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Use of ELISA, IFA, and Avidin-Biotin Staining for the Diagnosis of Bovine Genital Campylobacteriosis

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Abstract: Acid glycine extract (AGE) and whole cell (WC) antibodies were used in ELISA testing for the detection of *Campylobacter fetus subsp. venerealis* and *subsp. fetus* antibodies in the blood sera of 150 female cattle and 100 bulls which were one year old or older. It was observed that AGE antibody was more specific than WC antibody. A cross reaction was detected between *C. fetus subsp. venerealis* and *subsp. fetus.* Thus, it was concluded that for *Campylobacter* ELISA could only be used as a monitoring test.

In direct diagnosis of *C. fetus* with the Indirect Flurescent Antibody Test and Avidin-Biotin Staining, 36% and 31.2% positivity were detected in 125 samples of vaginal mucus. 42% and 40% positivity were detected with the same test in 100 samples of preputial fluid. The differences of 5% and 2% between the two positivity values were not found to be statistically significant. The AB method is suggested for direct diagnosis.

Key Words: *Campylobacter fetus,* ELISA, IFA, Avidin-Biotin.

Genital Campylobacteriosis'in Tanısında ELISA, IFA ve Avidin Biotin Yöntemlerinin Kullanımı

Özet: *Campylobacter fetus subsp. venerealis* ve *subsp. fetus* antikorlarının saptanması için, 150 inek ve bir yaş ve üzerinde 100 boğa kan serumlarına uygulanan ELISA'da asitglisin ekstrak (AGE) ve tüm hücre antijenleri (TH) kullanıldı. AGE antijeninin TH antijenine göre daha seçici olduğu görüldü. *Campylobacter fetus subsp. venerealis* ve *subsp. fetus* arasında kros reaksiyon saptandı. Camplobacter ELISA'sının sadece bir tarama testi olarak kullanılabileceği saptandı.

Campylobacter fetus'un direk tanısında 125 vaginal yıkantı sıvısında IFA yöntemi ile % 36 ve AB yöntemi ile % 31.2 pozitiflik, 100 prepisyum yıkantı sıvısında aynı testlerle sırasıyla % 42 ve % 40'lık pozitiflik saptanmıştır. Her iki yöntemin kendi arasındaki pozitiflik oranı arasındaki % 5 ve % 2'lik farklar istatistiksel açıdan önemsiz bulunmuştur. Direk tanıda Avidin-Biotin yöntemi önerilmiştir.

Anahtar Sözcükler: *Campylobacter fetus,* ELISA, IFA, Avidin-Biotin.

Introduction

Campylobacter fetus is an economically significant veterinary pathogen. Two subspecies are classified, *C. fetus subsp venerealis* and *C. fetus subsp fetus. C. fetus subsp. venerealis* causes veneral genital tract infections in cattle which can lead to infertility and abortion. The natural habitat of this organism is the bovine reproductive tract. In the bull, it is confined to the mucosa of the glans penis, prepuce and the distal portion of the urethra. Infected bulls show no clinical signs but become carriers and infect females at service. In heifers and cows, the sites of infection are within the lumen of the vagina, the cervix, uterus and oviducts. The disease is characterised by temporary infertility of female cattle as a result of subacute diffuse mucopurulent cervicitis, endometritis and salpingitis. Abortion occurs in a small percentage of infected cows. Transmission is known to occur by venereal means (1, 2, 3).

The other subspecies, *Campylobacter fetus subsp fetus,* is known to cause sporadic aboriton in cattle. This organism, which originates in the intestine and can cause occasional abortion in cattle, is not normally associated with bovine infertility (3). However, genital tropism and coital transmission of subsp. fetus in cattle was described by Agumbah and Ogaa (4). In addition, this subspecies has been reported to cause infertility in cattle and to persist in the genital tract of experimentally inoculated heifers (5).

Although the incidence of bovine venereal diseases has decreased considerably in countries where artificial

insemination and effective vaccination and control programmes are extensively practised, it has been shown that *C. fetus subsp. venerealis* continues to be a major microbial agent causing reproductive problems in many countries (2). In Turkey, *C. fetus subsp. venerealis* has been isolated both in preputial fluid and in aborted fetal material (6, 7).

