Adherence to and Chemokine Expression in HeLa Cells Infected by *Campylobacter fetus* subsp. *venerealis*

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**Abstract**

The adherence and invasion of *C. fetus* subsp. *venerealis* in HeLa cells and the chemokines induced during infection were investigated using the PN heifer-passaged strain and its parental strain, NCTC 10354ᵀ. Both *C. fetus* subsp. *venerealis* strains were adherent and non-invasive, being the PN strain significantly more adherent than its parental strain, NCTC 10354ᵀ. Cell adhesion appeared at 15 minutes post-infection with significantly higher levels between 120 and 180 minutes for both strains. The PN strain induced significant higher levels of mRNA for CXCL8 early, at 1h post infection, compared to those induced by its parental strain NCTC 10354ᵀ and by uninfected cells. Both PN and NCTC 10354ᵀ strains did not induce statistically significant levels of CCL2, but a late peak accumulation of CCL2 mRNA was observed at 12 h post infection for PN strain. The passage in heifers intensified the virulence of *C. fetus* subsp. *venerealis*, increasing its adhesive capacity and the chemokines expression induction by infected epithelial cells. The adhesion and induction of proinflammatory chemokines in genital epithelial cells are potentially important events in the immunopathogenesis of Bovine Genital Campylobacteriosis (BGC), since at the same time they trigger the initial immune response against *C. fetus* subsp. *venerealis* and mediate the tissue injury resulting from inflammation, which is involved in infertility in BGC. In conclusion, *C. fetus* subsp. *venerealis* adheres but does not invade epithelial cells and induces the expression of chemokines CXCL8 and CCL2.

**Keywords:** Bovine Genital Campylobacteriosis; Host-Pathogen Interactions; Cellular Attachment; Innate Immunity; Inflammation; Pathogenesis

**Abbreviations**

Ad: Adherent; BHI: Brain Heart Infusion; BGC: Bovine Genital Campylobacteriosis; *C. fetus* subsp. *venerealis*: *Campylobacter fetus* subsp. *venerealis*; C. fetus: *Campylobacter fetus*; CETEA: Ethics Committee in Animal Experimentation; CFU: Colony Forming Unit; FBS: Fetal Bovine Serum; Inv: Invasive; MOI: Multiplicity of infection; MEM: Minimal Essential Medium; PBS: Phosphate Buffered Saline; PN: Heifer-Passaged; RFLP: Restriction Fragment Length Polymorphism; UFMG: Universidade Federal de Minas Gerais

**Introduction**

*Campylobacter fetus* subsp. *venerealis* is an important pathogen for cattle. It is the agent of bovine genital campylobacteriosis (BGC), a disease that affects cattle in reproductive age and causes significant economic losses in infected herds [1,2]. In bulls, BGC is asymptomatic and the microorganism remains lodged in the epithelial surface of the glans penis, urethra and prepuce, whereas in cows, *C. fetus* subsp. *venerealis* colonizes the genital tract, leading to endometritis, early embryonic death and sporadic abortions [2,3].

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After the pathogen enter the genital tract, the next step toward to establishment of infection is the attachment to epithelial cells, colonizing the vaginal, cervical and uterine mucosal epithelia and oviduct, featuring an ascending infection [4]. Uterine colonization is of utmost importance to the pathogenesis of BGC, because uterine inflammation leads embryo loss and repeat-breeding [5,6]. The inflammatory process caused by attachment of a microorganism to a host cell involves the liberation of chemotactic factors [7]. These chemotactic factors, named chemokines, are produced by a great variety of cells, among which epithelial cells [7,8]. Chemokines are a group of structurally homologous cytokines that stimulate migration of leukocytes from blood into the tissues, marking the onset of the inflammatory response. Chemokines are normally classified in two main groups, the CC (also called β) chemokines (e.g. CCL2), in which the two defining cysteine residues are adjacent and the CXC (or α) group (e.g. CXCL8), in which these residues are separated by one amino acid [8]. Of all known chemokines, CXCL8 is one of the most important in the initial immunity against bacteria, which are pathogenic to mucosa surfaces [9]. This chemokine, produced by epithelial cells and cells of the mononuclear phagocyte system, is a potent neutrophil activator, and attracts these cells to the compromised site after infection [7,10].

