The use of enzyme-linked immunosorbent assay and immunoblotting for the detection of *Campylobacter fetus* immunoglobulins in the cervico-vaginal mucus of female cattle[1]

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ABSTRACT.- Pellegrin A.O., Miranda K.L., Figueiredo J.F., Barbosa E.F. & Lage A.P. 2011. **The use of enzyme-linked immunosorbent assay and immunoblotting for the detection of *Campylobacter fetus* immunoglobulins in the cervico-vaginal mucus of female cattle.** *Pesquisa Veterinária Brasileira 31(3):247-254*. Embrapa Pantanal, Rua 21 de Setembro 1880, Corumbá, MS 79320-900, Brazil. E-mail: aiesca@cpap.embrapa.br

An indirect enzyme-linked immunosorbent assay was developed to detect antigen-specific secretory IgA antibodies to *Campylobacter fetus* subsp. *venerealis* in bovine vaginal mucus with a protein extract of the *Campylobacter fetus* subsp. *venerealis* by the acid glycine extraction method. Mean optical density measurement (λ=450 nm) was 0.143±0.9. The most immunoreactive protein bands of the *Campylobacter fetus* subsp. *venerealis* or *Campylobacter fetus* subsp. *fetus* recognized by IgA in immunoblotting, using bovine vaginal mucus samples, migrate at 42.6 kDa. The protein that migrates at 93 kDa was recognized exclusively for *C. fetus* subsp. *venerealis*. A positive vaginal mucus sample of a cow from negative herd recognized antigens of *C. jejuni* subsp. *jejuni e C. fetus* subsp. *fetus*.

INDEX TERMS: *Campylobacter fetus*, Campylobacteriosis, ELISA, immunoglobulin A.

RESUMO.- [**Uso do ensaio imunoenzimático e imunoblotting para detecção de imunoglobulinas contra *Campylobacter fetus* em muco cérvico-vaginal de fêmeas bovinas.**] Foi padronizado um ensaio imunoenzimático do tipo indireto para detecção de imunoglobulina A (ELISA IgA) anti- *Campylobacter fetus* subp. *venerealis* em muco cérvico- vaginal bovino utilizando um extrato protéico de *Campylobacter fetus* subsp. *venerealis* produzido pelo método de extração ácida pelo tampão de glicina (0,2M; pH2,2). A média dos valores de densidade ótica (DO_{450}) foi de 0,143±0,09. As bandas protéicas dos antígenos de *Campylobacter fetus* subsp. *venerealis* e de *Campylobacter fetus* subsp. *fetus* melhor reconhecidas pela IgA do muco cérvico- vaginal migraram em 42,6 kDa mas a proteina evidenciada em 93 kDa foi reconhecida exclusivamente pelo *Campylobacter fetus* subsp. *venerealis*. Os anticorpos presentes na amostra de muco vaginal testada no "immunoblotting" que apresentou resultado positivo no ELISA IgA, reconheceu antígenos de *C. jejuni* subsp. *jejuni* e *C. fetus* subsp. *fetus.*

TERMOS DE INDEXAÇÃO: *Campylobacter fetus*, Campilobacteriose, ELISA, imunoglobulina A.

INTRODUCTION

Bovine Genital Campylobacteriosis (BGC) is a specific reproductive disease, which can cause embryonic death, resulting in irregular cycles, and, less often, abortions (Dekeyser 1986). Immunity against Bovine Genital Campylobacteriosis is mainly local, with the production of IgA class immunoglobulins in the vaginal and uterine mucosa, as investigated in naturally or experimentally infected bovine females (Corbeil et al. 1974b).

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The kinetics of immunity against *Campylobacter fetus* in the vaginal mucosa shows that IgM are the first immunoglobulins to appear in experimentally infected heifers, followed by IgA, and eventually by IgG1 (Corbeil et al. 1974a,b, Van Aert et al. 1977). In heifers infected by the vaginal route, the emergence of IgM and IgA is almost simultaneous, before IgG1, which appears around the 8th week post-infection. This emphasizes the importance of the immunity provided by IgA for the resistance against natural infection by *C. fetus*, as IgM, IgG1 and IgG2 develop in systemically vaccinated females (Corbeil et al. 1974a).

