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Evaluation of direct fluorescent antibody test for the diagnosis of Bovine Genital Campylobacteriosis

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ABSTRACT. The direct fluorescent antibody test (DFAT) for the diagnosis of Bovine Genital Campylobacteriosis was assessed for its detection limit, observer effect, sensitivity and specificity. In addition, the specificity of the fluorescent conjugate was tested against *Campylobacter* sp, *Arcobacter* sp, *Helicobacter* sp, *E. coli* and other bacteria from the preputial flora. Ten – fold dilutions of *C. fetus* subsp. v*enerealis* NCTC 10354 in PBS or preputial washings with or without centrifugation were used. All experiments were done in duplicate by three observers. Positive and negative controls were included in each assay. The detection limits of DFAT were 10^4 CFU/ml for PBS and non - centrifuged preputial washings and 10^2 CFU/ml for centrifuged preputial washings. There was no observer effect. The sensitivity and specificity of DFAT were 92.59% and 88.89%, respectively. The DFAT was observed to be sensitive, specific and the effect of experienced observers was minimal on test performance.

Key words: Bovine Genital Campylobacteriosis - direct fluorescent antibody test - *Campylobacter fetus*-preputial washing - diagnosis.

RESUMEN. La inmunofluorescencia directa (IFD) fue probada para el diagnóstico de la Campilobacteriosis Genital Bovina para el límite de detección, efecto del observador y sensibilidad. De la misma manera, la especificidad de la inmunofluorescencia directa fue probada contra *Campylobacter* sp, *Arcobacter* sp, *Helicobacter* sp, *E. coli* y otras bacterias originadas de la flora prepucial. *C. fetus* subsp. *venerealis* NCTC 10354 o lavados prepuciales con o sin centrifugación fueron diluidos diez veces en PBS. Todas las experiencias fueron hechas en duplicados con tres observadores. Controles positivos y negativos fueron incluidos en cada prueba. El límite de detección de la IFD fue 10^4 UFC/ml para PBS y lavados prepuciales sin centrifugación y 10^2 UFC/ml para lavados prepuciales centrifugados. No hubo efecto del observador. La sensibilidad y la especificidad de la IFD fueron 92.59% y 88.89%, respectivamente. Se observó que la IFD fue sensible, específica y el efecto de los observadores es mínimo en la ejecución de la prueba.

Palabras chave: Campilobacteriosis Genital Bovina-inmunofluorescencia directa - *Campylobacter fetus* - lavado prepucial - diagnóstico.

INTRODUCTION

Bovine Genital Campylobacteriosis is an infectious disease caused by *Campylobacter fetus* subsp. *venerealis* whose transmission occurs mainly along venereal paths. The disease causes infertility whose major sign is repeated return to service with prolonged interservice intervals.[2]

C. fetus infection in cattle has decreased in regions where artificial insemination and effective programs of vaccination and antibiotic therapy have been practiced, yet the disease continues to be an important cause of reproductive problems in many countries that have large herds at natural breeding,[5] such as Brazil.[12]

The diagnosis and control of Bovine Genital Campylobacteriosis are mainly done in bulls since they act as asymptomatic carriers of organisms and also are responsible by spreading the disease in the herd. The detection of bulls infected by *C. fetus* permits to establish the causes of reproductive disturbs in the herd which should be submitted to the appropriated method of control.[1] Currently available techniques used to diagnose *C. fetus* infection in these animals are isolation and identification of bacteria[1], polymerase chain reaction (PCR)[4,23] and direct fluorescent antibody test (DFAT).[3,15,18,19,26]

The DFAT has been used since 1960s for the diagnosis of *C. fetus* infection. It has been characterized as a convenient and quick method for detection of carrier bulls[2] and is prescribed by OIE for testing bulls for international trade.[24] However, until now, this technique had not been assessed for its accuracy and detection limit. Thus, the aim of this study was to evaluate the detection limit, the observer effect, the sensitivity and the specificity of previously described DFAT, and the specificity of the fluorescent conjugate against *Campylobacter* sp, *Arcobacter* sp, *Helicobacter* sp, *E. coli* and other bacteria from the preputial flora.

