InVet 2021, 23 (1): 1-14
ISSN 1514-6634 (impreso)
ISSN 1668-3498 (en línea)

ARTÍCULO DE INVESTIGACIÓN

EVALUATION OF DIRECT IMMUNOFLUORESCENCE TEST FOR *CAMPYLOBACTER FETUS* IN BULLS EXPERIMENTALLY INFECTED AND COMMENSAL BACTERIA FROM THE REPRODUCTIVE TRACT OF BULLS

EVALUACIÓN DE LA PRUEBA DE INMUNOFLUORESCENCIA DIRECTA PARA *CAMPYLOBACTER FETUS* EN MICROBIOTA GENITAL DE TOROS Y TOROS EXPERIMENTALMENTE INFECTADOS

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Recibido: 04-02-2021
Aceptado: 26-07-2021
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RESUMEN

Campylobacter fetus es una bacteria Gram negativa que incluye 2 subespecies de relevancia sanitaria para el bovino: *C. fetus* subsp. *fetus* (Cff) y *C. fetus* subsp. *venerealis* (Cfv), causantes de importantes pérdidas reproductivas. En el presente estudio, se utilizaron cuatro grupos de toros: un grupo (G1) para caracterizar la microbiota genital, y otros tres (G2, G3, G4) (n=10) para evaluar el test de inmunofluorescencia directa (IFD) para el diagnóstico de la campilobacteriosis bovina en esmegma prepucial (EP) de toros experimentalmente infectados con Cff y Cfv. Se identificaron siete cepas bacterianas de distinto género del G1. Otras 11 cepas bacterianas de laboratorio fueron incluidas: Cff, Cfv, Cfv biovar Intermedius, 3 *Campylobacter* no-*fetus*, 3 Gram negativo y 2 Gram positivo. Solo las cepas de *C. fetus* presentaron inmunofluorescencia positiva. La sensibilidad y especificidad de la IFD fue de 79 % y 100 %, respectivamente. La concordancia entre la IFD y el cultivo bacteriológico fue de 83 % (Kappa: 0,65). Se demuestra la alta especificidad de la IFD para la detección de *C. fetus* con un conjugado fluorescente elaborado en Argentina, y se destaca la no reacción cruzada contra 15 cepas bacterianas normalmente presentes en la microbiota prepucial del toro.

Palabras clave: (campilobacteriosis genital bovina), (rumiantes), (enfermedad venérea), (abortos), (infertilidad)

ABSTRACT

Campylobacter fetus is a Gram negative bacterium, with 2 relevant subspecies for cattle health: *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv) which cause important reproductive losses. In the present work, four groups of bulls were used: one group to characterize the genital microbiota (G1; n=3) and the other three to evaluate the direct immunofluorescence (DIF) test for the diagnosis of bovine campylobacteriosis in preputial samples (PS) from experimentally infected bulls with Cff (G2; n=3) and Cfv (G3; n=3), and controls (G4; n=4). Seven bacterial strains of different genera were identified in the PS from G1. Other 11 laboratory bacterial strains were included: Cff, Cfv and Cfv biovar Intermedius, 3 *Campylobacter* non-*fetus*, 3 Gram negative and 2 Gram positive. Only the *C. fetus* strains presented positive immunostaining. The sensitivity and specificity of the DIF test were 79 % and 100 %, respectively. Concordance between DIF test and bacteriological culture was 83 % (Kappa index: 0.65). The present study shows the high specificity of the DIF test for detection

of *C. fetus* using a fluorescent conjugate elaborated in Argentina, and highlights the relevance of no cross reaction against 15 bacterial strains normally present in bull preputial microbiota.

Keywords: (bovine genital campylobacteriosis), (ruminants), (venereal disease), (abortions), (infertility)