The diagnosis of bovine venereal campylobacteriosis is difficult, because of the low survival rate of the organism with conventional sampling procedures (2, 8, 9) and the absence of reliable sero-diagnostic methods (10). In serologic diagnosis of campylobacteriosis, the fluorescent antibody test, vaginal mucus agglutination, complement fixation and ELISA have been used by several researchers (9, 10, 11, 12, 13, 14, 15, 16, 17). In recent years, an enzyme-linked immunosorbent assay (ELISA) measuring antigen specific IgA antibodies in vaginal mucus has been used to diagnose bovine venereal campylobacteriosis (18, 19).

The aim of this study was to examine *C. fetus subsp. venerealis* and *subsp. fetus* antibodies in adult male and female cattle brought to the slaughter house from different districts and also in cattle bred on local farms. A further aim was to identify *C. fetus* in the vaginal and preputial washing fluids of these animals with avidin biotin staining (AB) and by an indirect fluorescent antibody (IFA), and to assess the utility of AB staining by comparing these two techniques.

Materials and Methods

The material used in this study was as follows;

1. The sera of 150 cows (one year old or more); blood samples were collected from 60 cows in the field and 90 cows that were brought to the slaughter house due to infertility.

2. Vaginal washing fluids were collected from 125 cows. Sixty-five of them were from the slaughter house and 60 were from the field.

3. The sera of 100 bulls (one year old or more); 14 of them were from the field and 86 were from the slaughter house.

4. Preputial washing fluids were collected from the 100 bulls above.

Bacterial strains

C. fetus subsp venerealis was kindly supplied by S. Hum, NSW Agriculture, Regional Veterinary Laboratory, Armidale, New South Wales, Australia. *C. fetus subsp fetus* (1419 EF 6828 A) was supplied by KÜKENS, University of Istanbul, Faculty of Medicine, Department of Microbiology, Turkey.

Collection and processing of vaginal mucus and preputial fluid

The collection and processing of vaginal mucus were adapted from the method of Lander (20). Preputial fluid was collected using the lavage technique from the prepuce of the bulls (21). The collected samples from the vagina and prepitium were centrifuged at 4000 rpm for 1 h and the deposit was washed three times in sterile phosphate-buffered saline (PBS, pH 7.2). Finally, the deposit was stored at 4°C until examination.

Preparation of whole cell atigens

C. fetus subsp. fetus and *C. fetus subsp. venerealis* were grown on Blood Agar No: 2 (Oxoid) with 7% defibrinated sheep blood at 37°C, in an atmosphere of 5% O_2, 10% CO_2 and 85% nitrogen for 72 h. Cells were harvested in 0.85% saline containing 0.4% formalin. The suspensions were kept at room temperature for 18 h and washed three times with washing solution (0.85% NaCl containing 0.2% formalin). The suspensions were adjusted to the McFarland No:1 neflometry standard and stored at 4°C (22).

Preparation of Acid-Glycine Extract Antigens

Bacteria were cultured on blood-agar plates as described above. Cells were collected and washed twice with sterile distilled water. Bacteria pellets were suspended in 0.2 M/glycine- HCL buffer (pH 2.2) at a concentration of 1g wet weight of cells in 25 ml buffer. The suspensions were stirred and incubated overnight at 4°C. Antigen extracts were centrifuged at 11.000g for 15 min. The supernatant was neutralized with NaOH and then dialyzed against distilled water at 4°C for 24 h (22, 23). Protein concentration was determined by the new Lowry method (24). The antigen extracts were stored at -20°C.

Antibody Production

Antigenic preparations were administered i.v. in New Zealand white rabbits. A typical injection schedule consisted of 0.5 ml antigen on the 1 st day, 1 ml antigen on the 4 th day, and 2 ml antigen on the 7 th, 10 th, 14 th and 16 th days. Bleeding was carried out on the 24 th day. The sera were collected and stored at –20 °C. Control non-immune sera were obtained before the first injection.