MATERIALS AND METHODS

Fluorescent conjugate – Antisera against *C. fetus* subsp. v*enerealis* NCTC 10354 were produced in three rabbits as described by Edwards & Ewing[6] with modifications. Briefly, 0.5 ml of *C. fetus* subsp. *venerealis* NCTC 10354 in

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Rev Latinoam Microbiol 2002; 44 (3-4): 118-123

PBS pH 7.2 at a concentration equal to tube 10 of MacFarland standard was emulsified in Freund's complete adjuvant and this mixture was intramuscularly inoculated in each rabbit. These animals were endovenously boosted with 0.5, 1.0, 2.0 and 4.0 ml of a similar suspension of *C. fetus* subsp. *venerealis* NCTC 10354 in PBS pH 7.2 without adjuvant, respectively, on days 6, 13, 20 and 27 after first inoculation. After 15 days of the last shot, animals were bled, IgG was purified[14] and conjugated with fluorescein isothiocyanate.[20] Optimum dilution of the fluorescein conjugate was determined by checkboard titration against *C. fetus* subsp. *venerealis* NCTC 10354.

Direct Fluorescent Antibody Technique (DFAT) - DFAT for the detection of *C. fetus* subsp. *venerealis* was done according to Mellick et al.[15] and Winter et al. [26] The material (20µl) was applied in duplicate on microscopic slides. In parallel, 0.01M phosphate-buffered saline (PBS) pH 7.2, and suspension of *C. fetus* subsp. *venerealis* strain NCTC 10354 were included, respectively, as negative and positive controls of the reaction. The material was air-dried and fixed in acetone at – 20ºC for 30 min. The fluorescent conjugate anti – *C. fetus* subsp. *venerealis* NCTC 10354 was added at 1/50 dilution and incubated for 30 min at 37ºC. The slides were washed three times in PBS pH 7.2 and one time in tap water and then mounted in buffered glycerol pH 9.2. The preparations were examined in an epifluorescent microscope (CBA, Olympus, Japan). Samples that showed at least one fluorescent bacterium presenting typical morphology of *C. fetus* in one of any duplicate were considered positive.

Bacterial suspensions-A total of six bacterial suspensions coded as A, B, C, D, E and F were prepared from *C. fetus* subsp. *venerealis* strain NCTC 10354 which was grown on Brain Heart Infusion agar (BHI-Difco – USA) supplemented with 10% horse blood for 48 h at 37ºC in microaerobic conditions (5% O_2, 10% CO_2, 85% N_2). The concentration of microbial cells was adjusted to 10^9 CFU/ml by MacFarland No. 3 standard and 1.0 absorbance at 595 nm. Then, serial ten-fold dilutions were prepared from each bacterial suspension. Viable counts were done by the drop counting method on BHI blood agar plates.[16] Nine dilutions from A, B and C bacterial suspensions were used in the evaluation of observer effect and detection limit of DFAT in PBS. The bacterial suspensions D, E and F and their first eight dilutions were used to inoculate preputial washing mixtures. The schematic model of the use of these bacterial suspensions is presented in Figure 1.

Preputial washing preparation – Fresh *C. fetus*-free preputial washings were obtained as described by Leite et al.[13] from 18 bulls, aged approximately nine months, from a Bovine Genital Campylobacteriosis–free herd. An aliquot of 1 ml from all preputial washings was tested by the DFAT for *C. fetus*[15, 26] and negative results were given for all animals. The preputial washings gave three mixtures (300 ml each) (M1, M2 and M3) and each mixture was divided in 10 aliquots of 27 ml. One aliquot of each preputial washing mixture did not receive inoculum and was centrifuged in order to evaluate the specificity of DFAT. Three milliliters of bacterial suspension D, E, and F and each of their first eight ten-fold dilutions were added to 27 ml of each aliquot of preputial washing mixtures. The inoculated aliquots of preputial washings were used without centrifugation for the evaluation of the detection limit and the observer effect of DFAT. The same inoculated aliquots of preputial washings were centrifuged to evaluate the detection limit, the observer effect and the sensitivity of DFAT (Fig. 1). Two steps of centrifugation were used. First, preputial washings were centrifuged at 600 x g for 10 min at 4ºC. The supernatant of the first step was submitted to 13000 x g for 30 min at 4ºC. The pellet of the second centrifugation step was resuspended in 500 µl of PBS pH 7.2 and analyzed by DFAT.[15,26]

Experimental design

Slides examination – All slides for DFAT were prepared in duplicate and blindly read by three observers, codified as I, II and III, which were responsible for the Bovine Genital Campylobacteriosis diagnosis at Escola de Veterinária da Universidade Federal de Minas Gerais.