INTRODUCTION

Campylobacter fetus is a Gram-negative microaerophilic bacterium that includes 2 relevant subspecies for cattle health: *C. fetus* subsp. *fetus* (Cff) and *C. fetus* subsp. *venerealis* (Cfv). An additional subspecies of Cfv was identified as biotype *intermedius* (Cfvi)[8]. *Campylobacter fetus* subsp. *fetus* is an inhabitant of the intestinal tract of cattle and sheep associated with sporadic abortions, whereas Cfv resides exclusively in the genital tract of cattle and causes the bovine genital campylobacteriosis (BGC), a venereal infectious disease responsible for reproductive failure, return to estrus, transient infertility and abortion. This disease is an important cause of cattle reproductive losses worldwide, being endemic in many developing countries[3,12]. The prevalence of herds infected by BGC varies between countries and regions within the same country, ranging from 1.5 to 37 %[3,10,12,13]. The highest incidences of BGC occur in developing countries, where natural breeding of cattle in extensive production systems is widely practiced[3]. In Argentina, since the late 1970s, the direct immunofluorescence (DIF) technique has been used for the diagnosis of campylobacteriosis in bulls, employing polyclonal antibodies against Cfv conjugated to fluorescein isothiocyanate (FITC)[19]. Due to the similarity of the antigenic structures of Cfv and Cff, the polyclonal antibodies do not discriminate between the two *C. fetus* subspecies in clinical samples. However, both subspecies are important causes of reproductive losses in livestock[3,11]. When this diagnostic technique began to be used in Argentina, the infection rates at bull and herd level were 13.5 and 32.5 %, respectively[19]. Nowadays, with the massive use of DIF in the network of veterinary diagnostic laboratories during the last 30 years, infection rates of 10 % and <2 % have been reached at herd and individual animal level, respectively[10,16].

There are advances in the diagnosis of BGC by using molecular methods such as Polymerase Chain Reaction (PCR)-based technics that allow the discrimination between species and subspecies of *C. fetus*. Although several authors observed failures in PCR accuracy, not being reliable for field samples[18, 22], at the present time it has gained special interest as a

confirmatory diagnostic technique. However, the DIF test remains as an important screening method for the identification and elimination of infected bulls, and it is listed by the World Organization for Animal Health (OIE) as a prescribed diagnostic method, being the main routine diagnosis test in some countries of South America[14, 18]. Depending on intrinsic factors of bull, sampling and laboratory technician, the DIF test varied in sensitivity and specificity between 69.4 and 92.6 % and from 88.9 to 94.4 %, respectively[3, 5, 6].The low sensitivity given by intrinsic factors of the bull such as sexual rest and appropriate time lapse between samplings, together with human factors associated with sampling method and laboratory expertise makes necessary to carry out at least 2 consecutive samplings in order to reduce false negative bulls in the herd[3, 18]. The aim of this study is to evaluate the specificity of DIF test for *C. fetus* against different *Campylobacter* non-*fetus* and non-*Campylobacter* strains, and to compare the performance of DIF with that of bacteriological culture for detection of *C. fetus* in preputial smegma from bulls experimentally infected with Cff and Cfv.

MATERIALS AND METHODS

Description of experimental group

Four groups of bulls, 5 year old, *Bos taurus,* Aberdeen Angus breed were used for this study. One group of 3 bulls tested negative for *C. fetus* (G1) was used as a source of bacteria from genital microbiota to check cross-reactivity of the fluorescent conjugate. The second group of 3 bulls (G2), third group of 3 bulls (G3) and fourth group of 4 bulls (G4) were used for experimental infection with *C. fetus*. Bulls of G2 and G3 were infected with Cff and Cfv, respectively, whereas bulls of G4 were used as negative controls, without being infected (aval CICUAE 163/2018).

Bull genital microbiota identification

Bulls of G1 (n=3) were randomly selected from a BGC-free herd and checked by 3 consecutive negative DIF analyses to confirm that they were negative for *C. fetus*. Bulls were kept in sexual rest for 30 days previous to sampling. Preputial smegma (PS) samples were taken by aspiration method from each bull, collected in Cary Blair (CB) and Stuart (SM) transport medium, and sent to the laboratory within 1 h after sample collection. They were inoculated in MacConkey agar (MCA), Columbia agar supplemented with 7 % of bovine blood (CBA) (Oxoid Ltd., Wad Road, Basingstoke, UK) and Skirrow agar (AS) enriched with antibiotics. The MCA, CBA and AS plates were incubated at 37 ºC under aerobic, 10 % CO_2

and microaerophilic (5 % O_2, 10 % CO_2 and 85 % N_2) atmospheres, respectively. Then, plates were observed at 24 h (MCA), every 24 h for 3 days (CBA) and every 48 h for 10 days (AS). Following the isolation of bacteria, they were identified by colony characteristics, Gram staining, microscopic morphology and biochemical reactions such as urease, indol, nitrate reductase, catalase and fermentation of carbohydrates. Genera were classified according to the Bergey's Manual of Determinative Bacteriology.

