Angus bulls naturally challenged with Cfv, Cfv biovar intermedius (Cfvi) or Cff were assessed through conventional and molecular diagnosis of preputial smegma (PS) samples collected with different devices. The Cfv- and Cfvi-bulls were positive until at least 9 months. Although Cff is not considered a venereal strain, this study demonstrated its transmission to bull from heifers experimentally infected, with evidence of colonization and persistence in the preputial cavity for 5 to 6 months. This finding suggests a potential risk of dissemination of the bacteria within herds. The results obtained by bacteriological culture or direct immunofluorescence (DIF) showed no significant differences, regardless the sampling device used (aspiration with Cassou pipette, metal and plastic scraper). On the other hand, the qPCR for *C. fetus* detection yielded better results with an in-house DNA extraction method than with a commercial kit (75% vs 66.6%). Furthermore, the molecular diagnosis by qPCR was more efficient than culture (66.6%) or DIF (56%). Bacteremia in whole blood samples was negative by qPCR and bacteriological culture in all samples. PCR-based methods are promising for the diagnosis of Bovine Genital Campylobacteriosis from clinical samples of PS. This study demonstrated the transmission of Cff from heifers to bull.

Introduction

Campylobacter fetus is a gram-negative motile bacterium, with two subspecies relevant for cattle health: *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv) (Michi et al. 2015). *C. fetus* subsp. *fetus* inhabits the intestine of cattle and sheep, but may migrate to the genital tract and cause sporadic abortions (Sahin et al. 2017). Although there are no reports of venereal transmission of Cff, the bacterium can be present in bull's genitalia (as evidenced by positive preputial samples) and in the vagina and cervix of heifers (Marcellino et al. 2015). On the other hand, Cfv, including the biotype *intermedius* (Cfvi), exclusively inhabits the genital tract of cattle and is the causal agent of Bovine Genital Campylobacteriosis (BGC) (Michi et al. 2015; Campero et al. 2017).

Bovine Genital Campylobacteriosis is a global venereal disease causing pregnancy loss (up to 20%) and with high prevalence in countries with extensive breeding systems, such as Argentina, Brazil, Colombia, Australia, France, New Zealand, Iran, Nigeria, among others (Van Bergen et al. 2005; Mshelia et al. 2010; Campero et al. 2017; Balzan et al. 2020).

Differential virulence of both subspecies (Cff and Cfv), is still controversial (Anderson 2007; Marcellino et al., 2015; Michi et al. 2015). In the male, the bacteria inhabit the preputial crypts and produce a chronic asymptomatic disease without affecting libido or fertility (Mshelia et al. 2007; Michi et al. 2015). Although the primary mode of transmission of BGC is the coitus, the bacteria can survive to cryopreservation in semen and, therefore, is also transmissible via artificial insemination (Givens and Marley 2008). The bull is the most important reservoir and disseminator of BGC and, in consequence, is

the main target for diagnosis. In this regard, preputial smegma (PS) is the sample of choice to identify the organism and, in turn, prevent and/or control the disease in a herd (Silveira et al. 2018; OIE 2021).

Researchers have assessed several methods for the collection of preputial smegma (PS) samples from bulls, regarding the identification of venereal pathogens, with different success rates (Tedesco et al. 1977; McMillen et al. 2006; OIE 2021). Routine diagnosis of *C. fetus* from PS includes direct immunofluorescence (DIF). This technique, however, cannot differentiate between subspecies and is more laborious, since it requires two or more preputial samplings to reach an adequate test sensitivity (Figueiredo et al. 2002; Silveira et al. 2018). The bacteriological culture (the gold standard), on the other hand, can differentiate subspecies, but requires special growth conditions that are not performed routinely (Mshelia et al. 2007; Chaban et al. 2013). To date, PCR protocols for *C. fetus* have become more sensitive and specific (van der Graaf-van Bloois et al. 2013; Guerra et al .2014; Del Piazzo et al. 2021; Mederos et al. 2022). However, proper subspecies differentiation is still a problem (van der Graaf-van Bloois et al. 2013; Silva et al. 2020; Polo et al. 2021).

Herein, we evaluated the diagnosis of *C. fetus* with DIF, bacteriological culture and qPCR, from PS of bulls naturally challenged with the three *C. fetus* subspecies. Concomitantly, three PS sampling methods were compared for DIF and bacteriological culture. In addition, *C. fetus* bacteremia was evaluated by bacteriological culture and qPCR in blood samples.

Material And Methods

Animals: naturally infected bull model

Three Aberdeen Angus bulls, of 4 to 5 years of age, were used in the study. Each bull was kept in a pen with a group of eight heifers experimentally infected (Fig. 1) with either Cfv, Cfvi or Cff, as previously reported (Garcia et al. 2021a). The strains used for the challenge of the heifers were Cff-C1N3, Cfv-97/608 and Cfvi-99/541 (Bacteriology Lab of INTA EEA Balcarce) (Calleros et al. 2017; Sahin et al. 2017). A negative control group (NCG) of two bulls with six non-challenged heifers was allocated in a separate paddock.

Sample collection

Nine PS samples were collected from each bull by three different devices using two sampling methods (aspiration and scraping): a) aspiration by artificial insemination pipette (Cassou pipette) and disposable plastic sheats with 2 ml of PBS; b) scraping with metal scraper; and c) scraping with flexible plastic disposable scraper. Before collecting the samplings, the veterinarians induced urination by preputial massage, with subsequent trimming of the hairs and cleaning of the preputial orifice with a cloth moistened with 70° ethanol. The area of the fornix was reached with all the devices.

