Quantitative analysis of *Campylobacter fetus venerealis* adhesion to bovine reproductive tract cell cultures

Análise quantitativa da adesão de Campylobacter fetus venerealis em culturas de células do aparelho reprodutor bovino

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Abstract

Campylobacter fetus is the etiological agent of bovine genital campylobacteriosis, a sexually transmitted disease which is associated with reproductive losses in bovines. *Campylobacter* colonizes the vagina and the uterus and then infects the epithelial cells of the endometrium. The objective of this work was to develop an *ex vivo* model to quantify the adhesion of *Campylobacter* to its natural specific target cells; this is a key step for the establishment of infection and studies regarding the adherence and cytotoxicity on the natural host cells are not available. The assays were carried out by seeding *Campylobacter fetus venerealis* on bovine vaginal and uterine epithelial cell cultures. HeLa cells were used as control. Bacterial adhesion was corroborated by optical microscopy and determination of the percentage of adherent bacteria was performed on immunochemically-stained slides. Results are presented as percentage of cells with adherent *Campylobacter* and as number of bacteria per cell. In comparison to the control HeLa cells, the statistical analysis revealed that primary cultures show a higher percentage of infected cells and a lower variation of the evaluated parameters. This primary culture model might be useful for studies on cytopathogenicity and adhesion of different field strains of *Campylobacter fetus*.

Keywords: *Campylobacter fetus*. Adhesion. Target cells. Model. Primary cultures.

Resumo

Campylobacter fetus é o agente etiológico da campilobacteriose genital bovina, uma doença sexualmente transmissível que está associada com perdas reprodutivas em bovinos. *Campylobacter* coloniza a vagina e o útero e então infecta as células epiteliais do endométrio. O objetivo deste trabalho foi desenvolver um modelo *ex vivo* para quantificar a adesão de *Campylobacter* às células-alvo naturais específicas; este é um passo fundamental para o estabelecimento da infecção e estudos acerca da adesão e citotoxicidade sobre as células do hospedeiro natural não estão disponíveis. Os ensaios foram realizados a través da semeadura de *Campylobacter fetus venerealis* em culturas celulares epiteliais vaginais e uterinas.Células HeLa foram utilizadas como controle.A aderência bacteriana foi confirmada por microscopia óptica e a determinação da porcentagem de bactérias aderidas foi realizada em lâminas tingidas imunoquimicamente. Os resultados são apresentados como porcentagem de células com *Campylobacter* aderente e como o número de bactérias por células. Em comparação com as células HeLa controle, a análise estatística revelou que as culturas primárias mostram uma maior porcentagem de células infectadas e uma menor variação dos parâmetros avaliados. Este modelo de cultura primária pode ser útil para estudos sobre citopatogenicidade e adesão de diferentes cepas de campo de *Campylobacter fetus*.

Palavras-chave: *Campylobacter fetus*. Adesão. Células-alvo. Modelo. Culturas primárias.

Introduction

Campylobacter fetus fetus (Cff) and *Campylobacter fetus venerealis (Cfv)* are causative agents of bovine genital campylobacteriosis. This venereal disease causes considerable economical losses in herds in Argentina and worldwide. The disease is characterized by infertility, early embryonic death, endometritis and, occasionally, by abortion in cows. *Cff* and

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Cfv are transmitted from the bull to the cow during coitus[1,2,3,4,5,6,7,8]. Thus, the bacterium firstly colonizes the vagina and then migrates to the uterus infecting the epithelial cells of the endometrium. The disease is self-limiting in females, although healthy carriers do occur. It is unknown how long infections may persist. However, several studies report persistence of the infection for up to two years[6,9,10,11,12,13,14].

The ability of *Campylobacter* to adhere to host cells plays an integral role in establishing the disease[9,15,16]. Thus, gaining knowledge on the mechanism of adhesion of *Campylobacter* to reproductive tract cells is essential to understand the pathogenesis of the disease. Moreover, it might provide the basis for the development of future studies on this sexually transmitted disorder.

It has been reported that human isolates of *Campylobacter* adhere to and produce cell damage by contact-dependent cytotoxic mechanisms in HeLa, HEp-2, CHO, INT-407 and others cell lines[17,18,19;20,21;22,23;24]. However, reports of *in vitro* adherence and cytotoxicity of *Cfv* on bovine vaginal and uterine epithelial cells are lacking.

The objective of this work was to develop an *ex vivo* model to quantify the adhesion of *Cfv* to its natural, specific target cells, since this is a key step for the establishment of infection and studies on the adherence and cytotoxicity on the natural host cells are not available.