Experimental infection of bulls

Bulls from G2 (Cff, n=3), G3 (Cfv, n=3) and G4 (negative control, n=4) were used for evaluating the sensitivity and specificity of DIF test from PS samples. All bulls were previously analyzed 2 times as negative for *C. fetus* spp. Bulls from G2 and G3 were challenged, receiving 2 preputial inoculations with an interval of 7 days. Each inoculation consisted of 2 mL inoculum of bacterial culture suspended in 2 mL of modified Brucella broth supplemented with antibiotics and with a concentration of 4.3×10^7 CFU/mL for Cff and 4.0×10^8 CFU/mL for Cfv. The type strain used was INTA C1N3 for Cff and INTA 97/608 for Cfv[3]. The G4 received 2 inoculations; each consisted of 2 mL of modified Brucella broth with antibiotics (placebo). Preputial samples were taken by aspiration with a sterile Cassou pipette, stored in formolated physiological solution (FPS) 1 % for DIF and, CB transport medium for bacteriological culture, and then submitted to the laboratory within 2 h after collection. For bacteriological culture, material from CB was inoculated into AS medium and incubated under microaerophilic conditions as previously described. Preputial samples were collected from all bulls (infected and negative control) immediately before the inoculation and every 7 days for 3 weeks. The day of first inoculation is noted as day "0" and re-inoculation as day "7". A sample of PS was extracted from each bull on day 7, 14, 21 and 28. A total of 24 post-inoculation samples were obtained from infected bulls (12 from G2 and G3) and 16 from negative control bulls (G4). Immediately after experimental assay all bulls were sent to slaughter to prevent spread of disease to other herds.

Direct immunofluorescence technique

The specificity of the fluorescent conjugate was tested against 18 bacterial strains. Eleven strains were obtained from the culture collection of the Bacteriology Laboratory of INTA-EEA Balcarce (Argentina). Six strains of *Campylobacter*: Cff (C1N3), Cfv (97/608), Cfvi (99/541)[2], *C. jejuni* (NCTC 11392), *C. bubulus* (NCTC 10355) and *C. hyointestinalis* (NCTC

11562); 3 Gram negative: *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Histophilus somni*; and 2 Gram positive: *Trueperella pyogenes* and *Staphylococcus coagulase-negative* were tested. All strains, with exception of *C. jejuni*, *C. bubulus* and *C. hyointestinalis* were isolated from previous clinical samples. The remaining 7 bacterial strains were obtained from *in vivo* samples collected from bulls of G1 (section *Bull genital microbiome identification*)**.** The samples stored in FPS 1 % that were subsequently analyzed by DIF as well as those used for isolation and further identification of strains were diluted (ten-fold dilutions) and their concentrations were adjusted to the turbidity standard of 1 McFarland units (approximately $3*10^8$ CFU/mL). For *C. fetus* detection by DIF test, a commercial FITC- labelled anti-*C. fetus* conjugate was used and, samples were processed according to the manufacturer's instructions (CONJUGADO-CAMPY®, Laboratorio Biológico de Tandil, Argentina). Briefly, 10 µl of each bacterial or smegma suspension at appropriate concentration was applied on microscopic slides of 12 wells. Slides were dried at 37 °C in oven and fixed with absolute ethanol at room temperature for 15 minutes, and then washed with distilled water and dried again at 37 °C. Conjugate was added at each well on the slides at 1/200 dilution and incubated in wet dark chamber at 37 °C for 1 hour. Slides were washed 3 times with PBS (phosphate buffered saline) pH 7.2 (10 minutes each wash) and one time with tap water. Cover slips were mounted with buffered glycerol pH 8 and examined with an epifluorescent microscope (Olympus CX31, Zhejiang, China in INTA Laboratory, Argentina and Zeiss Primo Star iLED, Germany, in Laboratorio Biológico de Tandil, Argentina). Dilutions up to 1/1200 were done in strains with positive fluorescence. The technique was done simultaneously in 2 laboratories (Venereal Disease Laboratory of INTA- EEA Balcarce, Argentina and Laboratorio Biológico de Tandil, Argentina) by 4 expert observers (2 per laboratory). Inter observer concordance was evaluated by Kappa of Fleiss coefficient statistical tool. Preparations showing fluorescent bacteria with the typical morphology of *C. fetus* were considered positive[14]. Kappa index concordance between techniques was evaluated.