The scraping samplings consisted of 30 forward and backward movements while scraping the penis and internal prepuce, whereas the aspiration method was performed with the pipette device and no scraping

(Fig. 2). Each sample was individually collected in Cary-Blair transport medium for bacteriological culture, in a tube with 1% phormolated physiological solution (FPS) for direct immunofluorescence (DIF) test and in a 1.5 ml microtube tube for qPCR analysis, and subsequently delivered to the lab within 2 h. Particularly, qPCR was performed with samples taken only with the aspiration method (which had been previously stored at -20°C until processed), because of its lower contamination.

Whole blood samples were collected from all three bulls on day 39, 132, 228 and 283, and processed by bacteriological culture and qPCR to evaluate possible bacteremia.

Bacteriological culture

The collected PS and whole blood samples were inoculated on modified 7% blood-Skirrow selective media agar (AS) plates (Oxoid, Hampshire, UK) enriched with 1.25 IU/ml polymyxin B sulfate, 5 mg/ml trimethoprim, 10 mg/ml vancomycin and 50 mg/ml cycloheximide (Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 37ºC under microaerophilic atmosphere (5% O_2, 10% CO_2 and 85% N_2) and observed every 48 h for 10 days (OIE, 2021). Bacterial genus was identified on the basis of colony characteristics (smooth, round, 1 to 3 mm in diameter, convex, white-gray, and translucent), and bacterial morphological characteristics, with darting motility in corkscrew, observed under a phase contrast microscope (OIE, 2021). These colonies were subcultured in Kligler's iron agar tubes to confirm *C. fetus* species identity based on catalase and oxidase enzyme activity (positive) and sulfhydryl acid production (negative).

Direct immunofluorescence for C. fetus

The DIF test was performed with a commercial fluorescent conjugate anti-*Campylobacter fetus* according to the manufacturer's instructions (CONJUGADO-CAMPY®, Laboratorio Biológico Tandil SRL, Argentina). Briefly, 10 µl of each sample was applied in microscopy slides of 12 wells. Slides were dried in a 37°C stove and fixed in absolute ethanol, at room temperature, for 15 min, and then dried in the stove again. Finally, the slides were washed with distilled water (5 s) and dried again at 37°C. The fluorescent conjugate anti-Cfv was added at a dilution of 1/200 and incubated in a wet dark chamber at 37°C for 1 h. The slides were washed three times in PBS (pH 7.2) for 10 min each wash, and finally washed once with tap water. Subsequently, the slides were mounted with buffered glycerol (pH 9.2), and observed under an epifluorescent microscope (Olympus CX31, Zhejiang, China) by experienced personnel Preparations with at least one bacterium presenting typical morphology and fluorescence characteristic of *C. fetus* were considered positive (OIE 2021).

Real Time-Polymerase Chain Reaction (qPCR) analysis

Two DNA extraction protocols for *C. fetus* were carried out: a slightly modified In-house method loaned by BIOTANDIL® (Laboratorio Biológico Tandil®) (LBT) and a commercial kit (Inbio Highway ®). Briefly, in the In-house-LBT, 500 µl of physiological solution was added to each PS samples and then the samples were homogenized and centrifuged at 12,000 xg for 5 min. The supernatant was discarded and the pellet resuspended with 100 µl Tris-EDTA (Inbio Highway®). Then, the samples were placed in a thermoblock at

100°C for 15 min under agitation, and then were centrifuged at 12,000 xg for 5 min. The supernatant was recovered and placed in a new 1.5 ml tube. The extraction with the commercial kit was carried out following the manufacturer's instructions (Inbio HighWay® DNA Puriprep-S kit, protocol for preputial smegma samples).

In the case of the whole blood samples, the same commercial kit for the DNA extraction was employed following the manufacturer's instructions for blood samples (Inbio HighWay® DNA Puriprep-S kit).

The qPCR was performed as described by Iraola et al. (2016) with a 10 µL reaction volume. The set of primers to amplify a sequence of 78bp from the 16SRNA gene were 16SFw (5'GCACCTGTCTCAACTTTC3') and 16SRv (5'CCTTACCTGGGCTTGAT3'), with a fluorescent TaqMan-MGB probe (16SPb: 5'-VIC-ATCTCTAAGAGATTAGTTG-MGB/NFQ-3'). This probe targets a polymorphic region of 19bp that discriminates the *C. fetus* strains from other *Campylobacter* species or other bacteria. A SsoAdvanced Universal Probes Supermix (Biorad®) (5 µL) was used: 1 µL (900 nM) of each of the forward and reverse primers, 1 µL of DNA template, 1.25 µL (250 mM) of the probe and 0.75 µL RNase-free ddH2O RNase-free ddH_2O. Negative (DNA from *C. jejuni*, *C. sputorum* subsp. *bubulus*, *C. coli* and *C. hyointestinalis* strains) and positive controls (DNA from Cff, Cfv y Cfvi strains), as well as a standard curve duplicate, were added in the assay. The standard curve was constructed with a serial 10-fold dilution from an aliquot of purified genomic DNA from *C. fetus* subsp. *fetus* strain 04-554 (range 50 pg/µL to 5 fg/µL).

The reaction conditions were as follows: polymerase activation and predenaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s, using StepOnePlus™ Real-Time (Thermo Fisher, Walhtma, MA, USA).

Statistical analyses

The full data set of samples were evaluated by Cohen's Kappa coefficient to measure agreement between results of the different *C. fetus* detection tests (qPCR, DIF and bacteriological culture) and between sampling devices (aspiration and metal and plastic scraper). Comparisons between diagnostic tests and between sampling devices for DIF test and bacteriological culture were performed by generalized mix linear model with binomial distribution, logit link function, by using Rcmdr as statistic software.

The analyses were performed considering bulls as independent (categorical or continuous) variable and the diagnosis tests or sampling devices as dependent variables. The significant difference was set at $P < 0.05$.