High-molecular-weight proteins of the outer membrane (OMPs), named S-layer (SAP) are considered important virulence factors for *C. fetus*, responsible for resistance to phagocytosis and avoiding bactericidal activity of serum immunoglobulins (Blaser 1983). In bovine isolates of Campylobacter fetus from bulls, cows and aborted fetus of brazilian herds, ten different patterns of SAP expression were demonstrated by identification of proteins with molecular mass of 97, 100, 127, and 149 kDa, obtained by an acid (glicin) method of extraction (Vargas et al. 2002).Experiments using mouse and bovine models sugest that S-layer proteins is a dominant virulence factor enabling persistence in the genital tract (Garcia et al. 1995) by inversion events resulting in expression of antigenically variant S-layers (Thompson 2002, Vargas et al. 2002) .S-layer proteins conferes resistance to systemic defenses (Pei & Blaser 1990, Blaser & Pei 1993) preventing the binding of complement factor C3b to the *C. fetus* surface (Blaser 1988).

Among the serological techniques used for the detection of the immune response, the enzyme-linked immunosorbent assay is widely accepted as very sensitive, and therefore, it is very convenient to be used in laboratories. However, its sensitivity and specificity essentially depend on the preparation of the antigen used in the test (Hirschl & Rotter 1990) and on the established cutoff (Smith 1994).

Several authors have used in the solid phase of the enzyme-linked immunosorbent assay acid extractable antigens (Blaser et al. 1983, Dunn et al. 1987), sonicated (Hewson 1985, Dunn et al. 1987) or by distilled water extraction (Garcia et al. 1995). These antigens can be classified as partially purified antigens, resulting in high sensitive and specific tests, as compared to non-purified antigens, which are less specific, or to highly purified antigens, which are less sensitive (Hirschl & Rotter 1990).

The use of an enzyme-linked immunosorbent assay for the detection of *C. fetus* subsp. *venerealis*-female carriers was previously described, and this test is considered more sensitive as compared to the mucus agglutination test for the detection of anti-Campylobacter fetus class G immunoglobulins in the early stages of infection (Hewson 1985). However, the latter has good specificity for the detection of specific immunoglobulins A in bovine females that aborted due to natural infection by *C. fetus* subsp. *venereal is* (Hum et al. 1991).

This study aimed at standardizing an enzyme-linked immunosorbent assay by comparing an antigen obtained by acid extraction with glycine buffer to a sonicated antigen employed in the solid phase for the detection of anti-*C. fetus* immunoglobulins A, and evaluating the humoral immune response in the cervico-vaginal mucus of virgin heifers.

MATERIALS AND METHODS

Animals. The negative controls were provided by samples of the cervico-vaginal mucus collected from 44 virgin heifers between 12 and 24 months of age. These heifers were selected from a Bovine Genital Campylobacteriosis-free herd, exclusively under artificial insemination (AI). The positive controls were provided by samples of the cervico-vaginal mucus collected from cows of herds with positive diagnosis of Campylobacteriosis that had aborted or had reproductive failure.

Samples. For cervico-vaginal mucus vaginal collection, a previously weighed tampon (Sempre Livre, Jonhson & Johnson, São Paulo) was introduced in the vagina of the heifers with the aid of an applicator coupled to a metal tube. The tampon remained on the wall of the vaginal cavity for at least 30min for saturation (Fernandes & Gomes 1992). The tampon was then removed, placed in a sterile container, and transported in ice to the laboratory, where it was weighted. Sample weight was recorded for later calculation of the amount of diluent (PBS + 0.05% Tween 20, pH 7.4) to be used in order to obtain an initial dilution of 1/10. After diluent addition, the tampon was pressed with a sterile glass bar for the removal of the cervico-vaginal mucus, which was then divided in aliquots and stored under refrigeration until use.

Reference samples and culture conditions. *Campylobacter fetus* subsp. *venerealis* NCTC 10354 (serotype A), *C. fetus* subsp. *fetus* ATCC 27354 (serotype B) and *C. jejuni* subsp. *jejuni* NCTC 11351 samples were used to produce the antigens. Samples were cultivated in BHI agar (Brain Heart Infusion, Difco, USA) with 10% equine blood for 48 hours at 37ºC, in microaerophilic atmosphere containing 85% N_2, 10%CO_2 and 5% O_2.

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Acid- extractable antigen. The procedure proposed by McCoy et al. (1975) was used for the production of the antigen by acid extraction. After cultivation colonies were removed from the plates with PBS (pH 7.4), and rinsed three consecutive times with this buffer in a centrifuge at 10,000 x g for 20 minutes at 4ºC. After the third rinsing, the sediment was suspended in glycine-HCl buffer (0.2 M, pH 2.2), at a proportion of 100ml of buffer per 4mg of wet bacterial mass, and submitted to agitation for 30 minutes. The suspension was again centrifuged at 13,000 x g for 20min, and the supernatant was dialized against deionized and distilled water for 48 hours, and later concentrated approximately 10 times in sucrose gradient. Protein was estimated by the method of Lowry (Sambrook 2001) using bovine albumin (Sigma, USA) as standard. Subsequently, the acid extraction in glycine buffer antigen was divided in aliquots and stored at -20ºC.