Although much has been studied on Campylobacter species associated with diarrhea, information about the host-pathogen interaction after C. fetus infection is scarce. Among Campylobacteriaceae family members, C. jejuni, bacteria that cause diarrhea in humans and animals, is the most studied [11,12]. C. jejuni has as one of the major virulence factors the ability to invade epithelial cells, since that the microorganisms are protected from immune response when inside the host cell and increase the damage caused in the host tissue and contributing to the development of diarrhea [11,13]. The ability of adherence and invasion of C. jejuni vary considerably among the isolates and strains with high invasion levels, usually isolated from diarrheic feces, are considered more virulent than those isolated from normal feces [14]. Moreover, C. jejuni reference strains and clinical isolates stimulated the expression of CCL2 (MCP-1) and CXCL8 (IL-8) by epithelial cells following infection [15-17].

The invasion ability of C. fetus subsp. fetus strains was previously reported, however it depends on its origin. Most fecal origin isolates are invasive, demonstrating capacity for intracellular replication and translocation [18-20] whereas strains of genital origin are noninvasive [20]. It is believed that fecal C. fetus subsp. fetus are likely able to invade epithelial cells due to the need to colonize the host’s intestine and evade the intestinal peristaltic movements and even spread to extra-intestinal sites and while during C. fetus subsp. fetus colonization of the genital tract, there is no need to invade but only to adhere to epithelial cells [18,20].

Adhesion of C. fetus subsp. venerealis, to HEP-2 [21], INT 407 [22], primary cultures from bovine vaginal and endometrial [23] and MDBK cells [24] has been analyzed, however, without investigating a potential cellular invasion. Cell invasion assays was performed in Caco-2 cells [25], an enterocyte-like intestinal line that due to its capacity to act as non-conventional phagocyte internalizes noninvasive bacteria [26,27], limits the discrimination between invasion or internalization. Despite these observations, none of the previous studies evaluated the temporal kinetics of attachment to epithelial cells and chemokines expression.

Therefore, considering that kinetics of C. fetus subsp. venerealis attachment to epithelial cells and chemokine induction by epithelial cells could play an important role in the immunopathogenesis of BGC, the aims of this study were to assess (i) the adherence and invasion of C. fetus subsp. venerealis in epithelial cells and (ii) the expression of inflammatory chemokines related to the innate immunity after infection in these cells.

Materials and Methods

Bacterial strains and culture conditions: C. fetus subsp. venerealis strain NCTC 10354T and the strain PN, the strain NCTC 10354T inoculated in a heifer and re-isolated, were used in this study. The reactivation of the type strain (C. fetus subsp. venerealis NCTC 10354) was performed by passing in heifer before the experiment. Seven days after intra-vaginal inoculation, the type strain was recovered from vaginal mucus and named PN. This experimental protocol was approved by the Ethics Committee in Animal Experimentation of UFMG (CETEA - Protocol 39/2007). The C. jejuni 84sp strain was used as invasive positive control in adherence and invasion assay, and C. coli 49sp was employed as a non-invasive negative control [28].

Bacterial strains were cultivated in Brain Heart Infusion (BHI) agar (Difco, Detroit, USA) supplemented with 10% defibrinated horse blood at 37°C in microaerophilic conditions (5% O2, 5% H2, 10% CO2, and 80% N2) for 48h [20]. Bacteria were suspended in sterile phosphate buffered saline (PBS) (0.01M, pH 7.2) (108 CFU/mL, equivalent to tube 2 of the MacFarland scale), after cultivation in BHI agar and
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centrifuged at 7,000 x g for 10 min at room temperature. The pellet was resuspended in Minimal Essential Medium (MEM) with 5% Fetal Bovine Serum (FBS) without antibiotics before inoculation in cell culture.