Results

According to the bacteriological culture and DIF tests (Fig. 3), the three bulls naturally exposed resulted infected with the respective strain corresponding to the group of infected heifers (Cfv, Cfvi y Cff) (García et al. 2021a).

The post breeding PS samples consisted of 81 samples (three bulls per nine sampling periods and per three devices, for the PS sampling) for the bacteriological and DIF tests.

The infection was persistent until day 283 for bulls infected with Cfv and Cfvi, while the bull infected with Cff was sustained for more than 5 months (day 165).

Table 1 details the number of positive samples to *C. fetus* by culture with the different devices for PS from each bull (27 PS samples per bull). Most samples were positive regardless the sampling device (67% for aspiration and plastic scraper; 63% for metal scraper). No significant differences were observed between the devices, when the samples were analyzed by bacteriological culture ($P = 0.940$). However, more samples were identified as positive in bulls infected with Cfvi (85.2% of the samples were positive) or Cfv (66.6%) than in the bull infected with Cff (44.4%) ($P = 0.011$). Including all the devices used, the bacteria were isolated by culture in 65.4% (53/81) of the samples. Considering only the venereal subspecies (Cfv and Cfvi), 75.9% (41/54) of the samples were detected by culture.

Table 1
Positive results of C. fetus of bacteriological culture from preputial smegma samples (n = 27 per bull) with three collection devices for the three bulls.

Device for collection of preputial smegma				
Bull	Aspiration	Metal scraper	Plastic scraper	Total (%)
Cfv	6	6	6	18[a] (66.6)
Cfvi	8	8	7	23[a] (85.2)
Cff	4	3	5	12[b] (44.4)
Total (%)	18 (66.6)	17 (62.9)	18 (66.6)	53 (65.4)
[a, b] Different letters indicates significant difference ($p < 0,05$).				

Table 2 shows the results of *C. fetus* detection by DIF according to the device used (27 PS samples per bull). The number of positive samples detected with this test was similar to those of the culture analysis, regardless of sampling device (62.9%, with aspiration; 59.2%, with metal scraper; and 66.6% with plastic scraper). No significant differences were observed between devices for DIF test ($P = 0.830$). Regarding the subspecies analysis, almost all the Cfvi samples (88.8%) were detected as positives, while around half of the Cfv (55.5%) and Cff (44.4%) samples were positively identified with this test. Indeed, like in the culture analysis, in this case there were also significant differences between the detection of positive samples from the bull infected with Cfvi and those from bulls infected with the other two subspecies (Cfv and Cff) ($P = 0.006$). Including all the devices evaluated, 62.3% (51/81) of the samples were positive. The analysis of the venereal subspecies (Cfv and Cfvi) yielded a higher positivity (72.2%; 39/54).

Table 2
Positive results of C. fetus of DIF from preputial smegma samples (n = 27 per bull) with three collection devices for the three bulls.

	Device for collection of preputial smegma			
Bull	Aspiration	Metal scraper	Plastic scraper	Total
Cfv	5	4	6	15[b] (55.5)
Cfvi	8	8	8	24[a] (88.8)
Cff	4	4	4	12[b] (44.4)
Total	17 (62.9)	16 (59.2)	18 (66.6)	51(62.9)
[a, b] Different letters indicates significant difference ($p < 0,05$)				

No significant differences were detected between the performance of the bacteriological culture and DIF ($P = 0.772$). However, the concordance between these two techniques (accounting all three sampling devices) for the 81 PS samples was moderate (kappa index: 0,463; IC:0,259-0,667).

The assessment of the qPCR detection was performed only with samples collected with the aspiration method to avoid inhibitions because of contaminations, in 8 sampling times per bull (24 PS samples). The two different DNA extraction methods used showed no significant differences (Table 3). The determined standard curve showing the logarithm of the copy numbers yielded a strong linear correlation (slope: -3.791, Eff%: 83.5%, and $R^2 = 0.995$) (Fig. 4). For qPCR, 75% (18/24) and 66.6% (16/24) of the samples resulted positive by IH-LBT and the commercial Kit DNA extraction method, respectively. Regarding only the venereal subspecies (Cfv and Cfvi), 87.5% (14/16) and 81% (13/16) of the samples were positive by IH-LBT and the commercial Kit DNA extraction method, respectively.

Table 3
Results from qPCR from preputial smegma samples (n = 24) from eight sampling periods from three bulls naturally infected with C. fetus (Cfv, Cfvi y Cff) with two DNA extraction methods.

	Time sampling (day)								Total positive qPCR
Bull	39	82	104	132	165	193	241	283	
Cfv	0/0*	1/0	1/1	0/1	1/1	1/1	1/1	1/1	6/6
Cfvi	1/1	1/0	1/1	1/1	1/1	1/1	1/1	1/1	8/7
Cff	1/1	1/0	1/1	1/1	0/0	0/0	0/0	0/0	4/3

Cfv: *Campylobacter fetus venerealis*, Cfvi: *Campylobacter fetus venerealis* intermedius, Cff: *Campylobacter fetus fetus*.

*DNA extraction method: In House LBT method/ Commercial InbioHighway kit method.

Concordance between DNA extraction methods used to analyze the PS samples through qPCR was quite adequate (kappa index: 0.77; IC:0,562-0,982). On the other hand, concordance between bacteriological culture and qPCR (kit commercial DNA extraction method) for the analysis of PS samples acquired through aspiration sampling was weak (kappa index: 0,386; IC:0,030 – 0,743). However, the concordance between DIF and qPCR (kit commercial DNA extraction method) with PS samples collected through aspiration sampling was adequate (kappa index: 0,622; IC:0,323-0,921).

No significant differences were obtained between culture, DIF test and qPCR ($P = 0.726$).

All the diagnostic tests (DIF, bacteriological culture and qPCR) identified the samples from bulls of the NGG as negatives throughout the assay.