Sonicated antigen. The sonicated antigen was prepared according to the protocol described by Hewson (1985),

reducing sample incubation time from 72 to 48 hours and using a standard sample of *C. fetus* subsp. *venerealis* (NCTC10354). The culture was collected from the medium with a phosphate buffer saline solution (PBS, pH 7.4), rinsed three times by centrifugation at 13,000 x g per 20min and the sediment collected . Finally, the sediment was re-suspended in 10ml of a buffer containing 0.5 M potassium chloride and 0.1% (vol/vol) cystein hydrochloride, sonicated for 6 30-sec cycles of 70-80 Hz power, with 1-min resting intervals, subsequently concentrated and divided in aliquots.

Enzyme linked immunoassay (ELISA). The acid extractable antigen was diluted in carbonate-bicarbonate buffer, pH 9.6 (Voller, 1979) and adsorbed to plated (Maxisorp, Nunc) for 18 h at 4ºC, in wet chamber. After rinsing, the plate was treated with PBS Tween 20 (0.05%) (PBST) with addition of 5% nonfat dry milk for 30min at 37ºC for unspecific site blocking. Incubation times and temperatures were: for the sample (cervico-vaginal mucus), 1h at 37ºC in humid chamber; for the conjugate, 1h at 37ºC in humid chamber; and for the substrate, 15 min at environmental temperature in dark room. PBST with addition of 1% nonfat dry milk was used in the rinsing stages. Checkerboard titration were used to optimisation and standardisation of reagents. Two antigen concentrations (500 and 250ng/100ul) and two dilutions of the peroxidase conjugated bovine anti-IgA (Bethyl) in 1/10,000 and 1/20,000 dilutions were tested with a 1/50 dilution of the cervico-vaginal mucus.

The chromogenic substrate used were 3, 3'5, 5'-Tetramethylbenzidine (TMB, Sigma, USA) and 1M H2SO4 as stop solution. Readings were made at a spectrophotometer with 450 nm filter. The optimal antigen and conjugated concentrations and sample dilution per well, determined by checkerboard titration against positive and negative control serum, were found to be of 250ng for the antigen, 1/10,000 for the anti-IgA conjugated, 1/50 for the mucus, respectively,

Polyacrylamide gel electrophoresis (SDS-PAGE). Ten mg of the antigen produced by acid extraction of each of the *Campylobacter* spp. (*C. fetus* subsp. *fetus*, *C. fetus* subsp. *venerealis*, *C. jejuni* subsp. *jejuni*) were used in each well, and the protein components were separated by electrophoresis according to the system described by Laemmli (1970). Low Range (BioRad, USA) or Broad Range (BioRad, USA) molecular weight standards were used and gels were stained by the method of Coomassie Blue (Sambrook et al. 1989).

After electrophoresis, proteins were transferred from the gel to a nitrocellulose membrane (Sigma, USA) using Tris glycine buffer containing 20% methanol in Transblot system (BioRad, USA) for 1h 30min at 100V (Bjerrum & Heegaard 1988). After transference, inespecific sites were blocked with Tris-NaCl Tween 20 (0.05%) (TBS) buffer containg 5% nonfat dry milk for 18 h at 4ºC. Several dilutions of the cervico-vaginal mucus were previously tested (1/10, 1/20, 1/25, 1/50), and the best dilution selected for immunoblotting was 1/50. For the recognition of the antigen protein, a pool of cervico-vaginal mucus of ten positive animals in the IgA ELISA test, diluted 1/10 in TBS was used as positive control, and a pool of cervico-vaginal mucus of ten negative animals in the IgA ELISA test, sampled among the virgin heifers, was used as negative control. The bovine anti-IgA Peroxidase (Bethyl, USA) conjugate was used in a 1/1000 dilution, and the substrate was 4-chloronaphtol (Sigma, USA). The reaction times used were 1h 30min for the antibody, 1h for the conjugate, and 10min for the substrate, and three 10-min rinsings of the membrane were performed with TBS buffer between each reaction phase.

Statistical analysis. The different antigen preparations were compared by analysis of variance using a randomized block experimental design ($P \leq 0.05$), whereas the acid preparations employing different antigens were compared by analysis of variance for a completely at ramdom experimental design ($P \leq 0.05$) (Sampaio 1998).

RESULTS

The optimal Elisa reagents concentrations and sample dilution per well, determined by checkerboard titration were found to be of 250ng for the antigen, 1/10,000 for the anti-IgA conjugated, 1/50 for the mucus, respectively.