**Cell lines and culture conditions:** The tests were performed in HeLa cells (ATCC - CCL2) kept in MEM (Sigma-Aldrich, St. Louis, USA) supplemented with 10% FBS (Life Technologies, Carlsbad, USA), 200 IU/mL penicillin G (Sigma, St. Louis, USA), and 50 µg/mL streptomycin (Sigma, St. Louis, USA). Cells were cultivated in plastic bottles kept in 5% CO₂ at 37°C for 24 to 48h. The confluent monolayer obtained in the surface of the bottle was trypsinized, diluted 1:3 in MEM with 5% FBS and antibiotics, and re-incubated in the same conditions.

**Multiplicity of infection (MOI):** The optimal ratio of *C. fetus* subsp. *venerealis* to epithelial cells was determined using *C. fetus* subsp. *venerealis* NCTC 10354T and HeLa cells. Bacteria (140 µL - 108 CFU/mL) were inoculated into duplicate wells of the 48-well cell plates (Corning, Bedford, USA). Infection of HeLa cells were performed at MOIs of 100, 500, 1000, and 10000 bacteria/cell. The gentamicin assay, as described by Konkel and Joens [29] (see adherence and invasion assay), was performed to evaluate the percentage of adherence and internalization for all MOI tested. Each MOI was tested twice, and all tests were performed in duplicate.

**Kinetics of adherence of *C. fetus* subsp. *venerealis* in epithelial cells:** The optimal incubation time for adherence of *C. fetus* subsp. *venerealis* to epithelial cells was determined using the strains NCTC 10354T and PN, and HeLa cells. Bacteria (140 µL - 108 CFU/mL) were inoculated into duplicate wells of the 48-well cell plates. The MOI used was determined in the previous assay (500 bacteria/cell). Infected monolayers were incubated for 15, 30, 60, 90, 120, 150 and 180 minutes to establish the adherence kinetics at 37°C and 5% CO₂. The gentamicin protection assay, as described by Konkel and Joens [29] (see adherence and invasion assay), was performed to evaluate the percentage of adherence and internalization for all incubation times tested. Each incubation time was tested twice for all bacterial strains, and all tests were done in duplicate.

**Adherence and invasion assay:** The gentamicin assay was performed using *C. fetus* subsp. *venerealis* strains NCTC 10354T and PN, and HeLa cells, as previously described [29]. Briefly, confluent cells were harvested by trypsinization and plated in a 48-well cell plate at a density of 2 x 10⁵ cells/well in MEM supplemented with 5% FBS, then incubated at 37°C for 18h in a 5% CO₂ atmosphere. After incubation, the medium was removed, and each well was washed ten times with 500 µL PBS. Bacterial strains (140 µL - 10⁸ CFU/mL) were inoculated into duplicate wells, containing HeLa cells. The multiplicity of infection used was 500 bacteria/cell. Infected monolayers were incubated for 3h at 37°C and 5% CO₂ to allow the bacteria adhere to the cells. After incubation, the medium was removed, and the wells were washed ten times with 500 µL PBS. Thereafter, 140 µL of MEM with 5% FBS and 250 µg/mL gentamicin were added in two of the wells, and the same volume of medium without any antibiotic was added to the other two wells for each bacterial strain and for each cell line tested. The plates were then incubated at 37°C in a 5% CO₂ for another 3h. After disposal of the medium, the wells were washed ten times with 500 µL PBS. Cells were lysed with 200 µL/well of 0.1% Triton X100 (LKB Bromma, Sollentuna, Sweden) for 10 minutes. The concentration of the bacterial suspensions was determined in duplicate by the drop count method in BHI agar with 10% defibrinated horse blood [30]. Each strain was tested twice, and all tests were performed in duplicate.