All the whole blood samples (n = 12, four period samplings per three bulls) were negative by bacteriological culture and qPCR.

Discussion

The bull is the main target for diagnosis and management of BGC. However, there are still difficulties to establish effective preventive and control strategies (Silveira et al. 2018).

As previously reported (Garcia et al 2021a), an experimental model mimicking natural infection can be exploited to study different diagnostic approaches.

The bulls infected with the venereal subspecies (Cfv y Cfvi) were positive, according to the diagnostic tests used, for at least 9 months. This result reinforces its known carrier status between breeding seasons (Campero et al. 2017; Silveira et al. 2018). Regarding the bull exposed to Cff contact, the infection was persistent for at least 5 months and allowed passive or active mechanical transmission by traditional (3 months) or continuous breeding systems. Importantly, this is also a risk for cattle reproductive health.

Although researchers have previously isolated Cff from preputial samples of experimentally and naturally infected bulls (Botelho et al. 2014; Marcellino et al. 2015), it is still unclear if this bacterium is a venereal pathogen in cattle. However, Cff is associated with infertility and induces abortion (Mshelia et al. 2010; Balzan et al. 2020; García et al. 2021b). These pieces of evidence suggest that bulls could have a significant role in the dissemination and maintenance of Cff within herds.

The results from our study demonstrated the transmission from heifers to bulls and the bacterial infection was characterized by colonization and persistence in the preputial cavity. Although this study includes the assessment of only one strain of Cff, our findings show that this subspecies should be considered in BGC diagnosis of bulls because of the potential risk in disseminating the bacteria among heifers or cows. In this line, differential diagnosis could not be mandatory.

The lower persistence of Cff could be due to its low adaptation in the prepuce, probably because of the lack of surface proteins, genomic islands and mobile elements that predominate in Cfv and enhance long

term survival in the urogenital tract (Gorkiewicz et al. 2010; Sprenger et al. 2017; Nadin-Davis et al. 2021). Particularly, the variant Cfvi had a higher rate of recuperation from PS than others variants. This could be associated to different genetic profiles regarding genes affecting colonization and attachment capacity (Nadin-Davis et al. 2021).

Bacteriological culture showed higher efficiency detecting the venereal strains (Cfv and Cfvi), with higher bacteriological recuperation of Cfvi. The detection through culture was more efficient in our study in relation to other reports (63–67% *vs*. 33–38%) (McMillen et al. 2006; Guerra et al. 2014).

Regarding the DIF test, there were significant differences between Cfvi in relation to Cff and Cfv. This may be explained by a higher concentration of the bacterium in the prepuce and may be associated with a more virulent strain. In effect, the higher detection rates of Cfvi through all the diagnostic tests reinforce the idea of a higher bacterium concentration.

Previous studies have reported detection variations, from 69.4–92.6%, with DIF test for Cfv (Figueiredo et al. 2002; Campero et al. 2017) and these percentage values are coincident with that of the present research (72%). In our study, the DIF detection rate decreased when Cff was included in the analysis (63%). The detection of this subspecies has not been previously assessed through this technique and thus we cannot make any comparison. Even though the detection rate of DIF is moderate (56–67%), this technique is still widely used because of its low cost, simplicity and fast processing (Campero et al. 2017; Silveira et al. 2018; Balzan et al. 2020).

No significant differences were detected between culture and DIF. This is in contrast with studies that showed significantly better results by bacteriological culture, which were associated with a low bacterial concentration or inadequate sampling (Marcellino et al. 2015). The moderate concordance between bacteriological culture and DIF opens the debate around the use of both tests in herds with endemic BGC, in order to reduce false negative bulls.

In accordance with this study, some researchers have suggested that the detection efficiency of PS collection by DIF or bacteriological culture seems to be independent of the devices used (Soto and di Rocco 1982; Ramos et al. 1986), while others have described better results with scraping devices (Tedesco et al. 1977; McMillen et al. 2006). According to McMillen et al. (2006), the scraping method would increase the possibility of isolating *C. fetus* at low concentrations within the preputial crypts, since friction against the mucosa generates detachment of epithelial cells along with the bacteria. As mentioned before, in the present study the results were independent of the device used. Thus, according to our results and those of some other researchers, the use of the device should be based on which is most practical for the veterinarian depending on the length of the preputial cavity. More studies should be made to resolve these discrepancies and found the most efficient device for this purpose.

Our study showed better results for the detection of *C. fetus* by qPCR (66.6%-75%) than through conventional diagnostic tests (culture and DIF; 66.6% and 56%, respectively). This is in accordance with previous studies (McMillen et al. 2006; Guerra et al. 2014; Del Piazzo et al. 2021; Polo et al. 2021;

Mederos et al. 2022). Variations in the molecular technique associated with genotyping failures, which may be due to different local strain variants, cross-reactions, and contaminants inhibiting the reaction (such as blood, urine, or pus) may limit the effectiveness of PCR, mainly in clinical samples (Willoughby et al. 2005; McMillen et al. 2006; Schulze et al. 2006; Spence et al. 2011; Polo et al. 2021). The inclusion of a TaqMan probe-based qPCR in the study was due to its high sensitivity (Iraola et al. 2016). Culture fastidious growth and lower capacity to process large volumes of samples, as well as the lower sensitivity of DIF, makes qPCR an adequate and promising diagnostic tool for *C. fetus* detection. The In-house DNA extraction method resulted in higher *C. fetus* detection. Indeed, its lower cost and fast processing makes this method a practical option.

The detection of subspecies of these bacteria continues to cause problems. This is due to the use of sequences or genomic island present both in Cff and Cfv, or even in other species, for PCR assays designed to discriminate between *C. fetus* subspecies. The use of these common sequences, needless to say, leads to false positives (Moolhuijzen et al. 2009; Abril et al. 2010; Spence et al. 2011; Silva et al. 2020; Polo et al. 2021).