ELISA procedure

The antigens (0.003 mg/ml) were diluted two-fold in 0.5 M carbonate buffer, pH 9.6, and 100 µl was added to

each well. The plates were incubated at 4°C overnight, washed with ELISA washing solution (PBS Tween, phosphate-buffered saline, pH. 7.4, 0.05% Tween 20) 3 times and dried by shaking. The positive and negative control sera were diluted two-fold in PBS Tween and 100 µl was added to each well. The plates were incubated at 37°C for 1 h and then washed as before. 100 µl of commercial rabbit anti-bovine lgG peroxidase conjugate diluted (1/800) in PBS was added to each well. After 1 h incubation at 37°C, the plates were washed and 100 µl substrate was added (40 mg orthophenylene-diamine in 100 ml phosplate-citrate buffer, pH 5, immediately activated by 40 µl 30% H_2O_2). The plates were incubated at room temperature for 10 min, after which the reaction was stopped using 50 µl of 1.25 M H_2SO_4. The optical density (OD) was measured on an ELISA reader (OT230, version, 1.53) at 490 nm.

Using the method described above, the sera of 150 cows and 125 bulls, all field animals, were tested with a previously determined antigen dilution. Each sample was tested in duplicate. Positive and negative controls were included on each plate. The optical density (OD) was measured and expressed as an ELISA value (EV) according to the formula:

$$EV: \frac{\text{OD of sample-OD of negative control}}{\text{OD of positive control-OD of negative control}} \times 100$$

The EV values of the sera lower than 40, between 40 and 50, and higher than 50, were taken to be negative, suspicious and positive respectively.

Avidin-Biotin Staining and Indirect Flurescent Antibody Test

The deposits of vaginal mucus and preputial fluids were analysed for the presence of *C. fetus subsp. venerealis* using Avidin-Biotin Staining and IFA tests. The deposits were resuspended in 0.5 ml. of phosphate-buffered saline (PBS), pH 7.2, and about 50 µl of the suspension was spread over an area of 1 cm^2 on a glass slide and allowed to dry (about 2 h).

Avidin Biotin Staining Procedure

The avidin biotin peroxidase test was performed using a commercial kit (ExtrAvidin Peroxidase Staining kit, Sigma). The procedure was modified from that provided with the kit. The modified procedure was similar to the procedure used by Can (25). The smears were fixed in 95% methyl alcohol at room temperature for 10 min. The slides were rinsed with PBS from a washing bottle and placed in a PBS wash bath for 5 min. Nonspecific background staining was then reduced by applying 3% normal goat serum in Tris buffer for 20 min in a humidified chamber at 35°C. Rabbit primary antibody was added at an optimal dilution of 1% in PBS with % Bovine Serum Albumine. After 1 h of incubation at 35°C in a humidified chamber, the slides were rinsed gently in a PBS washing bath for 5 min. Excess fluid was shaken off from the slides and carefully wiped as before. Secondary biotinylated Goat anti-Rabbit IgG (provided in the kit) was added for 30 min in a humidified chamber at room temperature and the slides were washed as before. Extra Avidin-Peroxidase (Extra-3 Sigma) was diluted at a 1:20 ratio in PBS and 100 µl was added for 30 min in a humidified chamber at room temperature, after which the slides were washed as before. The final step was the addition of a freshly prepared solution of 3.3 diaminobenzidin tetrahidrochloride (DAB: 6 mg DAB in 10 ml 0.05 M Tris buffer immediately activated by 100 µl 3% H_2O_2) for 10 min. The slides were washed in distilled water and counterstained with Harris hematoxylin.

Indirect Fluorescent Antibody Procedure

The IFA staining procedure and reagents were adapted from the methods of Dutta et al. (26) with slight modifications. The smears were fixed in 95% methyl alcohol at room temperature for 12 min. The slides were washed in distilled water and were then air-dried. Rabbit primary antibody was added at 1:100 dilution in PSB. The slides were incubated at 37°C for 30 min in a humidified chamber. The slides were washed in PBS for 5 min and then washed in distilled water for 3 min and air-dried. 10 ml of the secodary antibody fluorescein-labeled goat anti-rabbit lgG (Sigma, F 7256) was applied, and the slides were incubated at 37°C for 30 min in a humidified chamber. The slides were washed in PBS and then in distilled water as described above. Slides were air-dried and examined by use of an UV microscope.