Cutoff was calculated by the mean of the results obtained in cervico-vaginal mucus samples of 44 virgin heifers (0.143 OD_{450}) plus two standard deviations (0.09 OD_{450}). Therefore, samples presenting readings at 450 nm equal or higher than 0.323 were considered as positive.

For the comparison between sonicated and acid-extraction antigens, 35 samples of the cervico-vaginal mucus were collected at random and tested by IgA ELISA, using antigen preparations produced by acid extraction and sonicated extract. The results did not show significant differences ($P>0.05$) among the absorbance values of each antigen in the positive and negative herds, with a difference between means of 0.3806 and 0.3391, respectively, and a calculated least significant difference (LSD) of 0.150 OD_{450}, which was lower than the difference between the means of the two antigens (0.0415 OD_{450}). The antigen produced by glycine acid extraction was used to evaluate the humoral response of virgin heifers as it was easier to produce as compared to the sonicated antigen, and also because the results obtained with both antigens were similar.

In the solid phase of the comparison trial, antigens obtained by acid extraction of the species of *Campylobacter fetus* subsp. *fetus, C. fetus* subsp. *venerealis*, and *C. jejuni* subsp. *jejuni,* were used in different polystyrene "Maxisorb" plates with 96 wells (Nunc, USA). General means of obtained in optical density measurements were 0.253, 0.226, and 0.209, respectively, for *C. fetus* subsp. *venerealis*, *C. fetus* subsp. *fetus,* and *C. jejuni* subsp. *jejuni*, with a calculated least significant difference of 0.027. There was a significant difference ($P<0.05$) in the detection of immunoglobulins A when *C. fetus* subsp. *venerealis* was used as compared to the other samples, but this difference was not detected when *C. fetus* subsp. *fetus* and *C. jejuni* subsp. *jejuni* ($P>0,05$) were compared.

The profile of the sonicated antigen produced with the NCTC 10354 sample showed the presence of several protein bands with different molecular weights, which varied between 131.7 and 24.8 kDa. The most evident proteins migrated at 90.8, 57.5, 49.0, 45.5, 40.0, and 36.4 kDa, using Broad Range (BioRad, USA) as molecular weight standard (eletroforetic profile not showed).

In the antigen of *C. fetus* subsp. *venerealis* NCTC 10354 obtained by acid extraction with glycine buffer (pH 2.2), the most evident bands migrated in 93, 56.6, 42.8, 40.1, 32.8, 28.9, and 25.7 kDa regions (Fig. 1). In the antigen of *C.*

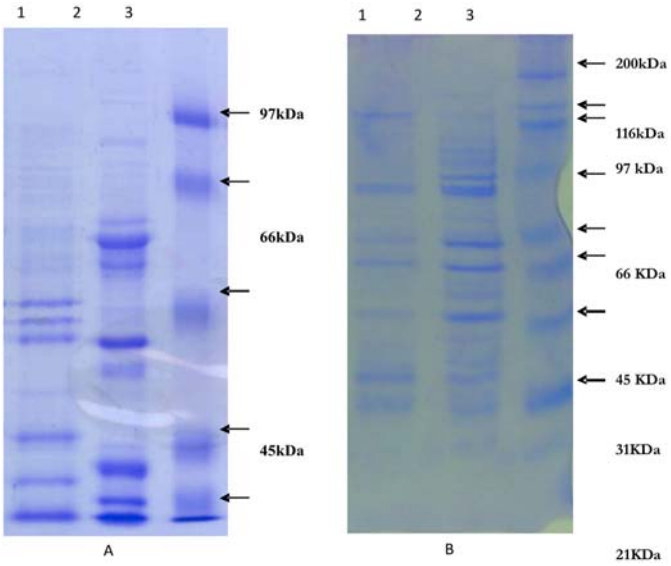

Fig.1. Gel with 12% acrylamide stained with Coomassie blue using 10g of an acid (glicin) extratable antigen per well. (A.1) *Campylobacter fetus* subsp. *venerealis*; (A.2) *C. fetus* subsp. *fetus*; (A.3) Broad Range Standard (BioRad); (B.1) *C. fetus* subsp. *venerealis;* (B.2) *C. fetus* subsp. *fetus;* (B.3) LowRange Standard (BioRad, Sigma).

fetus subsp*. fetus* ATCC 27374, 10 protein bands between 56.7 and 25.8 kDa were detected. The most evident bands appeared between 45 and 31 (42.8, 40.1, and 37.8 kDa) and immediately below (28.5 and 25.8 kDa). When these two antigens were analyzed together, it is possible to observe several proteins with similar molecular weights, migrating in 56.7, 42.8, 40.1, 25.8, and 25.7 kDa, among those that were visually different.