More studies are needed to know the implication of Cff in the reproductive performance in cattle. However, given the difficulties in subspeciation and its association with abortion, we suggest that all three subspecies should be diagnosed as etiological agents of BGC.

The differences on detection between samplings for either diagnostic test could be associated with variations of local immunity and bacterial concentration in the bulls, as well as strain differences (virulence factors) (Van Bergen et al. 2005; Mshelia et al. 2007). Because of these dissimilar factors and other variations, such as sampling and diagnostic test effectiveness, two or more scrapings are needed (Guerra et al. 2014; Del Piazzo et al. 2021; Mederos et al. 2022). Similarly, in our study concordance between tests varied from weak to strong, leading to false negative bulls.

Campylobacter spp., mainly Cff, is associated with bacteremia and extraintestinal infections in humans (Monno et al. 2004) and ruminants (Sahin et al. 2017). Here we were unable to detect any *C. fetus* in blood. This suggests different tropism associated with dissimilar *C. fetus* pathogenesis in bulls and humans, as suggested for Cff, which causes abortion in cows, ewes, alpacas, among others (Fenwick et al. 2000; Bidewell et al. 2016; Campero et al. 2017; Sahin et al. 2017).

Conclusion

The detection of the infection of bulls upon *C. fetus* challenge was similar with any of the diagnostic test evaluated. Thus, the easy implementation and low cost of DIF still make it an adequate test. In the case of culture, the availability of reference autochthonous strains in future research is highly important to confirm the etiology, as well as to develop and validate new diagnostic techniques. Finally, PCR-based methods are promising for the diagnosis of BGC from clinical samples of PS; however, the lack of infrastructure, equipment and trained personnel can be a limitation. Where BGC is endemic, as in

Argentina, more investment in infrastructure as well as laboratory training is necessary to establish control and eradication programs.

Declarations

Acknowledgments. We thank Martin Mayoral, Diego Herrera, Walter Bagazette, Abel Gulle and Cristian Gulle for their help in field sampling. We also thank Paula Nievas and Jorgelina Lomonaco for the preparation of culture media and material. This article is presented as a partial requirement for the first author to obtain a PhD Degree at Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina.

Funding This study was funded by the Instituto Nacional de Tecnología Agropecuaria (INTA) grant: Project PNSA-PD-I103 and Project PNSA-PD-I105; ANPCyT: project PICT2015-1541; CONICET grant: Project PIP11220150100316CO; project AGR 650/21.

Conflicts of interest The authors declare no conflict of interest.

Code availability Not applicable.

Author's contribution All authors contributed to the study conception and design. JAG, PF, AKG, MAM and CM performed laboratory analyses and JAG, JA and JFA performed the field assay included in the present study. JAG and MS carried out statistical analyses. JAG collected the data collection and prepared the manuscript preparation, under the supervision of AKG and FAP. All authors edited, read, and approved the final manuscript.

Ethics approval The experimental procedures were performed in compliance with the Comité Institucional para el Cuidado y Uso de Animales de Experimentación (CICUAE, INTA-CeRBAS, Argentina): Aval 163/2018.

Consent to participate All authors participated voluntarily in the research.

Consent for publication All authors give consent for publication.

References

1. Abril C, Brodard I, Perreten V (2010) Two novel antibiotic resistance genes, tet(44) and ant(6)-Ib, are located within a transferable pathogenicity island in *Campylobacter fetus* subsp. *fetus*. Antimicrob Agents Chemother 54(7):3052–3055
2. Anderson M (2007) Infectious causes of bovine abortion during mid- to late-gestation. Theriogenology 68:474–486
3. Balzan C, Ziech RE, Gressler LT, Vargas APC (2020) Bovine Genital Campylobacteriosis: main features and perspectives for diagnosis and control. Cienc Rural 50(3):e20190272.

