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# Association between virulence factors of *Escherichia coli*, *Fusobacterium necrophorum*, and *Arcanobacterium pyogenes* and uterine diseases of dairy cows

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ABSTRACT

The objective of this study was to evaluate the relationship between bacterial species-specific virulence factors (VFs) present in the uterus at 3 different stages of lactation (1–3, 8–10, and 34–36 days in milk (DIM)) and the incidence of metritis and clinical endometritis in dairy cows. The following VF genes were investigated: *plo* (pyolysin), *cbpA* (collagen-binding protein), and *fimA* (fimbriae expression) which are *Arcanobacterium pyogenes* specific; *fimH* (a type 1 pilus component), *Escherichia coli* specific; and *lktA* (leukotoxin), *Fusobacterium necrophorum* specific. Uterine swabs were collected from 111 postpartum dairy cows. PCR was used to detect the presence of *plo*, *cbpA*, *fimA*, *fimH*, and *lktA genes*. *A. pyogenes cbpA* was detected in only 5 samples and therefore was not subjected to further analysis. *E. coli* (*fimH*) was significantly associated with metritis and endometritis when detected at 1–3 DIM; *F. necrophorum* (*lktA*) was significantly associated with metritis when detected at 1–3 and 8–12 DIM and with endometritis when detected at 34–36 DIM; and *A. pyogenes* (*fimA* and *plo*) was associated with metritis (*fimA*) when detected at 1–3 DIM and endometritis (*fimA* and *plo*) when detected at 8–10 and 34–36 DIM.



## 1. Introduction

Post-partum uterine diseases – especially metritis, clinical endometritis, and subclinical endometritis – are important for both animal welfare reasons, contributing to cow discomfort and elimination from the herd, and economic reasons, profoundly affecting reproductive performance and reducing the profit potential of dairy farms (Bicalho et al., 2010). At the onset of parturition the physical barriers of the cervix, vagina and vulva are compromised, allowing environmental bacteria to ascend into the uterine lumen of 80–100% of all dairy cows (Foldi et al., 2006; Azawi, 2008). Nevertheless, not all cows will develop postpartum uterine disease (Sheldon, 2004; Sheldon and Dobson, 2004). The effectiveness of host defenses in staving off postpartum uterine disease is dependent on the immunological status of the cow, the species and virulence of bacteria, and the contamination load of the uterine lumen (Sheldon et al., 2006).

*Escherichia coli*, *Arcanobacterium pyogenes* and *Fusobacterium necrophorum* are considered to be important bacteria associated with uterine infection (Dohmen et al., 2000; Azawi, 2008). Bicalho et al. (2010) reported that six *E. coli* virulence factors (VFs) were associated with metritis and endometritis: *fimH*, *astA*, *cdt*, *kpsMII*, *ibeA*, and *hlyA*. The VF gene *fimH* was the most prevalent and cows with at least 1 *fimH*-carrying *E. coli* strain had a 4.6-fold increased odds of metritis when compared to *E. coli* negative cows. *A. pyogenes* possesses a number of VFs that contribute to its pathogenicity such as; pyolysin (*plo*), a

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collagen-binding protein (*CbpA*), and a component of fimbriae (*fimA*). Santos et al. (2011), investigated the association of the presence of the VFs *plo*, *fimA*, and *cbpA* with the incidence of metritis: the *plo* was indentified in 100% of the isolates, cbpA was detected in only one isolate, and *fimA* was significantly associated with metritis. *F. necrophorum*, has also been associated with metritis with the *lktA* (leukotoxin) VF being associated with its pathogenic action (Williams et al., 2005).

The objective of this study was to evaluate the relationship between the presence in the uterus of bacterial species-specific VFs of *A. pyogenes* (*plo*, *cbpA*, and *fimA*), *E. coli* (*fimH*) and *F. necrophorum* (*lktA*) detected at 3 different stages of lactation (1–3, 8–10, and 34–36 DIM) and the incidence of metritis and clinical endometritis.