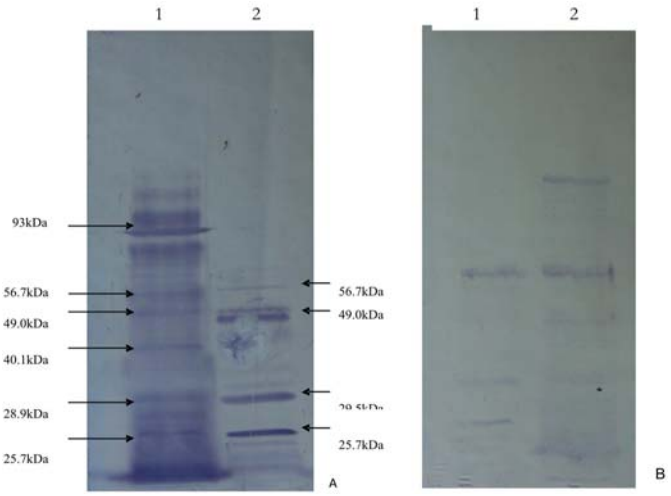

Fig.2. "Immunoblott" de proteínas de antígeno obtido por extração ácida de *Campylobatcer fetus venerealis* (1) e *C.fetus fetus* (2), utilizando 10mg de antígeno por canaleta e muco vaginal positivo ao Elisa IgA diluído 1:10 (A) e mistura de amostras de muco vaginal positivo ao Elisa IgA diluído 1:20 (B). Os imunoblotts foram visualizados utilizando um soro anti-IgA bovina (Bethyl) conjugado com peroxidase em diluição 1:1000 e revelação com 4-cloronaftol.

For the antigen *C. jejuni* subsp. *jejuni* NCTC1135, 11 different bands, from 84.5 kDa to 24.8 kDa were observed. The larger bands migrated in 84.5, 58.9, 53.6, 49.5, 37.4, 34.2, 26.4, and 24.8 kDa, although the analysis identified the presence of bands in in 41.7, 32.4 and 27.8 kDa (eletroforetic profile not showed).

Immunoglobulins A recognized in the cervico-vaginal mucus seven proteins of the antigen of *C. fetus* subsp. *venerealis*, which migrated in 93, 56.7, 49.8, 40.1, 36.0, and 28.9 kDa. An intermediate band was observed between 28.9 and 25.7 kDa, when the cervico-vaginal mucus was diluted at 1:10 (3-A), whereas only two main bands of 93 and 43 kDa (Fig.2) were present in a higher dilution (1:20). When the *C. fetus* subsp. *fetus* was tested against the cervico-vaginal mucus diluted at 1:20, six proteins were immunoreactive (56.7, 49.6, 42.6, 29.5 and 25.8 kDa), whereas when the sample was diluted at 1:10, only proteins with molecular weights of 42.6, 29.5, and 25.8 kDa were visualized in the nitro-cellulose membrane (Fig.2). The bands recognized in both antigens weighed 56.7 kDa, approximately 49 kDa (49.8, in *C. fetus* subsp. *venerealis* and 49.6 in C. fetus subsp. fetus), and 29 kDa (28.9 in *C. fetus* subsp. *venerealis*) and 29.5 in *C. fetus* subsp. *venerealis*). Several proteins weighting less than 25 kDa were also recognized for *C. fetus* subsp. *venerealis* (Fig. 2).

Cervico-vaginal mucus samples, positive in the IgA ELISA, collected from animals of a dairy herd with no history of BGC that used artificial insemination, were tested against acid-prepared antigens produced with samples of *C. fetus* subsp. *venerealis, C. fetus* subsp. *fetus*, and *C. jejuni* subsp. *jejuni*, by immunoblotting in three dilutions: 1:25, 1:50, and 1:100. These samples presented similar patterns for the recognition of IgA antibodies both for *C. fetus* subsp. *venerealis* and for *C. jejuni* subsp*. jejuni*. In 1:25 dilution, 56.7, 42.6, 29.5, and 25.7 kDa bands were observed in all three antigens, and a 42.6 kDa band in *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus,* which was probably represented by a 41.7 kDa band in *C. jejuni* subsp. *jejuni*. When dilution increased to 1:50, 56.7 and 42.6 kDa bands remained visible, but could no longer be observed. The highest immune reactivity occurred in the 42.6 kDa (Fig.2) protein of *C. fetus* subsp*. fetus*.