https://doi.org/10.1590/0103-8478cr20190272

4. Bidewell CA, Woodger NGA, Cook AJC, Carson TV, Gale SL, Chanter JI, Williamson SM (2010) *Campylobacter fetus* subspecies *fetus* abortion in alpacas (*Vicuna pacos*). Vet Rec 167:457–458
5. Botelho MPA, Hirsch C, Lage AP, da Rocha CMBM, Dorneles EMS, Cardoso PG, da Costa GM (2014) Prevalence of *Tritrichomona foetus* and *Campylobacter fetus* subsp. *venerealis* among bulls slaughtered in the state of Mina Gerais, Brazil. Ciencias Agrarias 39(5): 2039–2048
6. Campero CM, Moore DP, Odeón AC, Cipolla AL, Odriozola E (2003) Aetiology of bovine abortion in Argentina. Vet Res Comm 27:359–369
7. Campero CM, Cantón GJ, Moore DP (2017) Campylobacteriosis genital bovina. Abortos y otras pérdidas reproductivas en bovinos: diagnóstico y control. Ed. Hemisferio Sur SA, Buenos Aires, Argentina, pp 79–86
8. Calleros L, Iraola G, Méndez A, Morsella C, Paolicchi F, Silveyra S, Velilla A, Pérez R (2017) Assessing the intra-species genetic variability in the clonal pathogen *Campylobacter fetus*: CRISPRs are highly polymorphic DNA markers. J Microbiol Methods 132:86–94
9. Chaban B, Garcia Guerra A, Hendrick SH, Waldner CL, Hill JE (2013) Isolation rates of *Campylobacter fetus* subsp *venerealis* from bovine preputial samples via passive filtration on nonselective medium versus selective medium, with and without transport medium. Am J Vet Res 74(8):1066e9. https://doi: 10. 2460/ajvr.74.8.1066
10. Delpiazzo R, Barcellos M, Barros S, Betancor L, Fraga M, Gil J, Iraola G, Morsella C, Paolicchi F, Perez R, Riet-Correa F, Sanguinetti M, Silva A, Silveira C, Calleros L (2021) Accurate and fast identification of *Campylobacter fetus* in bulls by real-time PCR targeting a 16S rRNAgene sequence. Vet Anim Sci 11:100163
11. Fenwick SG, West DM, Hunter JEB, Sargison ND, Ahmed F, Lunsden JS, Collet MG(2000) *Campylobcater fetus fetus* abortions in vaccinated ewes.N Z Vet J48(5)
12. Figueiredo JF, Pellegrin AO, Foscolo CD, Machada RP, Miranda KL, Lage AP (2002) Evaluation of the direct fluorescent antibody test for the diagnosis of bovine genital Campylobacteriosis. Rev Latinoam Microbiol 44:118–123
13. García JA, Gioffré AK, Acuña J, Méndez MA, Morsella C, Aller JF, Paolicchi FA (2021a) Isolation of *Campylobacter fetus* subsp. *venerealis* from seminal vesicle of a naturally challenged bull. Vet Res Commun 45(4):447–452
14. García JA, Paolicchi F, Acuña Y, Fiorentino A, Malena R, Morsella C, Méndez A, Cantón G, Scioli V, Morrell E (2021b) Campylobacteriosis in beef fetuses: a descriptive study. XII Reunión Argentina de Patología Veterinaria y 14to Seminario Fundación CL Davis-SW Thompson. Congress, October 2021, INTA EEA Balcarce
15. Givens MD, Marley MSD (2008) Pathogens that cause infertility of bulls or transmission via semen. Theriogenology 70:504–507
16. Gorkiewicz G, Kienesberger S, Schober C, Scheicher SR, Gully C, Zechner R, Zechner EL (2010) A genomic island defines subspecies- specific virulence features of the host-adapted pathogen

Campylobacter fetus subsp. *venerealis*. J Bacteriol 192:502–517

17. Guerra AG, Chaban B, Hill J, Waldner CL, Hendrick SH (2014) Clinical sensitivity and specificity of a real-time PCR assay for *Campylobacter fetus* subsp. *venerealis* in preputial samples from bulls. Am J Vet Res 75:851–860
18. Iraola G, Pérez R, Betancor L, Marandino A, Morsella C, Méndez A, Paolicchi F, Piccirillo A, Tomás G, Velilla A, Calleros L (2016) A novel real-time PCR assay for quantitative detection of *Campylobacter fetus* based on ribosomal sequences. BMC Vet Res 12:286
19. Marcellino RA, Morsella C, Cano D, Paolicchi F (2015) Eficiencia del cultivo bacteriológico y de la inmunofluorescencia en la detección de *Campylobacter fetus* en fluidos genitales bovinos [Efficiency of bacteriological culture and the immunofluorescent assay to detect *Campylobacter fetus* in bovine genital fluids]. Rev Argent Microbiol 47(3):183–189
20. McMillen I, Fordyce G, Doogan VJ, Lew AE (2006) comparison of culture and a novel 5' taq nuclease assay for direct detection of *Campylobacter fetus* subsp. *venerealis* in clinical specimens from cattle. J Clin Microbiol 44(3):938–945
21. Mederos A, Galarraga D, van der Graas-van Bloois L, Buczinski S (2022) Performance of bovine genital campylobacteriosis diagnostic tests in bulls from Uruguay: a Bayesian latent class model approach. Trop Anim Health Prod 54:32
22. Michi AN, Favetto PH, Kastelic J, Cobo ER (2015) A review of sexually transmitted bovine trichomoniasis and campylobacteriosis affecting cattle reproductive health. Theriogenology 85(5):781–791
23. Monno R, Rendina M, Ceci G, Rizzo C, Luzzi I, Francavilla A, Ierardi E (2004) *Campylobacter fetus* bacteremia in an immunocompromised patient: case report and review of the literature. New Microbiol 27(3):281–285
24. Moolhuijzen PM, Lew-Tabor AE, Wlodek BM, Aguero FG, Comerci DJ, Ugalde RA, Sanchez DO, Appels R, Bellgard M (2009) Genomic analysis of *Campylobacter fetus* subspecies: identification of candidate virulence determinants and diagnostic assay targets. BMC Microbiol 9:86
25. Morrell E, Campero C, Cantón G, Odeón A, Moore PD, Odriozola E, Paolicchi F, Fiorentino A (2019) Current trends in bovine abortion in Argentina. Pesq Vet Bras 39:12–19
26. Mshelia GD, Amin JD, Woldehiwet Z, Egwu GO, Murray RD (2007) Bovine venereal campylobacteriosis: an overview. CAB Rev: Perspect Agr Vet Sci Nutr Nat Resour 2:14
27. Mshelia GD, Amin JD, Woldehiwet Z, Murray RD, Egwu GO (2010) Epidemiology of bovine venereal campylobacteriosis: geographic distribution and recent advances in molecular diagnostic techniques. Reprod Domest Anim 45:221–230
28. Nadin-Davis SA, Chmra J, Carrillo CD, Amoako K, Goji N, Ducepp MO, Devenish J (2021) A comparison of fourteen characterized mammalian-associated *Campylobacter fetus* isolates suggests that loss of defense mechanisms contribute to high genomic plasticity and subspecies evolution. PeerJ 9:e10586. DOI 10.7717/peerj.10586
29. OIE Terrestrial Manual (2021) Chap. 3.4.4. Bovine genital campylobacteriosis