The t-test was used for statistical analysis of the serological tests (27).

Results

A- Results of ELISA

As a result of the tests with optimal conjugate dilution, the working dilutions of the sera, AGE and TH were found to be 1/100, 1/500 and 1/100 respectively.

In a preliminary study conducted with *C. fetus subsp. veneralis* and *C. fetus subsp. fetus* positive sera together with their AGE and WC antigens; it was found that, the AGE and TH antigens of *C. fetus subsp. venerealis* give a high titer positive reaction with *C. fetus subsp fetus*

positive serum. It was also found that the AGE and TH antigens of *C. fetus subsp fetus* give a highly positive reaction with positive serum of *C. fetus subsp. venerealis.* As a result of this, when studying field sera samples, only the AGE and WC antigens prepared from *C. fetus subsp. venerealis* were used.

1) Sera from cows

a) Results using AGE antigen

Of the blood samples of 60 cows from local farms, 12 (20%) contained antibodies against *C. fetus subsp. venerealis.* In addition, of the blood samples of 90 cows, (brought for slaughter with infertility), 32 (35.5%) contained antibodies against *C. fetus subsp. venerealis.* It was found that 4 (2 by 2) of the samples collected from slaughterhouses and farms were suspicious.

With indirect ELISA using the AGE antigen of 150 randomly selected female cattle, it was found that 44 (29.3%) of them were positive and that 102 (68%) were negative for C. fetus antibody (Table 1).

b) Results using Whole Cell Antigen

Antibodies against *C. fetus subsp. venerealis* were detected in the blood samples of 20 (33.3%) cows out of a total of 60, and of 41 (45.5%) cows out of a total of 90. It was found that 5 of the samples collected from slaughterhouses were suspicious.

In detecting *C. fetus* antibodies using WC antigens and indirect ELISA, out of a total 150 cows, 61 (40.6%) were found to be positive and 84 (56%) were found to be negative (Table 1).

2- Sera from bulls.

a) Resuls with AGE antigen

Of the blood samples of 14 bulls (one year old or more) from farms, 1 (7.1%) contained antibodies against *C. fetus subsp. venerealis*. In the blood samples of a total of 86 bulls brought to be slaughtered (one year old or more), 15 (18%) contained antibodies against *C. fetus subsp. venerealis*. 2 of the blood samples collected from slaughterhouses were suspicious.

In detecting *C. fetus* antibodies using AGE antigens and indirect ELISA, out of a total of 100 bulls, 16 (16%) were positive and 82 (82%) were negative (Table 2).

b) Results using Whole Cell Antigen

Of the blood samples of 14 bulls from farms, 1 (7.1%) contained antibodies against *C. fetus subsp. venerealis.* Of the blood samples of a total of 86 bulls brouht to be slaughtered, 21 (24%) contained antibodies against *C. fetus subsp. venerealis*. It was found that 4 samples collected from the farms and 3 samples collected from the slaughterhouses were suspicious.

With indirect ELISA using the AGE antigen, of 100 randomly selected bulls, it was found that 22 were positive and 71 were negative for *C. fetus* antibody (Table 2).

B: The Results of the Indirect Fluorescent Test and Avidine Biotine Tests on Vaginal Mucus

1) Results of the IFA Test on vaginal mucus.

Samples showing fluorescing particles with the typical morphology of *C. fetus* were evaluated as positive. 45 (36%) out of a total of 125 vaginal mucus preparations were positive. Of 60 vaginal mucus preparations which were collected from local farms, 15 (25%) were positive; and of a total of 65 preparations collected from the slaughterhouses, 30 (46.1%) were positive. A total of 80

Table 1. The results of ELISA on the sera of 150 cows.