Material and Method

*Campylobacter fetus venerealis Cfv*27 isolated from the cervical mucus of a natural infected heifer was provided by the Clinical and Experimental Microbiological Laboratory of the College of Veterinary Science of UNCPBA, Tandil, Argentina. The strain was grown in an anaerobic jar at 37 °C on Skirrow medium (Merck) and incubated for three days in an atmosphere of 10% CO_2, 85% N_2 and 5% O_2[8,12]. The bacterial colonies were swept and washed with PBS pH 7.2, centrifuged and resuspended in minimum essential medium (MEM-EAGLE M0643 SIGMA-ALDRICH) to a final cell concentration of 4.5 (OD_{480}) . Gram staining was used to examine the morphology and purity of bacteria; only curved, spiral rods were selected. Spherical shaped bacteria were disregarded since this morphology is considered a sign of loss of *Campylobacter* viability. Stock cultures were maintained at -70 ºC in 15% (vol/vol) glycerol-tryptose phosphate broth (Merck 13811).

Uterine and vaginal cervix biopsies were taken from eleven adult venereal disease-free cows. After trypsin treatment of tissues, the cells were grown in MEM supplemented with 10% fetal calf serum (FCS-BIOCELL A15-042), penicillin 120000 UI and streptomycin 1g 100 mg/L. The cells were incubated in a humidified atmosphere at 37 ºC and 5% CO_2. Cellular growth was evaluated daily[25,26].

Cultures of HeLa cells were maintained in MEM supplemented with 10% fetal bovine serum and antibiotics. Cells were grown routinely in a humidified atmosphere at 37 °C and 5% CO_2. Confluent stock cultures were trypsinized, and new stock cultures were seeded in six-well plates at 10^5 cells/mL.

Confluent cultures of endometrial and vaginal cells were trypsinized and seeded on cover slides in six-well plates (CELLSTAR® Greiner Bio-One) at 14 x 10^5 endometrial cells/mL and 21 x 10^5 vaginal cells/mL.

To determine bacterial adhesion, semi-confluent monolayers of HeLa and bovine uterine and vaginal cells were inoculated with 250 µl of bacterial suspension in duplicated wells. Infected monolayers were incubated in a humidified atmosphere at 37 °C and 5% CO_2. The bacterial suspension was removed and collected at three hours post-incubation and bacterial viability was evaluated. Two wells of uninfected cells were used as negative control for each assay.

The cover slides were washed three times with PBS and cells were fixed with methanol for further analysis by Giemsa staining 10% (vol/vol) and immunochemistry.

Immunochemical techniques were previously used to determine the presence of extracellular bacteria[27,28]. Briefly, the infected monolayers were incubated with a primary anti-*Campylobacter fetus venerealis* polyclonal serum, followed by incubation with a biotinylated secondary antibody and revealed by a streptavidine-biotin system (Histomouse™-SP Kit, Zymed®Lab-SA System).

Bacterial adhesion was corroborated by optical microscopy and quantification to determine the percentage of adhesion (number of cells with *Cfv*27) and the number of *Cfv*27 per cell was performed on immunochemically-stained slides. Bacterial adhesion was analyzed in different planes with respect to the cellular nucleus.

The percentage of infected cells (cell with at least one bacterium bound), the average of *Cfv* in 100 cells and the average of *Cfv* per infected cell were estimated by analysis of variance by Levene test[29] using the Statistical Analysis Systems software, PROC GLM Version 9.1.3 ($p < 0.05$)[30].

Results

Binding of *Cfv*27 to HeLa, uterine and vaginal cells was assessed by Giemsa staining (Figure 1) and confirmed by immunochemistry (Figure 2).

Significant differences ($p = 0.0089$) were detected in the mean percentage of infected cells and between the primary and HeLa cell cultures ($p < 0.05$). However, significant differences between uterine and vaginal cell cultures ($p > 0.05$) were not observed (Table 1).

The mean and the variance for the average of bacteria in 100 cells and for the average of bacteria per infected cell are showed in tables 2 and 3, respectively. Marked heterogeneity of the variance was detected when both variables were analyzed. Compared with the primary cell cultures, a high variability in the mean adhesion of Campylobacter to HeLa cells was observed ($p = 0.0009$ and $p < 0.0001$, respectively).

Discussion

The ability of bacteria to bind to host tissues is important as it represents an early event in the establish-