RESULTS

Seven bacteria were identified from the PS samples from the 3 bulls of G1, resulting in 4 Gram positive and 3 Gram negative bacteria. These strains together with 11 laboratory strains, a total of 18 bacterial strains were included in the DIF assay (Table 1). Only Cff strain C1N3, Cfv strain 97/608 and Cfvi strain 99/541 presented immunostaining against the FITC at 1/200 dilution (Table 1, Figure 1), keeping the fluorescence intensity up to 1/1000 dilution and

decreasing by 1/1200. No cross-reactivity was observed with the rest of *Campylobacter* non-*fetus* strains such as *C. jejuni, C. bubulus* and *C. hyointestinalis* or with the other 12 Gram negative and positive strains which involve the following genera: *Proteus, Pseudomonas, Staphylococcus, Trueperella, Acinetobacter, Bacillus, Corynebacterium and Escherichia* (Table 1). Kappa coefficient resulted in a 100% concordance inter observers (Kfleiss: 1).

Table 1. Results of DIF test from 11 laboratory bacterial strains and 7 bacteria identified in preputial smegma samples collected from bulls of Group 1

Microorganism	**Bacteria origin**	**Gram**	**DIF**
C. fetus subsp. *fetus*	Laboratory	Negative	+*
C. fetus subsp.*venerealis*	Laboratory	Negative	+*
C. fetus venerealis biovar. *Intermedius*	Laboratory	Negative	+*
C. jejuni	Laboratory	Negative	-
C. bubulus	Laboratory	Negative	-
C. hyointestinalis	Laboratory	Negative	-
Histophilus somni	Laboratory	Negative	-
Proteus vulgaris	Laboratory	Negative	-
Pseudomonas aeruginosa	Laboratory	Negative	-
Staphylococcus coagulase negative	Laboratory	Positive	-
Trueperella pyogenes	Laboratory	Positive	-
Acinetobacter spp.	Preputial smegma	Negative	-
Acinetobacter calcoaceticus-boumannii	Preputial smegma	Negative	-
Bacillus thuringiensis	Preputial smegma	Positive	-
Corynebacterium renale	Preputial smegma	Positive	-
Escherichia coli	Preputial smegma	Negative	-
Staphylococcus haemolyticus	Preputial smegma	Positive	-
Staphylococcus chromogenes	Preputial smegma	Positive	-

DIF: direct immunofluorescence

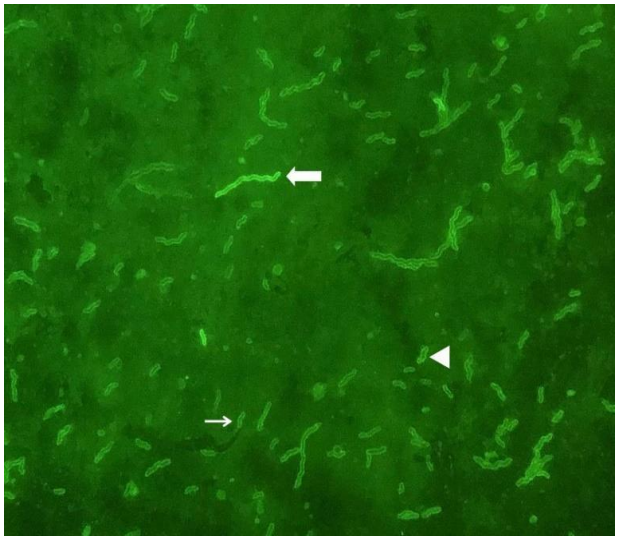

Figure 1. Direct fluorescence antibody test with polyclonal fluorescein isothiocyanate conjugated anti-*Campylobacter fetus* in bacterial suspension corresponding to *C. fetus* subsp. *fetus.* Note the different bacilli forms: large spiral form (thick white arrow), "S" form (white arrowhead) and seagull flight form (thin withe arrow), with intense fluorescence at the periphery of the bacterial structure.

Table 2 shows the results of DIF test and bacteriological culture in samples of PS from bulls experimentally infected with Cff (G2) and Cfv (G3). The G4 (negative control) resulted negative for the DIF test and bacteriological culture during all the sampling period. Among 24 readings from infected bulls, 19 were positive by DIF. It resulted in a sensitivity of 79 % and a specificity of 100 % of DIF for *C. fetus* detection on bull PS. The predictive positive value (PPV) and the negative predictive value (NPV) were 100 and 76 %, respectively. The PPV value was calculated as the number of true positive over true positive plus false positive results while NPV as the number of true negative over true negative plus false negative results. For bacteriological culture, of the 24 readings from the infected bulls, 20 were positive to *C. fetus*. A sensitivity and specificity of 83 % and 100 %, respectively wereachieved. The PPV and NPV values were 100 % and 76 %, respectively. Concordance between DIF and bacteriological culture was 83 % (Kappa index: 0.65) (0.412-0.885).