		Number of samples from Slaughterhouse	Samples from local farms	Total Positive	%
Positive	AGE	32	12	44	29.3
	WC	41	20	61	40.6
Suspicious	AGE	2	2	4	2.6
	WC	5	–	5	3.3
Negative	AGE	56	46	102	68
	WC	44	40	84	56

(64%) vaginal mucus preparations was found to be negative (Table 3).

2) Results of Avidine Biotine Test

When the typical morphological structure of *C. fetus* and the brown particles were seen, the results were taken to be positive (Fig. 1). Particles which did not exhibit the typical morphological structure were taken to indicate negative results. Out of a total of 125 vaginal mucus preparations, 39 (31.2%) were positive. Of 60 vaginal mucus preparations which were collected from local farms, 10 (16.6%) were positive, and 29 (44.6%) out of a total of 65 preparations collected from the slaughterhouses were positive. A total of 86 (68.8%) vaginal mucus preparations was found to be negative (Table 3).

When the two tests (IFA and AB) for detecting *C. fetus* were compared, out of a total of 125 vaginal mucus preparations, 37 were found to be positive in both tests. 8 samples were positive for only the IFA test and 2 samples were positive for only the AB test (Table 4).

In females, the number of positive results showed a 5% difference between IFA and AB. This difference was not significant (P>0.05).

C) The Results of the Indirect Fluorescent Test and Avidine Biotine Tests on the preputial fluid.

1) Results of the Indirect Fluorescent test

Thirt-seven (45%) of the preputial liquid preparations of 86 bulls from slaughterhouses were found to be positive, and 5 (35.7%) of the preputial liquid preparations of 14 bulls from local farms were positive. The overall positivity of 100 preputial liquid preparations from 100 randomly selected bulls was 42% on the IFA test (Table 5).

2) Results of Avidine Biotine test

When the typical morphological structure of *C. fetus* and the brown particles were seen, the results were taken to be positive (Fig. 2). Thirty-five (40.7%) of the preputial liquid preparations of 86 bulls more than one year old from slaughterhouses were found to be positive, and 5 (35.7%) of the preputial liquid preparations of 14 bulls from the local farms were positive. The overall positivity of 100 preputial liquid preparations from 100 randomly selected bulls was 40% on the AB test (Table 5).

When the two tests (IFA and AB) for detecting *C. fetus* were compared, 33 preputial liquid preparations

Table 2. The results of ELISA on the sera of 100 bulls.

		Number of samples from Slaughterhouse	Samples from local farms	Total Positive	%
Positive	AGE	15	1	16	16
	WC	21	1	22	22
Suspicious	AGE	2	–	2	2
	WC	4	3	7	7
Negative	AGE	69	13	82	82
	WC	62	9	71	71

Table 3. The results of IFA and AB tests on vaginal mucus from 125 cows.

Number of samples	IFA Number of positives	IFA Number of negatives	AB Number of positives	AB Number of negatives
60	15	45	10	50
65	30	35	29	36
125	45	80	39	86

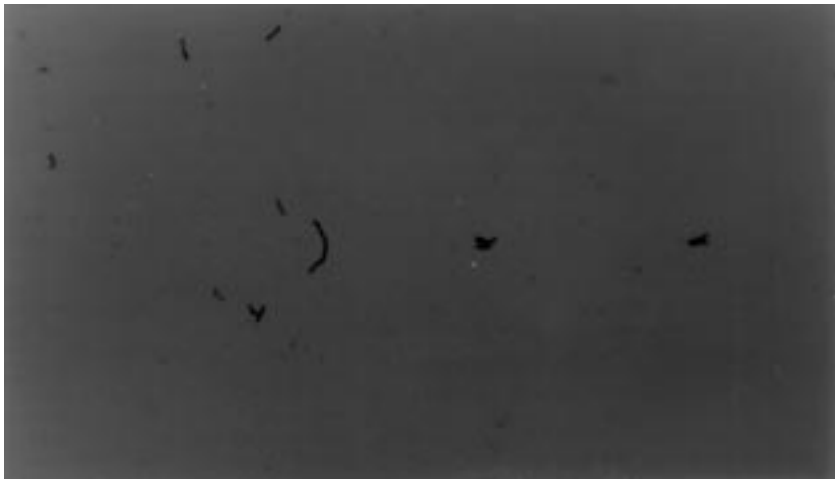

Figure 1. Avidin-Biotin staining of vaginal mucus for *C. fetus subsp. venerealis* (mag x 1200).