Limit of detection and observer effect – The limit of detection and the observer effect on DFAT were evaluated in three separated experiments: PBS inoculated with *C. fetus* subsp. *venerealis* strain NCTC 10354 (A, B and C suspensions) and preputial washings inoculated with *C. fetus* subsp. *venerealis* strain NCTC 10354 (M1, M2 and M3 mixtures) with and without centrifugation (Fig. 1). The observer effect in each experiment was analyzed using the Cochran's Q Test.[22] The evaluation of the detection limit of DFAT was done in each experiment, considering the readings of all observers. In order to find the final detection limit, the partial detection limit of each suspension was taken, i. e., the least concentration that all observers considered to be positive. Since three suspensions for each experiment were analyzed, three partial detection limits were taken. The higher concentration found among partial detection limits was regarded as the final detection limit because it indicated an agreement of all observers in all suspensions.

Influence of bacterial concentration on readings – Bacterial concentrations were grouped in three classes: class 1 - from 10^8 to 10^6, class 2-from 10^5 to 10^3, and class 3 - from 10^2 to 1 CFU/ml. The influence of bacterial concentration on readings was evaluated by the chi – square method.[21]

Rev Latinoam Microbiol 2002; 44 (3-4): 118-123

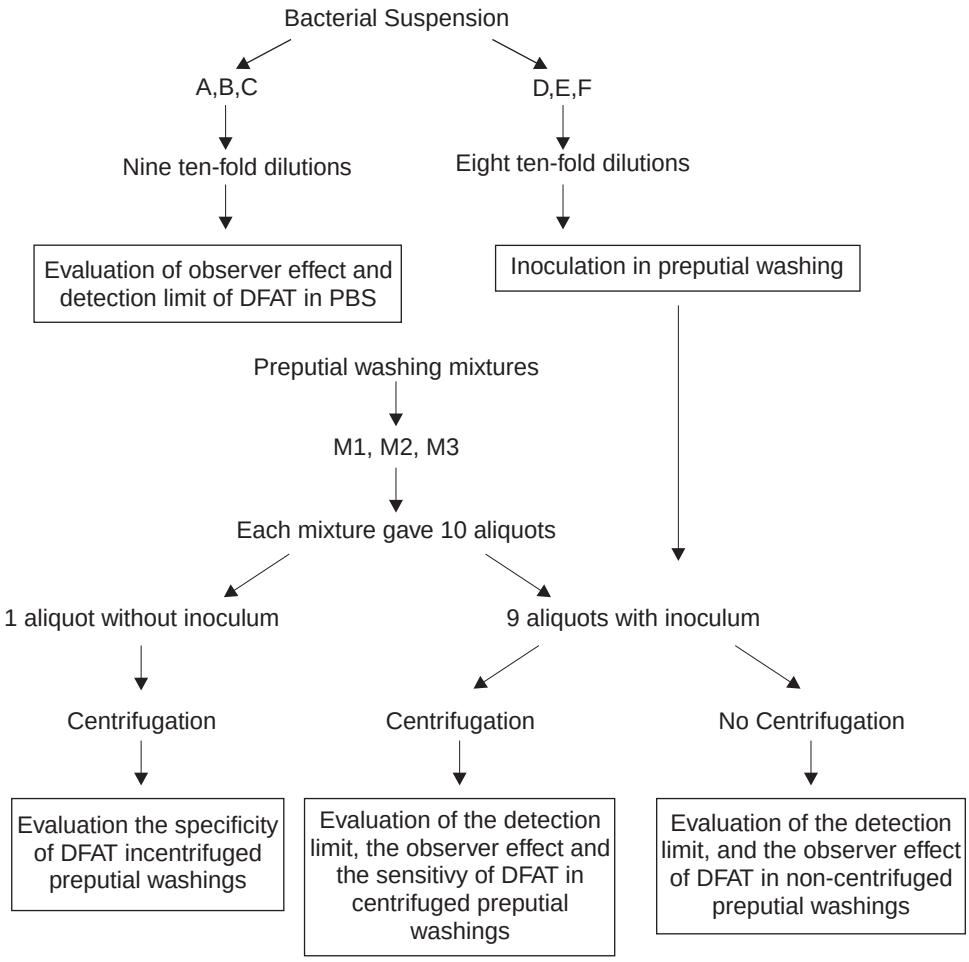

Figure 1. *Schematic model of the use of bacterial suspensions and preputial washings in DFAT evaluation.*

Specificity and sensitivity of DFAT – A total of 81 readings of centrifuged inoculated preputial washings (3 mixtures x 9 dilutions x 3 observers) were used for sensitivity analysis and 9 readings of centrifuged but non-inoculated preputial washings (3 aliquots x 3 observers) were used for specificity analysis of DFAT (Fig. 1). The statistical analysis was done in accordance to Henken et al.,[9] considering the inoculation of samples as the gold standard.

Specificity of the fluorescent conjugate – The specificity of the fluorescent conjugate was evaluated against reference bacterial strains listed in Table 1 and bacteria from the preputial flora which were isolated from preputial washings that did not receive inocula. All bacteria were isolated on BHI agar supplemented with 10% equine blood for 48 h at 37ºC in aerobic and microaerobic atmospheres. After growth, these bacteria were ressuspended in PBS pH 7.2 at a concentration adjusted to MacFarland No. 3 standard. From each bacterial suspension three serial ten-fold dilutions were made and 20 µl from each dilution were examined in duplicate by DFAT.

