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Bacterial invasion of the uterus and oviducts in bovine pyometra

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ABSTRACT

Pyometra is a common disease of cattle that causes infertility and thereby financial losses to the cattle industry. Bacteria involved in the development and progression of pyometra have been investigated by microbial culture but their tissue invading abilities, which is an important aspect of bacterial pathogenicity and development of lesions, have not been investigated. Bacterial invasion of the uterus and oviducts was studied in 21 cows diagnosed with pyometra at the time of slaughter by applying fluorescence *in situ* hybridization using probes targeting 16S ribosomal RNA of *Fusobacterium necrophorum*, *Porphyromonas levii*, *Trueperella pyogenes* and the overall bacterial domain *Bacteria*. *Fusobacterium necrophorum* and *P. levii* were found to invade the endometrium, especially if the endometrium was ulcerated, and penetrated deep into the lamina propria. These species co-localized within the tissue thus indicating a synergism. *Trueperella pyogenes* did not invade the uterine tissue. In addition to endometrial lesions, most cows with pyometra also had salpingitis but without significant bacterial invasion of the oviductal wall.

1. Introduction

Endometrial bacterial contamination develops in up to 90% of cows after parturition [1]. Bacterial contamination can progress into a postpartum infection, leading to conditions such as endometritis, metritis, and pyometra [2]. The diseases are associated with reduced reproductive performance, reduced milk production, milk withdrawal and increased costs for treatment leading to economic losses [3–12]. The annual costs of bovine postpartum uterine infections were estimated to be around €1.4 billion in the EU and around $650 million in the United States in 2009 [12].

Pyometra accounts for 2%–5% of the clinical cases of uterine diseases in cows [13,14] and is characterized by inflammation of the endometrium and accumulation of a purulent exudate in the uterine lumen in the presence of a persistent corpus luteum and a closed cervical canal [15,16]. Cows that ovulate early in the postpartum period, when bacterial contamination is still present in the uterus, are at higher risk of developing pyometra [17]. If endometritis is present after ovulation and during diestrus, the inflammation may inhibit the endometrial prostaglandin production thus preventing corpus luteum regression [16,17]. This inhibition causes a permanent closure of the cervical canal and subsequent accumulation of pus in the uterine lumen [16,17]. Affected cows show no systemic signs of illness, but are infertile [18]. Pyometra is usually treated by injection of $PGF_{2\alpha}$, which induces regression of the corpus luteum leading to relaxation of the cervix and expulsion of the intrauterine exudate with subsequent estrus as a result [19].

The bacterial flora in cases of bovine pyometra has been determined using conventional culture methods [17,20–23] and recently by culture-independent methods [24]. The most frequently isolated bacteria cultured from cases of pyometra are *Trueperella pyogenes* and Gram-negative bacteria [20,21], especially *Fusobacterium necrophorum* [17]. In addition to these frequently isolated bacteria, *Escherichia coli*, *Bacteroides melaninogenicus,* streptococci, staphylococci, *Pasteurella haemolytica*, *Bacillus* spp. and *Diphtheroides* spp. have been cultured [17,21]. The central role of *T. pyogenes* has been shown experimentally by inducing pyometra in postpartum cows either by inoculation of a monoculture or together with *F. necrophorum* and *B. melaninogenicus* [22,23]. By using next generation sequencing, we found bacteria belonging to the families *Fusobacteriaceae* (includes *F. necrophorum*), *Mycoplasmataceae*, *Bacteroidaceae*, *Porphyromonadaceae* and *Pasteurellaceae* in cases of bovine pyometra with *Fusobacteriaceae* as the most frequently found bacterium [24]. *Actinomycetaceae* and *Enterobacteriaceae*, which include *T. pyogenes* and *E. coli*, respectively, were however

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only a minor part of the operational taxonomic units [24].

Culturing and sequencing methods are useful to provide information on bacteria present in cases of pyometra. We have previously applied bacterial sequence analysis to the luminal content and endometrium of cows with pyometra [24], while other studies have focused only on the luminal content [17,20,24] and therefore not provided insight into the invasive properties of the bacteria present. Assessment of invasive properties is important as invasiveness may be associated with development of chronic endometrial lesions influencing the prognosis for fertility. Furthermore, treatment with antibiotics after restoration of cyclicity by $PGF_{2\alpha}$ may be more effective if targeting tissue invading bacteria.

Therefore, we used fluorescence *in situ* hybridization (FISH) to study the localization of bacteria in the stroma of the uterus and oviducts in cases of bovine pyometra and to determine to which extent *F. necrophorum*, *T. pyogenes* and *Porphyromonas levii* invade these structures.