Table 2. Results of bacteriological culture and DIF test of 24 preputial samples collected from bulls experimentally infected with *Campylobacter fetus venerealis* (n=3) and *Campylobacter fetus fetus* (n=3).

	Inoculation Day 0		**Re inoculation Day 7**		**Day 14**		**Day 21**		**Day 28**	
Strain	Culture	DIF	Culture	DIF	Culture	DIF	Culture	DIF	Culture	DIF
Cfv 1	-	-	-	+	+	+	+	+	-	+
Cfv 2	-	-	+	-	+	-	+	-	+	+
Cfv 3	-	-	+	+	+	+	+	+	-	+
Cff 1	-	-	+	-	+	+	-	-	+	+
Cff 2	-	-	+	+	+	+	+	+	+	+
Cff 3	-	-	+	+	+	+	+	+	+	+

Cfv: *Campylobacter fetus venerealis*; Cff: *Campylobacter fetus fetus*; DIF: direct immunofluorescence

DISCUSSION

The present study shows a high specificity of the DIF technique for detection of *C. fetus* in clinical samples and cultures using a fluorescent conjugate elaborated in Argentina.

The FITC-anti *C. fetus* conjugate did not react with *C. jejuni*, C. *bubulus* and *C. hyointestinalis,* and non-*Campylobacter* bacteria. This finding emphasizes the relevance of no cross- reactivity of fluorescent conjugate with bacteria isolated and identified from bull smegma since they are part of the normal microbiota of preputial cavity as well as external contaminants, mainly from feces[15], therefore they can be present in samples taken for diagnosis. The PPV value of 100 % indicated no false positive results while the NPV value of 76 % indicated a relatively low rate of false negative results. The 100 % of inter-observer concordance showed that there was no misinterpretation of the test results as can occur with inexperienced operators.

Although bacteriological culture of *C. fetus* is considered the gold standard for diagnosis of BGC, it has some limitations that affect its efficacy. The low number of organisms and the presence of numerous contaminants can impair the isolation[5]. Non-pathogenic contaminants such as *Proteus* spp and *Pseudomona*s spp have a rapid proliferation and inhibit the growth of *C. fetus.* In fact, microbial contaminants growing on plates can cover 75-100 % of the surface,

despite improved media[9]. This observation highlights the importance of using the DIF test with high specificity, sparing elimination of false positive bulls and keeping false negatives. This test also had a high sensitivity, 79 %, which seems to be better than 69 % reported in Campero *et al.*[3]. However, other authors have reported sensitivities higher than 90 % in bull PS[5, 6]. The comparison of DIF results with those of bacteriological culture showed that there was a good concordance (83 %) between both techniques, indicating that the two tests can be feasible and complementary for the diagnosis of campylobacteriosis.

The variability in sensitivity of diagnostic tests is influenced by several factors such as sampling by the veterinarian, intrinsic factors of the bull related to variation in bacterial concentration and expertise of observers[3,18]. The negative results recorded in the inoculated bulls in some samplings after inoculation suggest that the interval of 7 days between samplings or the presence of contaminants in the microbiome could have diminished the bacterial load, generating false negative readings. The microbiome has organisms that are common in soil, cow vagina, respiratory tract and feces[23] which could affect the infection level. However, by day "28", sensitivity was the highest possibly because the preputial colonization by *C. fetus* was consolidated and the microbial population increased. Infection is a complex process that occurs through different stages that involves different molecular mechanisms. First is adherence of bacteria to host epithelial cells, next translocation to deeper layers of the mucosa and finally circulation in blood stream[20].