DISCUSSION

The antigen profile, demonstrated by electrophoresis in polyacrylamide gel both for the sonicated antigen and the acid-extraction antigen, demonstrated similarities as to protein migration around 97 kDa or below (93 kDa), and in 63, 42.6, 29, and 25 kDa. In the immunoblotting test, these proteins were immunoreactive, and were recognized by the IgA present in the tested vaginal mucus, when the acid extract of *Campylobacter fetus* subsp. *venerealis* was used. This confirms the similar results (P>0.05) obtained in the enzyme-linked immunosorbent assay, which compared the sonicated and the acid-extracted antigens as to the detection of anti-*Campylobacter fetus* immunoglobulins A in the cervico-vaginal mucus. Therefore, this suggests that

the sonicated agent may be an alternative that can be used in the solid phase of enzyme-linked immunosorbent assays aiming at the detection of these immunoglobulins.

A comparison of the sensitivity and the specificity of several antigen preparations (Lewalla-Guruge et al. 1992) for the detection of anti-*Helicobacter pylori* IgG antibodies concluded that these test properties were equivalent both for sonicated and acid-extracted antigens, which was confirmed by this study.

The cutoff was determined by the mean of the optical density measurements of 44 samples of the cervico-vaginal mucus of virgin heifers, with ages between 12 and 24 months, derived from herds with no history of the disease. The anti-*C. fetus* subsp. *venerealis* IgA Elisa standardized in the present study was interpreted using the value of the cutoff plus two standard deviations, in order to increase test specificity, and to decrease the number of false negative results. This procedure were employed because samples from positive animals were not available due to the lack of success in the isolation of *C. fetus* subsp venerealis in suspected herds. The interpretation of the results of a enzyme-linked immunosorbent assay mainly depends on the cutoff established for the test and the aim of the test (Smith 1994). Similar procedures were employed by Hewson (1985) and Hum et al. (1991) for the establishment of test cutoffs. Test cutoff was determined by Hewson (1985) as the mean of OD_{450} values of 54 females with a positive diagnosis of Bovine Genital Campylobacteriosis by isolation, plus three standard deviations. Hum et al. (1991), on the other hand, expressed test results in ELISA Values (EV), based on DO_{450} measurements corrected for the OD_{450} values of positive (OD_{450}= 1.430) and negative (OD_{450}= 0.180) controls plus two standard deviations. Their aim was to detect abortions due to *C. fetus* subsp. *venerealis*. Another cutoff was later established by the same authors, being higher due to the use of three standard deviations, aiming at achieving higher test specificity for the diagnosis of *C. fetus* subsp. *venerealis* female carriers in herds (Hum et al. 1994).

McFadden et al (2005) performing a screening for campilobacteriosis in 125 beef cow herds in New Zealand established a cutoff for positive result of 33EV (Elisa value), determinated as the sum of the mean EV of the negative controls plus three standard deviations, the same adopted by Hum et al. (1994). The results obtained as previously described (Hewson 1985, Hum et al. 1991, Hum et al. 1994, McFadden et al. 2005) cannot be fully compared to the results obtained in the present study. In the assay standardized by Hewson (1985) and Cobo et al. (2003) a sonicated antigen was used, measuring the IgG-mediated local response, whereas Hum et al. (1991) and McFadden et al. (2005) used a crude antigen, which is considered less specific than the semi-purified antigens (Hirschl & Rotter 1990), such as the antigen preparation by acid extraction used in this study. The authors considered the specificity of the test unsatisfactory to for detecting infection under field conditions, probably due to cross reaction to Campylobacter species other than *C. fetus* subsp. *venerealis,* from prepucial washings from bulls of three tested herds. In this work, culture of *C. fetus* was not performed, but this possibility cannot be discounted.

The results found in IgA Elisa demonstrated that immunoglobulins A, present in the cervico-vaginal mucus of healthy and infected females, recognize antigen proteins of the *Campylobacter sp*. The virgin heifers used to establish the test cutoff were 12 to 24-months-old and derived from herds under AI service. Although they were not under the risk of a *C. fetus* infection, the mean OD_{450} measurements obtained by IgA ELISA was 0.143, with a standard deviation of 0.09, indicating the occurrence of a substantial amplitude in optical density measurements. Data presented by Hum et al. (1994) also showed a considerable amplitude of OD_{450} values, with 10 EV with a standard deviation of 15 EV in the values detected by IgA ELISA.

The role of outer membrane antigens in enzyme-linked immunosorbent assays have been determined by immunoblotting using monoclonal or polyclonal sera for the identification of immunoreactive proteins (Hirschl & Rotter 1990). In the present study, instead of polyclonal sera, the sample tested by immunoblotting was a cervico-vaginal mucus pool, as one of the objectives of this study was to standardize the test for IgA-mediated local response to *C. fetus* subsp*. venerealis*. The importance of IgA in the local immune response to *C. fetus* subsp*. venerealis* was discussed for naturally infected females, females that aborted, and those that only maintained persistent infection by Wilkie et al. (1972), Corbeil et al. (1975), Winter (1982), Hum et al. (1991, 1994).