30. Polo C, Garceia-Seca T, Hernández E, Fernández V, Rodríguez Lázaro D, Goyache J, Domínguez L, Pérez-Sancho M (2022) Evaluation of PCR assays for *Campylobacter fetus* detection and discrimination between *C. fetus* subspecies in bovine preputial wash samples. Theriogenology 172:300–306
31. Ramos ADA, Guida HG, Andrade VLB (1986) Comparison of three technics for collection of preputial samples for the diagnosis of Campylobacteriosis. Pesqui Agropecu Bras 21(3):303–309
32. Sahin O, Yaeger M, Wu Z, Zhang Q (2017) *Campylobacter*-Associated Diseases in Animals. Annu Rev Anim Biosci 8(5):21–42
33. Schulze F, Bagon A, Muller W, Hotzel H (2006) Identification of *Campylobacter fetus* subspecies by phenotypic differentiation and PCR. J Clin Microbiol 44(6):2019–2024
34. Silva MF, Duarte A, Pereira G, Mateus L, Lopes-da-Costa L, Silva E (2020) Assessment of *Campylobacter fetus* subsp. *venerealis* molecular diagnosis using clinical samples of bulls. BMC Vet Res 16:410
35. Silveira CS, Fraga M, Giannitti F, Macias-Rioseco M, Riet-Correa F (2018) Diagnosis of Bovine Genital Campylobacteriosis in South America. Front Vet Sci 14(5):321
36. Soto P, di Rocco MM (1982) Diagnóstico de Campylobacteriosis (vibriosis) en toros por la técnica de anticuerpos fluorescentes. Gac Vet 44(367):37–43
37. Spence RP, Bruce IR, McFadden AM, Hill FI, Tisdall D, Humphrey S et al (2011) Cross-reaction of a *Campylobacter fetus* subspecies *venerealis* real-time PCR. Vet Rec 5:168131
38. Sprenger H, Kienesberger S, Pertschy B, Pöltl L, Konrad B, Bhutada P, Vorkapic D, Atzmüller D, Feist F, Högenauer C, Gorkiewicz G, Zechner EL(2017) Fic Proteins of Campylobacter fetus subsp. venerealis Form a Network of Functional Toxin–Antitoxin Systems. Front. Microbiol 8:1965. doi: 10.3389/fmicb.2017.01965
39. Tedesco LF, Errico F, Del Baglivi PL (1977) Comparison of three sampling methods for the diagnosis of genital vibriosis in the bull. Aust Vet J 53:470–472
40. Van Bergen MA, Linnane S, van Putten JP, Wagenaar JA(2005) Global detection and identification of *Campylobacter fetus* subsp. *venerealis*. Rev Sci Tech 24: 1017-26
41. van de Graaf-van Bloois L, van Bergen MAP, van der Wal FJ, de Boer AG, Duim B, Schmidt T, Wagenaar JA (2013) Evaluation of molecular assays for identification *Campylobacter fetus* species and subspecies development of a *C. fetus* specific real-time PCR assay. J Microbiol Methods 95:93–97
42. Willoughby K, Nettleton PF, Quirie M, Maley MA, Foster G, Toszeghy M, Newell DG(2005) A multiplex polymerase chain reaction to detect and differentiate *Campylobacter fetus* subspecies *fetus* and *Campylobacter fetus* species *venerealis*: use on UK isolates of *C. fetus* and other *Campylobacter* spp. J Appl Microbiol 99:758–76

