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Presence and localization of bacteria in the bovine endometrium postpartum using fluorescence *in situ* hybridization

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ABSTRACT

The aim of this study was to investigate bacterial invasiveness of the bovine endometrium during the postpartum period. Fluorescence *in situ* hybridization was applied to endometrial biopsies using probes for *Fusobacterium necrophorum*, *Porphyromonas levii*, *Trueperella pyogenes*, *Escherichia coli* and a probe for bacteria in general (the overall domain *Bacteria*) to determine their tissue localization. Holstein cows were sampled at three time points postpartum (T1: 4–12 days postpartum, T2: 24–32 days postpartum and T3: 46–54 days postpartum). At T1, cows were clinically scored as having a uterine infection based on presence of a brownish, fetid vaginal discharge or as normal if having normal lochia. An endometrial biopsy was taken from all cows at T1 (n = 57). Endometrial biopsies were taken from the same cows at T2 and T3 if allowed by the size of the cervical canal and if the cow had not been inseminated. Fifty and 39 biopsies were obtained at T2 and T3, respectively. The biopsies were evaluated for inflammation and for presence and localization of bacteria. When analyzed by the probe for the entire domain *Bacteria*, bacteria were found in most biopsies irrespectively of time (T1: 79.0%, T2: 82.0%, T3: 89.7%). *Fusobacterium necrophorum* and *Porphyromonas levii* were often present in the endometrium at T1 (61.1% and 47.8%, respectively), but the prevalence decreased significantly over time. *Trueperella pyogenes* and *Escherichia coli* were less prevalent at T1 (8.8% and 10.5%, respectively) and their prevalence also decreased significantly over time. *Fusobacterium necrophorum* and *Porphyromonas levii* were often co-localized intraepithelially or in the lamina propria. *Trueperella pyogenes* and *Escherichia coli* were located only on the endometrial surface. Due to the high prevalence of tissue invasiveness, these findings emphasize the importance of *Fusobacterium necrophorum* and *Porphyromonas levii* in postpartum uterine disease of cattle and indicate that tissue invasiveness is an important aspect of the pathogenesis.

1. Introduction

Uterine inflammation commonly develops in dairy cattle during the postpartum (pp) period and constitutes a complex of associated and partly overlapping disease entities such as puerperal metritis, clinical endometritis and subclinical endometritis [1]. The terms reflect how deep into the uterine tissue the inflammation has developed as endometritis refers to an inflammation of the mucosa (endometrium; epithelium and lamina propria), while metritis refers to an inflammation that involves the myometrium also [2]. Bovine pp metritis is generally regarded as a progression of an endometritis as it develops from the internal surface of the uterus [3]. Mostly, the depth of an acute inflammation of the uterine wall is reflected in the severity of the clinical signs, i.e. metritis is clinically more severe than endometritis [4].

A mixture of bacterial species invades the uterine lumen during the early pp period in most cows [5], but the cows are usually able to overcome such bacterial contamination of the uterine lumen without developing disease. However, in some cows, the contamination progresses into an infection. Bacteriological studies including both conventional culturing [6–10] and culture-independent methods [11–15] have shown that a wide range of bacteria can be found in the uterine lumen during the pp period. Opportunistic pathogenic bacteria such as *Fusobacterium*

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necrophorum, *Trueperella pyogenes*, *Escherichia coli* and *Bacteroides* spp. are frequently isolated from uterine lavage, swabs, and biopsies from pp cows [8–10,14–19]. These bacteria are considered of major importance in causing endometritis and metritis and although the causes of progression into disease are multiple, they involve the overall balance between the immune system and bacterial pathogens [20,21].

The role of each bacterial species in the development of pp uterine disease is mainly unknown and is difficult to determine as many factors may influence their pathogenicity. Such factors could be e.g. interaction between different species, quantities of each species, various strains, etc. All the different components are creating a meshwork of pathogen and host factors that finally determines the outcome of a pp uterine contamination. One way to investigate the pathogenic role of a bacterial species is to assess its ability to invade the uterine tissue. Tissue invasiveness is often regarded as a potent marker for pathogenicity, although not being definitive as some bacteria perpetrate their pathogenicity by other mechanisms such as producing toxins. The aim of the present study was to use fluorescence *in situ* hybridization (FISH) to investigate the invasiveness of *F. necrophorum*, *Porphyromonas levii*, *T. pyogenes* and *E. coli* in the endometrium of cows at different times pp.