RESULTS

The concentrations of nine dilutions from bacterial suspensions A, B and C varied from 10^8 to 10^0 CFU/ml and the concentrations of bacterial suspensions D, E and F and their first eight dilutions varied from 10^9 to 10^1 CFU/ml, respectively. The bacterial concentrations of nine dilutions of M1, M2 and M3 mixtures varied from 10^8 to 10^0 CFU/ml, respectively.

The detection limits of DFAT in PBS, non-centrifuged and centrifuged preputial washings were 10^4, 10^4 and 10^2 CFU/ml, respectively (Table 2). The bacterial concentration influenced the results in all experiments (Table 3) (PBS: $\chi^2 = 7.78$, df = 2, $P<0.05$; non-centrifuged preputial washings: $\chi^2 = 36.57$, df = 2, $P<0.001$; centrifuged preputial washings: $\chi^2 = 12.96$, df = 2, $P<0.01$). In the three experiments analyzed no statistically significant difference was found in the results of the observers' readings (PBS: Q = 2.80, df = 2, P = 0.24; non-centrifuged preputial washings: Q = 2.88, df = 2, P = 0.23; centri-

Rev Latinoam Microbiol 2002; 44 (3-4): 118-123

Table 1. Reference bacterial strains used in the analysis of the specificity of fluorescent conjugate against *C. fetus* subsp. *venerealis*.

Species	Strains
Arcobacter skirrowii	LMG[1] 6621
A. butzleri	LMG 15919
Campylobacter coli	NCTC[2] 11366[T]
C. fetus subsp. *fetus* serotype B	ATCC[3] 27374[T]
C. fetus subsp. *fetus* serotype B	ADRI[4] 553
C. fetus subsp. *fetus* serotype B	ADRI 1810
C. fetus subsp. *fetus* serotype A	ADRI 1811
C. fetus subsp. *fetus* serotype A	ADRI 1812
C. fetus subsp. *venerealis*	NCTC 10354
C. fetus subsp. venerealis	ADRI 510
C. fetus subsp. *venerealis*	ADRI 528
C. fetus subsp. *venerealis*	ADRI 534
C. fetus subsp. *venerealis*	ADRI 1832
C. hyointestinalis subsp. *hyointestinalis*	LCDC[5] 17398 (= LMG 12686)
C. hyointestinalis subsp. *lawsoni*	LMG 14432[T]
C. jejuni subsp. *doylei*	LMG 8843[T]
C. jejuni subsp. *jejuni*	NCTC11351[T]
C. lari	NCTC 11352[T]
C. sputorum biovar *paraureolyticus*	LCDC 6577
C. sputorum biovar *fecalis*	NCTC 11415
C. sputorum biovar *paraureolyticus*	LCDC 6939
C. sputorum biovar *sputorum*[6]	LMG 6447
Escherichia coli	ATCC 25922
Helicobacter fenneliae	LMG 13306