Figures

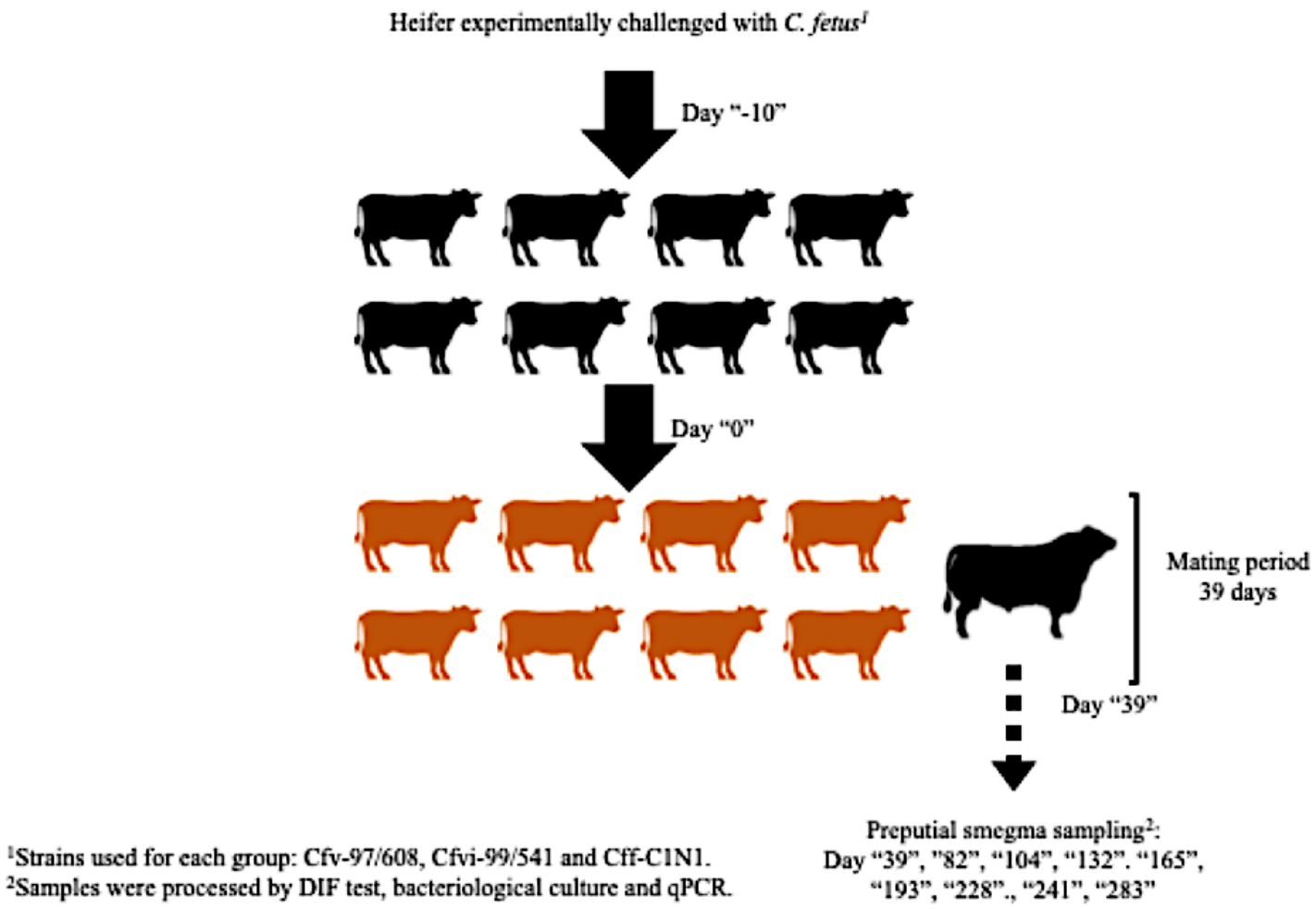

Figure 1

Schematic representation of the overall experimental procedure. Three groups of heifers (n=8 per group) were experimentally challenged with the respective *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *venerealis* biovar *intermedius* and *C. fetus* subsp. *fetus* strains, and then were kept in the same pen with one bull each for 39 days. Postmating preputial smegma sampling is shown. Dark heifers: *C. fetus* non-infected animals; orange heifers: animals experimentally challenged with the respective *C. fetus* strain (García et al, 2021a).

Figure 2

Preputial smegma sampling from bulls collected with three different devices: aspiration with Cassou pipette (left), and scraping with metal and plastic scraper (right).

Figure 3

Preputial smegma sample from Cfv-infected bull collected at day 104. Colonies of *Campylobacter fetus* spp. picked and observed under a phase contrast microscopy with a characteristic spiral shape (left), or subjected to direct immunofluorescence test with anti-*C. fetus* antibodies showing characteristic bacilli forms (spiral form, “S” form and seagull flight form) and intense periphery fluorescence (right). 100x

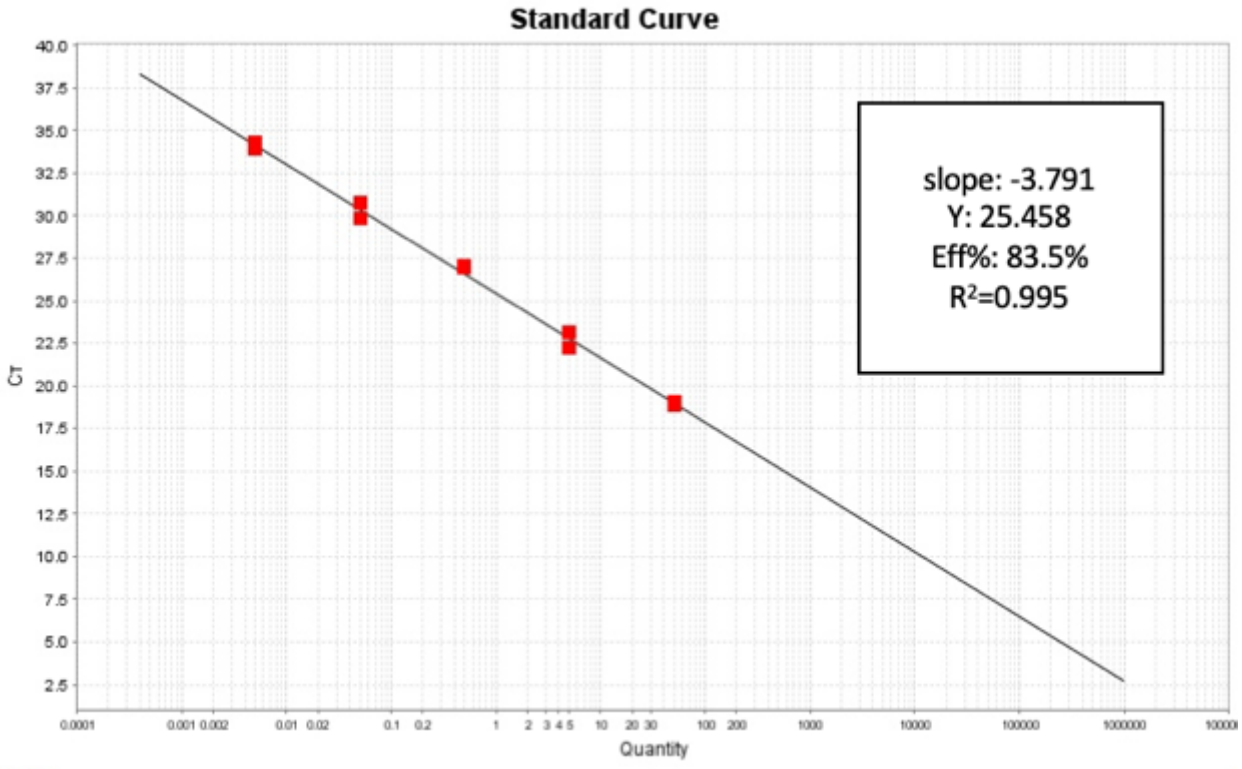

Figure 4

Standard curve of TaqMan-MGB probe real-time PCR for *C. fetus* detection. DNA Concentration ranging from 50 pg/µL to 5 fg/µL. The determination coefficient (R^2), amplification efficiency (Eff%), slope and Y-intercept (Y) are indicated.