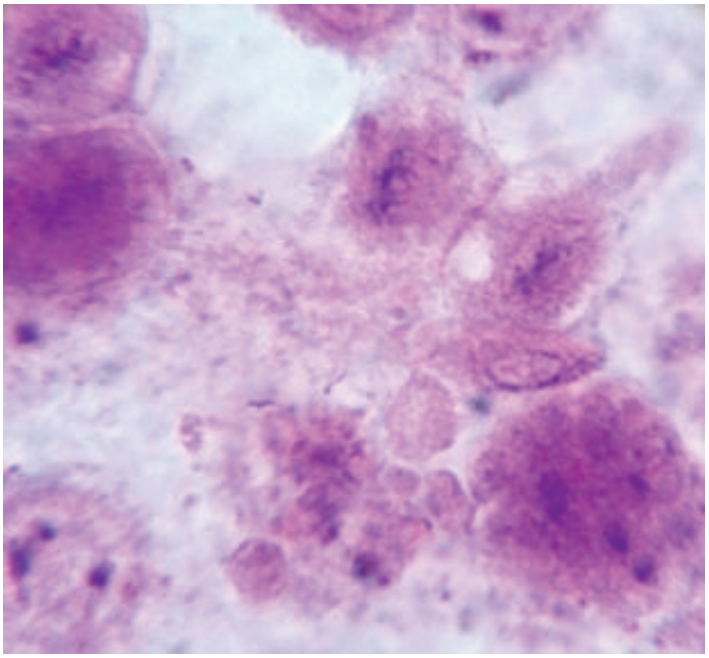

Figure 1 - *Cfv27* adhered on bovine vaginal cell. Cells were stained with 10% (vol/vol) Giemsa. Magnification 40X

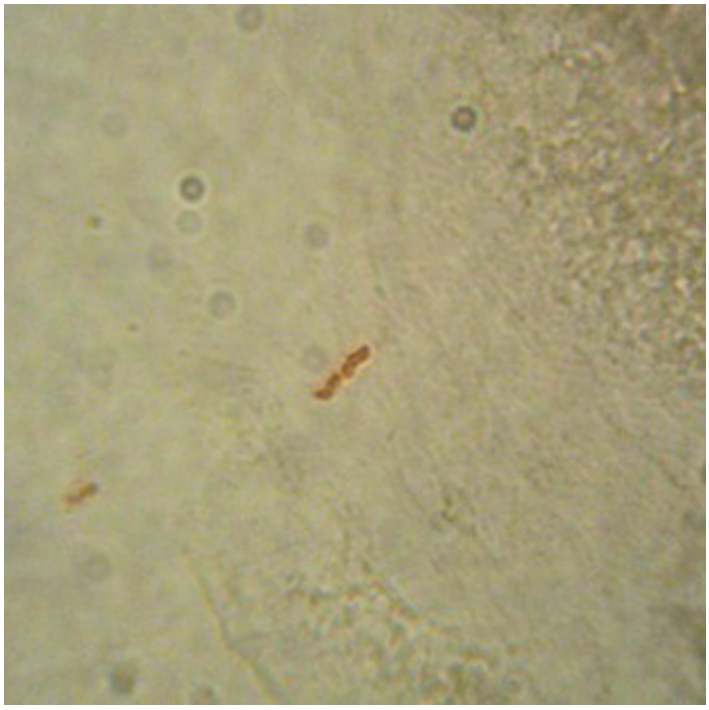

Figure 2 - *Cfv*27 adhered on bovine vaginal cell. Immunochemistry. Magnification 100X

Table 1 - Percentage of infected cells of *Cfv* 27 in primary and HeLa cell cultures

Cell culture	*n*	*Mean*	*Std Error*
HeLa	9	63.41a	4.45
vaginal	11	76.62b	1.73
uterine	11	77.31b	3.27

Different letters indicate significant differences ($p < 0.05$)

Table 2 - Simple statistics for the average of *Cfv*27 adhered to 100 cells

Cell culture	*n*	*Mean*	*Std Error*	*Variance*
HeLa	9	1.996	0.248	0.555
vaginal	11	1.690	0.043	0.020
uterine	11	1.756	0.084	0.078

Table 3 - Simple statistics for the mean of *Cfv*27 per infected cell

Cell culture	*n*	*Mean*	*Std Error*	*Variance*
HeLa	9	3.079	0.214	0.414
vaginal	11	2.219	0.024	0.006
uterine	11	2.280	0.031	0.010

ment of the *in vivo* microorganism-cell relationship. Sometimes, such binding process is also a requirement for pathogenicity when the microorganisms are exposed to the immune response.

Understanding the capability of adherence of *Campylobacter* to the host cell is significant for a thorough knowledge of the initial steps involved in the pathogenesis of bovine genital campylobacteriosis.

Different cell lines and animal models[23,31,32,33,34,35,36,37,38] have been used in studies of cell-*Campylobacter* interaction. However, it would be important to develop studies using the natural target cells of *Cfv* (the epithelial cells of the bovine reproductive tract). In the assays described here, bacterial adhesion was observed and confirmed in monolayers of bovine uterine and vaginal cells and in HeLa cultures (used as control). The results reflect the ability of *Cfv27* to adhere to different cell cultures. With the *Campylobacter* strain used in this study, the differences detected between control and bovine primary cell cultures, demonstrate that this *ex vivo* model is more efficient for bacterial adherence quantification, as determined by a higher percentage of adhesion and a lower variance between assays in the primary cultures.

According to our results, these primary cell cultures might also be useful to study different factors involved in the mechanisms of bacterial binding to the host cell[15,39, 40,41,42,43,44]. Moreover, this model provides an alternative to analyze the pathogenic effects of *Campylobacter fetus venerealis* on the bovine reproductive tract tissues.

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