T – Type Strain
1 – LMG – Laboratorium voor Microbiologie – Rijksuniversiteit Gent – Belgium
2 – NCTC – National Culture Type Collection – United Kingdom
3 - ATCC – American Type Culture Collection – United States of America
4 - ADRI – Animal Diseases Research Institute - Canada
5 – LCDC – Laboratory Center for Disease Control – Canada
6 – Formerly type strain of "C. sputorum biovar bubulus" (= old "*C. bubulus*")

fuged preputial washings: Q = 4.66, df = 2, P = 0.09). However, in all experiments, observer I had a better performance on readings than the others, followed by the observer II and III (Table 4).

Among 81 readings from inoculated preputial washings, 75 were positive by DFAT and among 9 readings from non-inoculated preputial washings, 8 were negative by DFAT. Those results showed a sensitivity of 92.59% and a specificity of 88.89% of DFAT for *C. fetus* subsp. *venerealis* detection on preputial washings.

The specificity of the fluorescent conjugate against bacterial reference strains and other bacteria from the preputial flora was the same for all observers. No fluorescent bacteria were seen in preparations from *Campylobacter* sp other than *C. fetus*, *Helicobacter* sp, *Arcobacter* sp, *E. coli* or bacteria from preputial flora. The conjugate prepared against *C. fetus* subsp. v*enerealis* strain NCTC 10354 reacted only with *C. fetus* subsp. *venerealis* strains ADRI 510, ADRI 528, ADRI 534 and ADRI 1832 and NCTC 10354 and with *C. fetus* subsp. *fetus* serotype A strains ADRI 1811 and ADRI 1812.

DISCUSSION

The detection limit is one of the most important points in test evaluation because it influences sensitivity. The detection limit of DFAT in PBS shows the ability of the technique for *C. fetus* subsp. *venerealis* detection performed without contaminants and with the exact bacterial concentration of samples. Its results were similar to the ones found with preputial washings without centrifugation and only moderately high numbers of bacteria could be detected.

Table 2. Detection limits of DFAT for *C. fetus* subsp. *venerealis* detection in different experiments.

Experiments	Partial detection limits (CFU/ml)						Final detection limits (CFU/ml)
	Suspensions			Mixtures			
	A	B	C	M1	M2	M3	
PBS	10^4	10^3	10^0	-	-	-	10^4
Non-centrifuged preputial washing	-	-	-	10^4	10^3	10^4	10^4
Centrifuged preputial washing	-		-	10^2	10^1	10^0	10^2

Table 3. Percentage of accurate readings in DFAT for *C. fetus* subsp. *venerealis* detection in different classes of bacterial concentrations.

Experiments	Classes of Concentrations (CFU/ml)		
	10^8 to 10^6	10^5 to 10^3	10^2 to 1
PBS	100%	92.59%	77.77%
Non-centrifuged preputial washing	100%	85.18%	29.62%
Centrifuged preputial washing	100%	100%	77.77%

Rev Latinoam Microbiol 2002; 44 (3-4): 118-123

Table 4. Percentage of accurate readings in DFAT for *C. fetus* subsp. *venerealis* detection by observers.

Experiments	Observers		
	I	II	III
PBS	96.29%	88.88%	85.18%
Non-centrifuged preputial washing	77.77%	70.37%	66.66%
Centrifuged preputial washing	96.29%	92.59%	88.88%

This was probably due to the lack of centrifugation steps in both procedures. Moreover, in non-centrifuged preputial washings, cell debris could have impaired either the DFAT reaction or the reading by the observers.