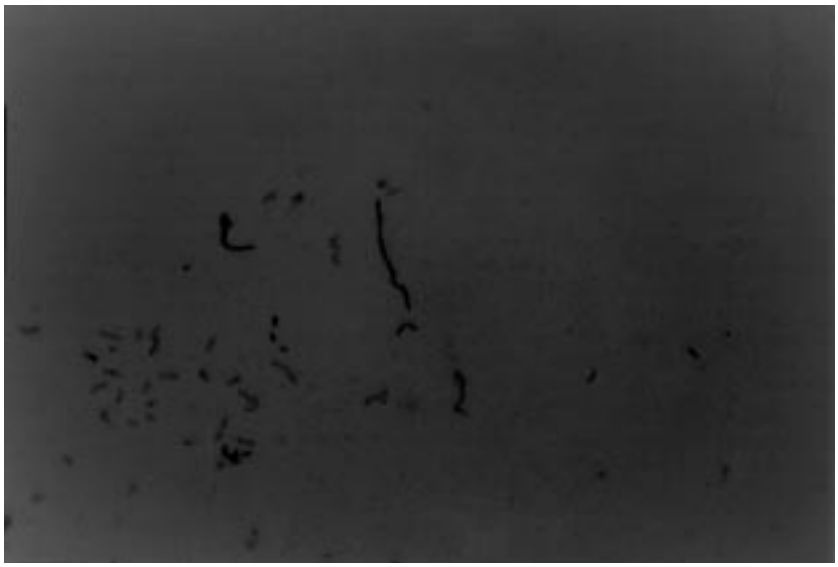

Figure 2. Avidin-Biotin staining of preputial liquid preparations for *C. fetus subsp. venerealis* (mag. x 1200).

were found to be positive in both tests, 4 preputial liquid samples were positive only for the IFA test and 2 samples were positive only for the AB test (Table 6).

In males, the number of positive results showed a 2% difference between IFA and AB. This difference was not significant (P>0.05).

Table 4. Comparison of the positive percentages of IFA and AB.

Number of samples	%	IFA	AB
37	(29.6)	+	+
8	(6.4)	+	–
2	(1.6)	–	+

Discussion

Various methods of antigen preparation have been used to prepare specific antigens from Campylobacters for the diagnosis of Campylobacteriosis in animals (10, 17, 22). McCoy et al. (28) first described the extraction of micro-capsule from *C. fetus* using 0.2 M glycine, pH: 2.2, and the extracted antigen was called acid-glycine extract (AGE). Further studies managed to purify this antigen and characterize it as S-layer after biochemical analysis (29, 30). Yardımcı et al. (22) compared ELISA results in which two types of antigens of Campylobacter acid-glycine extract and whole cell (WC) were used. Their study showed that the acid-glycine extract gave better results because of the method of antigen preparation, which revealed the antigenic determinant. It was also

Table 5. The Results of Indirect Fluorescent Test and Avidine biotine Tests on the preputial fluid of 100 bulls.

Number of samples	Number of positives	IFA Number of negatives	AB Number of positives	Number o negatives
14	5	9	5	9
86	37	49	35	51
100	42	58	40	60

Table 6. Comparison of the positive percentages of IFA and AB.

Number of samples	%	IFA	AB
38	(38)	+	+
4	(4)	+	–
2	(2)	–	+

emphasized that AGE antigens from *C. fetus subsp. fetus* and *C. jejuni* crossreacted.

In the present study, WC and AGE antigens of Campylobacter were used in ELISA and the results were compared. A preliminary study showed that the AGE antigen is more useful in that it can be used in higher dilutions. Sera from male and female cattle were analysed by ELISA for Campylobacter antibodies using two types of antigens, WC ad AGE. The results of ELISA showed that the same false positives were found when WC antigen was used. The results of the test also indicated that AGE antigens were more specific than WC.