Strains were considered non-adherent and non-invasive (Ad-/Inv-) when no bacterial growth was detectable during bacterial counts made from the wells with and without gentamicin. Adherent but non-invasive strains (Ad+/Inv-) showed bacterial growth in wells without gentamicin treatment, but bacterial counts were equal or inferior to the negative control counts (strain 49sp) in the wells treated with antibiotic. Conversely, invasive strains (Ad+/Inv+) had bacterial growth in wells without gentamicin treatment and bacterial enumeration in wells treated with gentamicin were higher than the negative control counts. The percentages of adherence and invasion were calculated as previously described [31]. The percentage of adherence for each strain was calculated from the bacterial populations of the wells without gentamicin as follows: % adherence = ([# intracellular bacteria + adherent bacteria/mL]/[# bacteria in inoculum/mL]) x 100. The percentage of invasion for each strain was defined using the bacterial count of the wells treated with gentamicin, by the formula: % of invasion = ([# intracellular bacteria/mL]/[# bacteria in inoculum/mL]) x 100.

**Expression of chemokines by epithelial cells infected by *C. fetus* subsp. *venerealis:*** The expression of chemokines following *C. fetus* subsp. *venerealis* infection was performed using the strains NCTC 10354T and PN, and HeLa cells. HeLa cells (4 x 10⁵ cells/mL) were cultured in 6 wells plates (3.5 mL/well) containing MEM supplemented with 5% FBS and incubated at 37°C and 5% CO₂ for 18h. The cells were infected with suspensions of the strains *C. fetus* subsp. *venerealis* NCTC 10354T and PN, with a MOI of 500 bacteria/cell. Infected cells were incubated for 0, 0.5, 1, 2, 4, 6, 8, and 12h at 37°C and 5% CO₂. All experiments were performed in duplicate.

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At each incubation time, the mRNA was extracted from the cell monolayer with Trizol® Reagent (Invitrogen, USA) following the manufacturer’s instructions. cDNA strands were synthesized from 1.5 μg of total RNA using Reverse Transcription System kit (Promega, USA). The PCR was performed with 200 μM of each dNTP (Thermo Fisher Scientific, Carlsbad, USA), 1.0 U Taq DNA polymerase (Phoneutria, Belo Horizonte, Brazil), and 10% enzyme buffer (500 mM KCl, 100 mM Tris-HCl pH 8.4, 1% Triton X-100) (Phoneutria, Belo Horizonte, Brazil) up to a final volume of 20 μL. Primers used to amplify the CCL2 and CXCL8 chemokines and the housekeeping gene (β-actin), and PCR conditions for each one are listed in the table 1. PCR products were visualized in 1% agarose gel (Life Technologies, Carlsbad, USA) stained with ethidium bromide (0.5 mg/mL) (Sigma-Aldrich, St. Louis, USA) after electrophoresis at 100V for 40 minutes. Images of the gels were captured by the Image Master VDS (Pharmacia Biotech, Uppsala, Sweden). The density of the bands was measured using the software Image Master TotalLab (Pharmacia Biotech, Uppsala, Sweden). Data obtained in the Image Master TotalLab were submitted to normalization based on the expression of β-actin. The identity of the amplified fragments was confirmed by restriction analyses (RFLP).

### Table 1: Primers and PCR conditions for amplification of chemokines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers 5’ 3’</th>
<th>Accession numbers</th>
<th>Amplicon size (bp)</th>
<th>Annealing temperature [Primer]</th>
<th>[MgCl₂]</th>
<th>Number of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>F - CTTGGAGGCTTCTCTGATT&lt;br&gt;R - TCAAAAACCTTCCACAACC</td>
<td>NM_000584</td>
<td>254</td>
<td>50.5°C 1 µM 3.5 mM</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>F - ATAGCAAGCCACCTTCTGTC&lt;br&gt;R - TGGATCTCGAACGCCATTC</td>
<td>NM_002982</td>
<td>223</td>
<td>55°C 2.5 µM 3.5 mM</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>F - TCAACCACACTGTGGCCATCTACGA&lt;br&gt;R - CAGCGCAACCGCTTATTGGCAATGG</td>
<td>NM_001101</td>
<td>295</td>
<td>60°C 0.5 µM 3.5 mM</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