As consequence of moderate sensitivity, it is recommended that at least 2 consecutive scrapings per bull should be done before the diagnosis of an animal as negative. It is known that the DIF test does not differentiate between the two *C. fetus* subspecies; however both have clinical relevance diminishing cattle reproduction rates associated with infertility and abortion[3, 11]. Particularly, the subspecies Cff is common in the gastrointestinal tract and, occasionally it can colonize the genital tract of male and female cattle for a variable period during which the sexual transmission of the microorganism is possible[1]. For this reason, Cff identification in bulls is an important diagnosis to take into account when control or preventive measures are defined to limit abortions, therefore it must not be dismissed. Particularly, the Bacteriology Laboratory of INTA-EEA Balcarce (Argentina) has a collection of 137 strains of *C. fetus* recovered from aborted bovine fetuses tissues (n=93), cervico-vaginal mucus (n=33) and preputial smegma (n=11) during 28 years (1990-2018). Of the 137 strains, 85 % were classified as Cff and only 35 were analyzed by PCR, with 67 % of coincident results between molecular subspeciation approaches and phenotypic typing[3].

Currently, DIF is widely used as a routine diagnostic test for BGC in South America such as Uruguay, Paraguay, Argentina, Brazil and Colombia[18]. According to OIE, DIF is considered a suitable diagnostic method to apply in BGC control programs, being easy, fast, economic as well as highly sensitive and specific.

The development of several molecular methods, among them the PCR assay, has enhanced the BGC diagnostic and subspeciation of *C. fetus*[21]. Although this technique is an improvement, it has its limitations causing a cross reaction with *C. hyointestinales*, a common resident in cattle feces[19], and fails to identify the subspecies of *C. fetus*[24]. Additionally, the PCR protocols need to be standardized and validated prior to the implementation in routine to demonstrate the performance against the reference method under South American conditions[18].

The BGC is endemic in many developing countries with consequences on cattle productivity and economy. Particularly in Argentina, a 10 % reduction in weaning rate of infected herds and annual losses of $165 million have been reported[7]. The first evidence of BGC circulation occurred in 1968 when *C. fetus* was identified. However, with the implementation of a national program for control and eradication (1983-1996) of venereal diseases that included the use of DIF test and the application of productive management rules as elimination of positive bulls, the incidence of BGC decreased[4, 17] (unpublished data, Laboratorio Biológico Tandil). The percentage of BGC infection in beef cattle herds decreased from about 50 % to 15-18 % between 1983 and 1999, and up to 7.2 % in 2008[4, 16, 19]. In the area of Cuenca del Salado (Buenos Aires province), the number of positive establishments (with at least one *C. fetus* positive animal) decreased from 5.7 % to 4.2 % between 2001-2004 and 2005-2009 and, for the same time period, the number of positive bulls decreased from 0.65 % to 0.5 %[16]. Along with these results, an unpublished study of Laboratorio Biologico Tandil in Buenos Aires province, found a decrease from 1.04 % to 0.37 % on individual prevalence over 133962 bulls during the period 2003-2013.

The specificity of DIF for detection of *C. fetus* was not extensively evaluated but some studies have been reported[5, 18]. To our knowledge there are not enough experiments designed to test for cross-reaction including bacteria growing on PS collected from *in vivo* bull, most of the studies use reference strains from culture collection. This allows to "simulate" the preputial microbiota from clinical samples. Cross-reactivity may cause misinterpretation of the results,

therefore checking the antibody that specifically reacts with *C. fetus* for cross-reaction with closely related bacteria is crucial for validation of diagnostic tests.

To characterize the preputial microbiota of bulls may lead to a better comprehension of the establishment and persistence of *C. fetus*[6], so further studies are needed to identify the bacterial communities residing in preputial cavity and investigate possible interferences with diagnostic tests. To explore preputial microbiota of bovines can provide insights that help explain failure and success of diagnostic test using directly preputial samples.

CONCLUSION

The results reported in this study point out that DIF is a highly specific test since Cff and Cfv were detected and no cross-reactions were observed with bacterial strains isolated from bull genital microbiome and culture collection. The DIF technique demonstrated comparable performance to the cultured reference method in preputial smegma samples; however the high specificity together with other advantages such as rapid and easy detection and low cost make the DIF an effective alternative and important tool for the diagnosis of campylobacteriosis in bulls.

Although there was no effect of operators in the readings of assay, it is advisable to have a well-trained observer with certain experience to discriminate other possible fluorescent structures and, also use positive and negative control for all assays.

Declaration of competing interest. The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

We thank Martin Mayoral, Walter Bagazette, Diego Herrera, and Abel and Cristian Gulle for their help in field sampling. This publication is presented as a partial requirement for JAG to obtain a Doctor Degree at Facultad de Ciencias Agrarias, Universidad Nacional de Mar del Plata, Argentina.

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