In female cattle, local immunity is induced by natural infection of *C. fetus subsp. venerealis* and this immune response is higher than that of a systemic infection. On the other hand, in carrier males, the induction of local immunity is reduced because of the lack of the adhesion of the bacteremia to the mucosal surfaces. As a result, the amount of locally produced lg in males is smaller than that in females (3). Some authors have reported the presence of cross-reactivity between *C. fetus subsp. venerealis* and *C. fetus subsp. fetus* (2, 3).

In a previous study, 400 dairy cows were analysed to determine the prevalence of *C. fetus* and this prevalence was found to be 47% (11). A similar study by the same authors over a period of a year showed that this prevalance was 22% in 790 cattle (12).

In this study, ELISA was performed using the positive sera of *C. fetus subsp. venerealis* and *subsp. fetus,* and the titers obtained from both sera were similar. The sera of 150 randomly selected cows were analysed by ELISA for the presence of antibodies of Campylobacter using AGE and WC atigen. In the sera of 44 (29.3%) cows, AGE antigens and in the sera of 61 (40.6%) cows, WC antigens were found to be positive. In the sera of 16 (16%) of the randomly selected bulls, AGE antigens and in the sera of 22 (22%), WC antigens were found to be positive. At this stage, it is difficult to say that these antibodies are specific for *C. fetus subsp. venerealis* because of the cross-reactivity between the two species of camplylobacters. Importanly, the seroprevalance in bulls was lower than in the females tested. Detection of antibodies by ELISA in the sera can be used as a screening test for campylobacteriosis, but it is not specific.

Fluorescent antibody tests have been widely used for the rapid and direct screening of preputial samples for the presence of *C. Fetus* (2, 3, 9). At present, the test is specific for *C. fetus,* but is unable to distinguish between *C. fetus subsp venerealis* and *fetus.* However, fluorescent antibody techniques can differentiate *C. fetus* from *C. sputorum subsp. bubulus.* The use of indirect fluorescent antibody may be more sensitive and suitable when only a limited supply of the conjugated reagent is available (2). In a previous study, 59 (96.7%) of 61 bulls experimentally infected with *C. fetus subsp. venerealis* were positive on FA tests when preputial samples were tested (9). In a study carried out in Argentina, 22% of 11300 bulls were found to be positive for *C. fetus* when the IFA test was used (31). In many laboratories, FAT and IFA tests are commonly used with culturing in the diagnosis of *C. fetus.* In one study, 22 of the preputial samples of a total of 50 bulls were found to be positive with FAT (32). When these 22 preputial samples (positive) were cultured, *C. fetus subsp. fetus* was isolated from 17 of them.

Avidine-Biotine is one of the immuno-enzymatic methods used for the expression of cells or cell or cell products in tissue samples (25). McRill et al. (33), showed quick diagnosis in pure cultures and animal tissues using salmonella serotypes and the PAP technique. They emphasized that this test can be used in clinical samples, and the major advantages are specificity and the

use of a light microscope. Ericson et al. (34), described the use of Avidine-Biotine in the diagnosis of *C. coli* and *C. jejuni* in intestinal adenomatesis in the blue fox.

The diagnosis of *C. fetus* using Avidine-Biotine or any other immuno-peroxidase method has not been reported in the literature. In this study, a total of 125 vaginal mucus samples were tested and 45 (36%) were positive using the IFA test and 39 (31%) were found to be positive using the AB test. 42 (42%) out of 100 preputial samples were found to be positive using the IFA test and 40 (40%) were found to be positive using the AB test. When the test were compared, 5% and 2% statistical differences were found in the females and males respectively. These differences were not taken into account in terms of the methods used. The two methods, IFA and AB, were not very different from each other; but the use of light microscopy in the latter was a major advantage.

In conclusion, the use of ELISA in detecting Campylobacter antibodies in blood sera was found to be useful. In screening *C. fetus* in vaginal mucus and preputial samples. AB was found to be the most useful test. Although it does not show differences from IFA or FAT tests in procedure, the use of light microscopy is a major advantage. Therefore, this test can be used in the laboratory together with culturing.

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