**Statistical analysis**

Analysis among the different MOIs tested and among times tested in kinetics of adherence experiments and in the expression of chemokines by epithelial cells infected by *C. fetus* subsp. *venerealis* were performed by one-way ANOVA followed by Student-Newman-Keuls test [32]. In the kinetics of adherence assay and chemokines expression analyses between *C. fetus* subsp. *venerealis* strains within the same time were performed by unpaired t-test [32]. Statistical analyses were performed with aid of GraphPad Prism 5.00 software (GraphPad Software, La Jolla, USA). Significance was defined in all cases at P < 0.05 [32].

**Results**

**Effect of MOI on adherence of *C. fetus* subsp. *venerealis* to HeLa cells:** Percentage of adherence and count of adherent (CFU) *C. fetus* subsp. *venerealis* NCTC 10354° to HeLa cells in each MOI tested is shown in figure 1. *C. fetus* subsp. *venerealis* NCTC 10354° showed significantly higher percentages of adherence to HeLa cells when the multiplicity of infection used was 500 bacteria/cell (Figure 1A). There was no significant difference in the number of adherent *C. fetus* subsp. *venerealis* NCTC 10354° to HeLa cells among the MOIs tested (Figure 1B).

**Adherence kinetics of *C. fetus* subsp. *venerealis* in HeLa cells:** Adherence of *C. fetus* subsp. *venerealis* NCTC 10354° and PN to HeLa cells is shown in figure 2. Adherence to cells first appeared after 15 minutes of incubation, with significant levels in 120, 150, and 180 minutes of incubation for both strains. However, adherence kinetics was significantly different between strains, with PN strain resulting in significantly higher percentage of adherence than its parental strain, NCTC 10354°, in most incubation times tested. There was no invasion of the HeLa cells in any incubation time by any strain.

**Expression of chemokines by epithelial cells infected by *C. fetus* subsp. *venerealis***

**CXCL8:** The accumulation of CXCL8 mRNA in HeLa cells infected with the strains *C. fetus* subsp. *venerealis* NCTC10354° and PN are shown in figure 3A. PN strain induced significantly higher levels of CXCL8 mRNA at 1h post infection compared to its parental strain NCTC 10354° and non-infected cells. There was no significant difference between parental strain NCTC 10354° and non-infected cells in neither of the tested times.

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Figure 1: Percentage of adherence (A) and colony forming unit - CFU (B) of C. fetus subsp. venerealis NCTC 10354T to HeLa cells in each multiplicity of infection (MOI). Bars show the mean and standard error. Lowercase letters indicate statistical differences among MOI tested (One-way ANOVA followed by Student-Newman-Keuls test) (P < 0.05). The data accounts for at least two repetitions performed in duplicate.

Figure 2: Adherence of C. fetus subsp. venerealis NCTC 10354T (A) and PN (B) to HeLa cells in different post-infection incubation times. Bars show the mean and standard error. Significant differences (P < 0.05) between strains (on same time) are indicated by uppercase letters (unpaired t-test), and lowercase letters indicate statistical differences among times tested in same strain (One-way ANOVA followed by Student-Newman-Keuls test). The data accounts for at least two repetitions performed in duplicate.

CCL2: The level of CCL2 mRNA in HeLa cells after infection with *C. fetus* subsp. *venerealis* NCTC 10354ᵀ and PN is shown in figure 3B. HeLa cells infected with PN and parenteral NCTC 10354ᵀ strains did not exhibit significant difference in CCL2 mRNA accumulation compared with non-infected cells in any time post infection. However, a late peak of mRNA accumulation at 12h post infection with PN strain was observed.