Immunoblotting performed with acid-extracted antigens of *C. fetus* subsp*. venerealis* NCTC 10354 and *C. fetus* subsp. *fetus* ATCC 27374 samples revealed the presence of proteins with similar molecular weights for both subspecies. There was a coincidence of protein migration around 49 kDa to 49.8 kDa for *C. fetus* subsp*. venerealis,* and 49.6 kDa for *C. fetus* subsp. *fetus* antigen proteins, which are probably similar (Fig.2). This protein that migrated around 49 kDa showed correspondence in all electrophoresis profiles, varying from 49.4 kDa (in *C. jejuni* subsp. *jejuni)* to 49.8 kDa (in *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus).*

Proteins with molecular weights around 29 kDa (28.9 and 29.5) were shown both in *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* as well as proteins weighing below 25.7 in both antigens.

The study of protein profiles of several *C. fetus* subsp. *fetus* and *C.* subsp. *jejuni* antigen preparations revealed a isomeric form of OMP de 43 kDa (or 45 kDa). In *C. jejuni*, this monomer can vary between 43 and 45 kDa, depending of the studied samples, but it was always present in the samples analyzed by Dunn et al. (1987), representing 70% of the capsular component of the outer membrane. The observation of the profile of *C. fetus* subsp. *fetus* obtained in the present study allows us to assume that these proteins are similar. However, this can only be asserted by the use of bi-dimensional polyacrylamide gel electrophoresis.

Immunoglobulins A present in the cervico-vaginal mucus

detected a large number of proteins of the acid-extracted antigen. Among the protein panel of *C. fetus* subsp. *fetus*, 28.9 and 25.7 kDa proteins were detected, but not in *C. fetus* subsp. *venerealis* by immunoblotting. .Studies indicate that proteins migrating between 29 and 31 kDa are superficially exposed and very antigenic, and can be extracted at low pH. These proteins were observed in all *C. jejuni* samples studied by Dunn et al. (1987). Trust & Logan (1984) previously suggested that these microcapsular components have good specificity, allowing the detection of *C. jejuni* and *C. coli* in human clinical specimens, and also its use for the production of a specific vaccine (McCoy et al. 1975, Logan & Trust 1986, Dunn et al. 1987).

In this study, two low molecular weight proteins, of about 21 and 31 kDa, were strongly immunogenic in the immunoblotting of *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* glycine-extracted antigens and cervico-vaginal mucus of positive females derived from suspected/positive herds. Dunn et al. (1987) suggest that these proteins represent a capsular component of *C. jejuni*, which is actually represented by several proteins (Trust & Logan 1984), similar to those observed in the protein profile of *C. jejuni* subsp. *jejuni*.

The false positive results in animals with no clinical signs of the disease and derived from herds with no history of BGC may be attributed to the recognition, in the IgA ELISA test, of microcapsular proteins weighing about 31 kDa (28.9 and 25.7 kDa). Immunoglobulins A may be present in healthy animals, which had contact with *C. fetus* subsp. *fetus* , serotype B (Hum et al., 1994). This is clear when we consider that the *C. fetus* subsp. *fetus* ATCC 27374 sample belongs to serotype B. The genus *Campylobacter*, particularly the species *C. fetus*, *C. jejuni,* and *C. coli,* has several wall antigens with similar molecular weights (Logan & Trust 1983, 1986).

A protein migrating at approximately 63 kDa, which possibly is the flagellar antigen, was observed in the electrophoresis profile of the three subspecies studied here. However, immunoblotting results indicated a weak recognition by the anti-*Campylobacter fetus* IgA present in the tested vaginal mucus when the antigen produced with a sample of *C. fetus* subsp. *venerealis* was used. This protein was also not recognized by the sample of *C. fetus* subsp. *fetus,* when the mucus of positive females was used. Although this protein is common to the three subspecies, it is possible that it is an antigen specifically recognized by antibodies produced against *C. fetus* subsp.*venerealis* in this study. However, Logan & Trust (1983) asserted that it is recognized by immunoglobulins G in the immunoblotting performed with *C. jejuni* proteins and Zhao et al. (2010) developed and evaluated an highly specific and sensitive indirect ELISA for the detect of IgG antibodies against *C. fetus* in cattle sera using as the test antigen a recombinant (r) SapA-N and SapA-C codified proteins. The Elisa results indicated that the immunological activity of rSAP-N was highter that that of rSAP-C, but both proteins appeared as bands of approximately 66,2 kDa on a 12% polyacrylamide gel.