## 2. Materials and methods

### 2.1. Farm, management and sample collection

Uterine swabs were collected from 111 post-partum dairy cows that were housed on a commercial dairy farm located near Ithaca, NY. Samples were collected from April 2010 through June 2010. Reproductive management utilized a combination of Presynch (Moreira et al., 2001), Ovsynch (Pursley et al., 1995), Resynch (Fricke et al., 2003), and detection of estrus, with 25–30% of cows bred via TAI and the remainder bred after detection of estrus solely by activity monitors (ALPRO; DeLaval, Kansas City, MO). Uterine secretion samples were collected from each cow three times during the study period (at 1–3 DIM, at 8–10 DIM, and at 34–36 DIM). Two uterine sample collection methods were used: uterine swab for the first and second sample and uterine lavage for the third sample. At 34–36 DIM the uterus has usually involuted and uterine fluid volume has decreased. Thus, at this point, performing uterine lavage is probably a better way of sampling. Furthermore, uterine lavage was also used at 34–36 DIM for the diagnosis of endometritis. Uterine swabs were collected as follows: cows were restrained and the perineum area was cleansed and disinfected with 70% ethanol. Then, a sterile swab (Har-Vet™ McCullough Double-Guarded Uterine Culture Swab, Spring Valley, WI) covered by a sterile pipette (inside a plastic sheath) was introduced to the cranial vagina. The pipette was manipulated through the cervix into the uterus, the sheath was then ruptured, and the swab was exposed to uterine secretion. The swab was pulled inside the pipette and kept in transportation medium at 4 °C until it was processed in the laboratory. Uterine lavage samples were collected as described by Gilbert et al. (2005). Briefly, cows were restrained and the perineum area was cleansed and disinfected with 70% ethanol. A plastic infusion pipette (inside a plastic sheath) was introduced to the cranial vagina. The sheath was subsequently ruptured, and the clean pipette tip was manipulated through the cervix into the uterus. A total of 40 ml of sterile saline solution was injected into the uterus, agitated gently, and a sample of the fluid aspirated. The volume of recovered fluid ranged from 5 to 15 ml. Samples were kept in ice prior to laboratory processing.

This project proposal was reviewed and approved by the Cornell University Institutional Animal Care and Use Committee (# 2011-0111).

### 2.2. Case definition

Puerperal metritis was clinically defined as an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge, with signs of systemic illness (decreased milk yield, dullness or other signs of toxemia) at 8–10 days after parturition and diagnosed by one of the veterinarians of the research team. Clinical endometritis was evaluated at 34–36 DIM by visual inspection of a uterine lavage sample obtained as described by Gilbert et al. (2005). In this way we were able to ensure that visible signs of inflammation (purulent or mucupurulent exudate) emanated from the uterus, rather than from another site. All of the uterine lavage samples were visually scored by one investigator, who assessed the presence of a purulent or mucopurulent secretion in the uterine lavage sample. The score ranged from 0 to 2, with 0 indicating absence of a purulent or mucopurulent secretion in the lavage sample, 1 indicating a bloody but not purulent sample, and 2 the presence of pus in the lavage sample. Cows with a score of 2 were considered as diagnosed with clinical endometritis. Body condition scores were recorded at the time of each uterine lavage using a five-point scale with a quarter-point system as described by Edmonson et al. (1989). Additionally, farm recorded calving ease score 1–5 (1 and 2 were non-assisted parturitions and 3–5 were assisted partitions with increasing degree of difficulty), stillbirth parturition, and retained placenta incidence were used as risk factors.

### 2.3. DNA extraction, PCR, gel electrophoresis and sequencing

Swab samples were immersed in 1 ml of phosphate-buffered saline (PBS) into a 15-ml Falcon tube and vortexed to disperse any mucus, bacteria, cells, or transport culture medium. Isolation of total DNA was performed from 400 μl of the suspension by using a QIAmp DNA minikit (Qiagen, Santa Clara, CA) according to the manufacturer's instructions for DNA purification from blood and body fluids. Some convenient modifications, such as addition of 400 μg of lysozyme and incubation for 12 h at 56 °C, were included to maximize bacterial DNA extraction. Total DNA was eluted in 100 μl of sterile DNase/RNase-free water (Promega, Madison, WI). DNA concentration and purity were evaluated by optical density using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

PCR was used for the amplification of specific VFs genes' parts. Among the VF genes that contribute to the pathogenic potential of *A. pyogenes*, three were amplified: *plo*, *cbpA* and *fimA*. To categorize *E. coli*, the *fimH* gene was chosen. The leukotoxin gene (*lktA*), which appears to be unique for *F. necrophorum*, was used as that bacterium's VF. Details regarding the primer sequences, annealing temperatures, and size of amplicons can be found in Table 1. Presence of known and putative *A. pyogenes, E. coli* and *F. Necrophorum* VF genes was assessed independently. Thermal cycling parameters were adjusted according to