![Figure 3: Expression of CXCL8 (A) and CCL2 (B) by HeLa cells infected with C. fetus subsp. venerealis NCTC 10354T, C. fetus subsp. venerealis PN and non-infected control cells. The results are expressed as mean. Significant differences (P < 0.05) between strains (NCTC 10354T vs. PN) (on same time) are indicated by uppercase letters (unpaired t-test), and lowercase letters indicate statistical differences among times tested in same strain (One-way ANOVA followed by Student-Newman-Keuls test).](image)

**Discussion**

The determination of the most appropriate MOI is a crucial step in studies of bacterial-cell interaction, since it influences the infection efficiency and levels of cell invasion [12]. MOIs of 100 to 500 are widely used for *Campylobacter* infection studies, including research with *C. fetus*, since that lead to the highest numbers of intracellular bacteria possible [12,18,25] and therefore, increase the sensitivity of cell invasion detection. In our study, the use of MOI of 500 triggered a significant *C. fetus* subsp. *venerealis* NCTC 10354ᵀ adhesion in HeLa cells and therefore, it was chosen to maximize detection of a possible cell invasion.

Adherence to host cells is often the first step in the establishment of mucosal bacterial infections and an important step in the *Campylobacter* pathogenesis, since it allows bacteria cell to withstand the mechanical clearing mechanisms of the host and establishing extracellular persistence, representing thereby an early event in the establishment of an *in vivo* niche [13,18,21,33]. The *C. fetus* subsp. *venerealis* adhesion to epithelial cells detected at 15 minutes post infection with significantly greater adherence at 120 minutes and without invasion in any infection time, suggests that in the host *C. fetus* subsp. *venerealis* can rapidly bind to epithelium, however, without invading their cells, persisting as a mucosal extracellular pathogen in the bovine reproductive tract. In fact, histopathological data supports this hypothesis [5,34,35], since tissues of infected cows did not present deep lesions, indicating that *C. fetus* subsp. *venerealis* does not invade endometrial tissue. The pathogen remains in the lumen of the uterus above the epithelium and in its preferred microenvironment, the lumen of the uterine glands [5,6,34].

The inability of *C. fetus* subsp. *venerealis* to enter into a non-phagocytic genital cell line such HeLa, verified in our study, supports the concept that has been shared by different research groups [24,36-38], that *C. fetus* subsp. *venerealis* is non-invasive but an extracellular bacterium of the bovine genital tract.

Indeed, a recent study of our research group showed [20] that *C. fetus* subsp. *fetus* of genital origin also did not invade HeLa cells, suggesting that the inability to invade genital epithelial cells is shared by both subspecies *C. fetus*. Together, these findings demonstrate that during *C. fetus* colonization of the genital tract, there is no need to invade but only to adhere to epithelial cells.

Another important event involved in host colonization is the ability to induce proinflammatory chemokines in epithelial cells, mainly CXCL8 and CCL2, considered as pathogenicity determinant in *C. jejuni* and *H. pylori* infections, since they contribute to mucosal injury [15-17,39]. The induction of CXCL8 and CCL2 chemokines by *C. fetus* subsp. *venerealis* in infected epithelial cells observed in the present study evidences that these chemotactic factors act primarily on the initial immune response against this pathogen, recruiting inflammatory cells and may provide a link between infection and its consequences involving inflammation. Although the inflammatory infiltrate acts beneficially in favor of the host against infection [5,35], it secondarily promotes tissue injury and makes the uterus very inflamed and therefore an environment incompatible with the survival of the embryo. Therefore, inflammatory response of the uterine mucosa to *C. fetus* subsp. *venerealis* is considered a direct cause of the reproductive disorders in BGC [4,6,34,40]. Furthermore, combination between endometritis and the direct effect of the pathogen on the conceptus [40,41], or restriction of oxygen supply to the embryo, due to the decrease in the oxygen tension promoted by the bacterium [42], have also been incriminated as causes of infertility in BGC.