2. Materials and methods

2.1. Animals

Reproductive tracts of dairy and beef cows with pyometra were collected at a slaughterhouse and sent to the University of Copenhagen for examination from August 2012 to August 2013. Cooling was initiated at the slaughterhouse shortly after slaughter and the organs were kept at 4 °C during storage and transportation. Inclusion criteria were: 1) the cow was slaughtered more than 21 days postpartum, 2) the uterus was distended with pus, 3) the cervical canal was closed and 4) a corpus luteum was present in one or both ovaries.

2.2. Sampling

Before opening the uterus, the serosal surface was wiped with 70% ethanol and flame sterilized at the incision point. A full thickness specimen of about 1 × 1 cm was taken from the dorsal aspect of the base of the left uterine horn using sterile forceps and a scalpel. The uterine sample and the entire oviducts were fixed in 10% neutral buffered formalin. An endometrial tissue sample (0.5 × 0.5 cm) and 1 mL of luminal exudate were also sampled and used for 16S rRNA polymerase chain reaction combined with next generation sequencing. These results have been published separately [24].

2.2.1. Histology

The formalin fixed specimens were trimmed and from the oviducts, the ampullae were isolated. The tissues were processed by routine methods, embedded in paraffin, sectioned at 3 μm and stained with hematoxylin and eosin. Selected specimens were also stained by Luna's method for eosinophilic granula.

2.2.2. FISH

Sections for FISH were mounted on SuperFrost®Plus slides (Menzel-Gläser, Germany), and 16S rRNA oligonucleotide probes targeting *F. necrophorum*, *P. levii*, *T. pyogenes* and the overall bacterial domain *Bacteria* (Eub-338) were used (Table 1). Positive and negative controls were included in each hybridization. Positive controls consisted of sections containing the specific bacterial species and processed with the respective probes, while negative controls were not processed with the probe.

The hybridizations were performed in Shandon hybridization racks (Thermo Scientific, UK), using 99 μL *in situ* hybridization buffer (1 M Tris (pH 7.2), 5 M NaCl, 10% SDS, H_2O) added 1 μL probe. The slides were incubated at 46 °C for 16–24 h, rinsed 3 × 3 min with *in situ* hybridization buffer and 3 × 3 min with *in situ* washing solution (1 M Tris (pH 7.2), 5 M NaCl, H_2O), and finally dipped in MilliQ water 10 to 15 times at room temperature.

The slides were mounted with Vectashield® (Vector Laboratories, INC. Burlingame, CA, USA), and evaluated using an Axiomager M1 epifluorescence microscope with a 100-W HBO lamp. Filter sets 43 and 38 were used to visualize Cy3 (excitation/emission peak at 552/570 nm) and FITC (excitation/emission peak at 495/520 nm), respectively. Images were obtained using an AxioCam MRm version 3 FireWiremonocrome camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

The slides were evaluated by one examiner (CCK) on the day of hybridization at ×400 magnification. The presence and the specific localization of bacteria within the tissue or on the surface of the mucosa were recorded.

2.3. Data management

Calving date, date of slaughter and parity were extracted from the Danish Cattle Data Base.

3. Results

3.1. Study population

Twenty-one cows were included in the study. Twenty reproductive tracts were examined within 48 h of slaughter whereas one tract was examined 96 h after slaughter. The left oviduct and ovary from one cow was lost during slaughter. The parity ranged from one to six, with a mean parity of 2.9. The cows were on average 290 days after parturition [range 26–1093 days]. One cow had been treated for endometritis (76 days before slaughter) and three others for retained fetal membranes (100, 112 and 462 days before slaughter). Details on treatment were not available.

3.2. Histology

3.2.1. Uterus

Most of the specimens had a single layer of columnar epithelium through which neutrophils migrated. Endometrial polyps (Fig. 1A) were present in 38.1% (8/21) of the specimens, and 47.6% (10/21) had endometrial ulceration characterized by loss of epithelium, deposits of fibrin and massive infiltration with neutrophils and mononuclear cells in the adjacent lamina propria. Ulceration with development of granulation tissue (Fig. 1B) was present in 19.0% (4/

Table 1
Oligonucleotide probes[a] used for locating bacteria in uterine and oviductal tissue in cows with pyometra.

Target bacteria	Name of the probe	Target sequence (5′-3′)	Target region	Fluorochromes	References
Domain *Bacteria*	S-D-Eub-338	5′ gctgcctcccgtaggagt 3′	16s	FITC	[25,26]
Fusobacterium necrophorum	S-S-F.necrop-183	5′ gattcctccatgcgaaaa 3′	16s	FITC	[27]
Porphyromonas levii	P.levii-443	5′ tacctacgtttactcgcc 3′	16s	Cy3	[28]
Trueperella pyogenes	S-S-A.pyogenes-a-A	5′ gcacataccgtcacaaaa 3′	16s	FITC	

[a] Eurofins MWG Operon, Ebersberg, Germany.