Evaluating acid extracts of *C. fetus* samples, Dunn et al. (1987) assume that the bands between 50 and 63 kDa represent breakdown products of the flagellar antigen. The electrophoresis profile of *C. fetus* subsp. *venerealis* and *C. fetus* subsp. *fetus* samples indicated the presence of one and two bands, respectively, with similar molecular weight (Fig.1). In the immunoblotting, the recognition of this *C. fetus* subsp. *venerealis* band was weak, possibly indicating protein breakdown. However, a definite band, of 56,7 kDa was recognized in *C. fetus* subsp. *fetus*. This disparity can only be elucidated by bi-dimensional gel electrophoresis.

The role of the protein migrating at 97 kDa, is a SAP antigen (Blaser et al. 1985, Tu et al. 2004), was not evident in the IgA ELISA performed, and was recognized only for *C. fetus* subsp. *venerealis*. It may have contributed to test specificity for the detection of animals truly infected by this subspecies, and for the lasting immunity against *C. fetus* subsp. *venerealis*, allowing the resistance to reinfection. Blaser et al. (1985) and Blaser et al. (1987) showed that this protein migrates up to 100 kDa, it is associated to the resistance of *C. fetus* to human antibodies, and it is labile when extracted by acid. It can be spontaneously lost when the bacterium is cultivated *in vitro* (Winter et al. 1978, Blaser et al. 1987), which may have occurred with the *C. fetus* subsp. *fetus* sample (Fig.1.).

In sheep, Grogono-Thomas et al.(2000) suggested that the 97 kDa protein of the S layer is essential for the colonization and passage of *C. fetus* subsp. *fetus* through the placenta, but that it is not directly responsible for fetal injury. After the abortion, a lasting immunity against *C. fetus* subsp. *fetus* is produced by systemic antibodies that are specific against the S layer proteins.

The phenomenon of persistent infection of females by *C. fetus* subsp. *venerealis* seems to be related to changes in SAP proteins, which migrate around 97 kDa, as suggested by several authors (Garcia et al. 1995, Grogono-Thomas et al. 2000, Vargas et al. 2002, Tu et al. 2004), and that were strongly recognized in the immunoblotting assay performed. Therefore, these proteins may be an interesting alternative for testing, as they allow the identification of females with persistent *C. fetus* subsp. *venerealis* infection. In addition, if we also take into account the relevant participation of these proteins in the mechanism that *C. fetus* uses to evade the immune system, these proteins are potential candidates for the production of mucosal vaccines aiming at the control of bovine genital campylobacteriosis.

A vaccine produced from *C. fetus* ssp. *fetus* which protected against ovine abortion comprised of a bacterial extract containing a ''rather loosely attached capsular envelope protein'' was later characterized as a surface microcapsule which mediated protection against phagocytosis The antigen was purified and characterized as a protein with an apparent molecular mass of 98 000 existing as a complex with lipopolysaccharide (Beveridge et al., 1997).Vaccines to promote immunity against *C. fetus* subsp. v*enerealis* and *Tritrichomonas foetus* has been

tested (Cobo et al. 2004) and the author observed an intravaginal instillation of *C. fetus* subso *venerealis* and *T. foetus* had been so sucessful to imunize the heifers using three different routes intravenous, subcutaneous and into the vaginal submucosa associated with a subcutaneous route. So, it seems that the route of antigen entrance in the organism is important to enhance the serological response, but only determination of systemic IgG, for both organisms were performed and no experiment were performed with intravaginal route only to define what is the most important (Cobo et al. 2004). For the serological test to *C. fetus* subsp. *veneralis* immune response these authors used a sonicated antigen whose procedure of extraction were similar to the used in this work but eletroforetic profile of extracted proteins were not presented.

The use of immunoblotting in cervico-vaginal mucus samples for the diagnosis of bovine genital campylobacteriosis in herds with no history of the disease could be a promissing tool for the elucidation of false positive results obtained in the ELISA, but it is a laborious and expensive test. An alternative test, could be the dot blot assay has been developed for serodiagnosis of several agents or related species, providing an objective evaluation and a convenient, time-saving, and inexpensive method (Cardona-Castro et al. 2000, Pinheiro et al. 2006). The standardized assay using an acid extractable antigen has a great potential for the identification of animals infected by *C. fetus*. It is best indicated for herd diagnosis due to the appearance of false positive results in herds with no history of the disease. Therefore, the diagnosis must be supported by the reproductive history of the herd and by clinical signs indicating the presence of Bovine Genital Campylobacteriosis.

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