Moreover, the expression of significant early CXCL8 levels (1h post infection) corroborates the early peak accumulation of CXCL8 found by others [39] and suggests that CXCL8 may be an important mediator of the acute inflammatory response that contributes to uterine injury during *C. fetus* subsp. *venerealis* infection. Since CXCL8 is a potent neutrophil chemotactic factor [7,8,10], its early release into the uterus will promote massive recruitment of neutrophils into the tissue, which explains the predominance of neutrophilic infiltrate found in the endometrium in the early stages of BGC. Polymorphonuclear leukocytes, especially neutrophils, are the first cells that infiltrate into the tissue and the most numerous in the early stages of BGC [5,35,40,43]. Neutrophils are the first type of leukocyte to be recruited from the blood into injured tissue by bacteria and thus, early neutrophil recruitment reflects early and abundant CXCL8 production by tissue cells in response to infection [7,10].

In contrast to the earlier expression of CXCL8, the CCL2 monocyte/macrophage chemoattractant chemokine has shown to be late induced during the infection by *Campylobacter* spp, with substantial CCL2 levels detected 24h after the infection with *C. jejuni* [16,17]. This naturally late dynamics of CCL2 explains the non-significant levels found until the last time point infection tested (12h). Although significant levels of CCL2 were not detected, the peak accumulation observed at 12h post infection indicates a potential for an increase up to 24h, as demonstrated by others [17], since a trend can be observed in the graph (Figure 3). The significant expression of CCL2, which is still to be demonstrated, could explain the predominance of mononuclear cells such as macrophages in inflammatory infiltrate present at the later stages of BGC. In the late stages of uterine infection by *C. fetus* subsp. *venerealis* the neutrophils decrease while mononuclear leukocytes, such as macrophages and lymphocytes increase [5,35,40,43].

In addition to the factors already discussed, we must consider two other events involved in the immune response to CGB infection. First, the effect of adaptive immunity components, namely IgM, IgG and local IgA, which in turn orchestrate the *C. fetus* uterine clearance. IgA acts on immobilization whereas IgG opsonizes *C. fetus* and facilitates its phagocytosis [44]. Second, the concurrent presence of other microorganisms such as bovine virus diarrhoea virus [45], *Brucella abortus* [46], *C. sputorum* bv. *sputorum* [47] and *Trichomonas foetus* [48] with *C. fetus* may also influence the dynamics of the immune response, making the cattle more resistant or more susceptible to *C. fetus* and thus exacerbating pathological lesions.
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Another interesting aspect of BGC pathogenesis revealed by our findings is the significantly higher adherence of the PN strain compared to its parental strain NCTC 10354\(^1\), the type strain, showing that *in vivo*-passaged bacteria increases the adhesion potential and virulence of laboratory-adapted strains that are often less virulent [29]. This phenomenon, now observed in *C. fetus* subsp. *venerealis*, has also been observed in the closely related bacteria, *C. jejuni* [49] and *Arcobacter butzleri* [50]. Moreover, the passage of *C. fetus* subsp. *venerealis* in the host besides improving the PN adhesion capacity also increased the bacterial potential of inducing chemokines. The greater induction of chemokines by the PN strain compared to its parental strain reflects the higher adhesion and virulence potential of this strain, which was able to trigger higher levels of stimulation in epithelial cells and consequently high levels of induction of chemokine expression. In fact, a previous study also showed that more adherent strains stimulate higher production of chemokines in comparison to laboratory-adapted strains [15]. Importantly, this benefit of PN reactivation was essential in the present study, since it allowed to show significant differences in the induction of chemokines, which would be hidden and could not be proved only with the use of the NCTC 10354\(^1\) reference strain. These findings highlight the importance of the use of host-passaged bacterial strains in chemokines expression studies to avoid the loss of potential information induced by laboratory subculture over time.

**Conclusion**

In this study, we showed that *C. fetus* subsp. *venerealis* adhere but does invade HeLa cells and induces the expression chemokines CXCL8 and CCL2, being the *in vivo*-passaged PN strain more virulent than its parental strain NCTC 10354\(^1\). These findings suggest that adhesion and induction of chemokines in epithelial cells are potential *C. fetus* subsp. *venerealis* virulence factors mediating inflammation and tissue injury, thus playing an important role in the pathogenesis of BGC.

**Bibliography**


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