2. Materials and methods

2.1. Study herd

The study was conducted in a Danish dairy herd with 1230 Holstein cows, located in north-eastern Jutland, with an average yield of 9700 kg energy corrected milk (ECM). The cows were kept in a loose housing system and fed total mixed ratio *ad libitum*. A total of 57 cows were included, with parity ranging from 1 to 5, and a calving date between April and July 2012. The herd was declared free from infection with bovine virus diarrhea virus based on quarterly bulk tank milk analyses. Based on the national cattle health status, the herd was also declared free from bovine brucellosis, infectious bovine rhinotracheitis and a wide range of other infectious diseases. The herd was considered free from bovine genital campylobacteriosis and trichomonosis, which have not been diagnosed in Denmark since 1995 and 1990, respectively [22].

2.2. Study design

The present cohort study was part of a larger project describing the microbiome of the bovine pp uterus [12]. All cows (n = 57) were sampled during days 4–12 pp (recruitment day, T1) and again at days 24–32 pp (T2) and days 46–54 pp (T3), if allowed by the diameter of the cervical canal or unless the cow had been inseminated (n = 7). Consequently only 50 and 39 cows were re-sampled at T2 and T3, respectively (Table 1). Thirty-five cows were sampled at all three time points, while three, 15 and four cows were sampled only at T1, T1/T2 and T1/T3, respectively. Recruitment of cows at T1 was done simultaneously with the herd veterinarian's (KMK) weekly routine examination of pp cows.

Table 1
Cows grouped according to vaginal discharge or uterine content characteristics at T1 and number of cows re-sampled at T2 and T3, with performed fluorescence *in situ* hybridization (FISH) examination.[a]

Discharge characteristics at T1	Number of cows included at		
	T1	T2	T3
Normal lochia	47	43	31
Brown and fetid	10	7	8
Total number of cows	57	50	39

[a] Due to the limited size of some of the biopsies, histopathological examination was not possible on all biopsies subjected to FISH. Sections for histology were available from 54, 49 and 36 biopsies at T1, T2 and T3, respectively.

Two groups of cows were included: 1) Cows (n = 10) with a presumed severe uterine infection based on the findings of a brownish, fetid vaginal discharge at T1 (uterine infection [UI] group) and 2) Cows (n = 47) with normal lochia selected at random among the other pp cows at T1 (normal puerperium [NP] group).

At T1 to T3, each cow underwent a thorough gynecological examination that included an inspection of the vulva, vaginal exploration, and transrectal palpation of the internal genital organs.

The study was approved by the Danish Animal Experiments Inspectorate (Permission no. 2011/561-90) and performed according to Danish legislation. The study was done with the consent of the herd owner.

2.3. Sampling

2.3.1. Biopsies

After careful washing of the vulvar labiae, perineum and surrounding skin with Lactacyd® (Sanofi-Aventis, Paris, France) and drying with a clean paper towel, a sterile divisible biopsy punch (Kruuse, Langeskov, Denmark) covered with a single use plastic chemise was inserted into the cervix whilst fixed by hand transrectally. The chemise was penetrated within the cervical canal, and the biopsy punch manipulated into the uterine body. The dorsal surface of the uterine body was transrectally gently pressed ventrally while the forceps was manipulated dorsally and an endometrial biopsy was taken from the dorsal part of the uterine body. The biopsy punch was retracted with the biopsy enclosed within the instrument. The biopsy punch was disinfected with 70% ethanol prior to opening and the biopsy was removed using sterile tweezers. The biopsy was divided into two; one half was fixed in 10% neutral buffered formalin while the other was put into RNAlater (Ambion, Austin, TX, USA). Biopsies in RNAlater were kept for DNA extraction and next generation sequencing, the results of which have been reported by Knudsen et al. [12].

2.3.2. Histology

Endometrial tissue for histology was fixed in 10% neutral buffered formalin, processed by standard histology methods and paraffin embedded. Sections of 3 μm were made and stained by hematoxylin and eosin (H&E). The biopsies were examined by one observer (CCK), blinded to the study groups. The level of inflammation was recorded as none, mild to moderate or severe and the cellular infiltrate characterized as being mainly neutrophils (representing suppurative inflammation), mainly mononuclear cells (representing non-suppurative inflammation) or an almost equal mixture of these. Fig. 1 provides examples of grading and inflammation.

2.3.3. FISH

Four 3 μm thick serial sections were mounted on SuperFrost® PLUS slides (Menzel-Gläser, Germany) and stored at 5 °C until hybridization. The slides were deparaffinized (2 × 3 min in xylene) followed by 2 × 3 min in 99% ethanol immediately before hybridization. Hybridization was 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 and incubated at 46 °C for 16–24 h. The slides were then 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 (around 21 °C), placed in a slide-cassette and dried at 46 °C for 1 h.

Probe details are shown in Table 2. Initially, a single section of each biopsy was hybridized with Eub-338 [23] targeting 16S rRNA