DFAT readings in centrifuged preputial washings showed the best results due to high rate of accurate readings among different classes of bacterial concentration among observers and the lowest detection limit. These results can be ascribed to the two step centrifugation process of samples that allow the elimination of cell debris and the concentration of bacteria in a small volume, improving the detection of *C. fetus*. The detection limit of DFAT found in centrifuged preputial washings in the present study was higher than that found by Eaglesome et al.[4] using PCR for detection of *C. fetus* subsp. *venerealis* in bovine semen (3 CFU/ml). However, it was similar to the findings of Philpott[18] that found a detection limit of DFAT in preputial washing of 50 to 100 CFU/ml.

The low subjectivity and detection limit of DFAT provide some advantages of this test over isolation of *C. fetus.* Although the isolation of the *C. fetus* from preputial secretions is considered the gold standard for diagnosis of Bovine Genital Campylobacteriosis,[10] low number of organisms per sample can impair the diagnosis due to the numerous contaminating bacteria which are normally present in preputial flora[19,25] and which do not constitute a problem in DFAT as shown by the present study.

The fluorescent conjugate did react against neither *E. coli* nor other bacteria from preputial flora. That was similar to the findings of Mellick et al.,[15] Dufty[3] and Philpott,[18] but it was different from the findings of Philpott,[19] who saw brilliantly stained diplococcus and rods in the samples.

The specificity of the fluorescent conjugate used in DFAT against *C. fetus* demonstrates the efficiency of the technique, since some of bacteria evaluated in this study are very similar to *C. fetus* subsp. *venerealis* and they can be naturally found in preputial cavity of healthy bulls,[8] aborted fetuses[7] and in bovine intestinal tract.[25]

Moreover, the specificity of the fluorescent conjugate used in this study discloses other advantages of DFAT over the isolation technique: first, its high efficiency, even in the presence of contaminants[19,20,26] and second, its ability to identify and differentiate *C. fetus* from another *Campylobacter* sp without the use of biochemical tests.[20,26]

The absence of previous data on specificity and sensitivity of DFAT in the diagnosis of Bovine Genital Campylobacteriosis limits our inferences from the values found in this study. DFAT, however, was observed to be quite sensitive and specific for the diagnosis of *C. fetus* infection. The presence of false-negative results (lost of sensitivity) probably occurred due to the low bacterial concentration of the samples, since all false-negative readings were done in samples with bacterial concentration equal to or lower than 10^1 CFU/ml. However, the exactness of the observers readings (high rate of correct readings) confirms the proposal of Winter et al.[26] in which the difficulties of preparation of specimens and interpretation of findings can be overcome almost entirely by well trained technicians.

Just one false-positive reading (lost of specificity) was found and it occurred in only one of the duplicates. Despite of the presence of few specimens for evaluation of the specificity of DFAT in this experiment, the value found, 88.89%, can be an estimate of the specificity of this technique in order to be discussed in future experiments.

Among all reference bacterial strains tested, the fluorescent conjugate just reacted against *C. fetus* strains. This specificity was similar to that found by Mellick et al.,[15] Dufty[3] and Ruckerbauer et al.[20] Moreover, in this study, it was found that the conjugate which was prepared against a *C. fetus* subsp. *venerealis* serotype A strain reacted just against *C. fetus* serotype A strains as mentioned by Dekeyser[2], recognizing all *C. fetus* subsp. *venerealis* strains but only *C. fetus* subsp. *fetus* serotype A strains.

The absence of reaction of the fluorescent conjugate against *C. fetus* subsp. *fetus* serotype B has no influence on the diagnosis of Bovine Genital Campylobacteriosis and on the epidemiological studies of *C. fetus* subsp. *venerealis*, which is far more important than *C. fetus* subsp. *fetus*, due to its genital tropism.[11] Besides, the *C. fetus* subsp. *fetus* infections are not considered to be endemic, but sporadic.[17]

In this study, the DFAT showed low detection limit in preputial washings, good sensitivity and specificity and low influence of experienced observers. These results corrobo-

Rev Latinoam Microbiol 2002; 44 (3-4): 118-123

rate the use of DFAT as an important and supportive technique for the control of Bovine Genital Campylobacteriosis.

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