Relationship between bacteriological findings in the second and fourth weeks postpartum and uterine infection in dairy cows considering bacteriological results

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ABSTRACT

The uterine lumen in early postpartum dairy cows is contaminated with different bacteria. The most relevant uterine pathogens are *Escherichia coli* and *Trueperella pyogenes*. Prevalence of α -hemolytic streptococci and coagulase-negative staphylococci (CNS) is also high; however, these pathogens are considered opportunistic. The overall objective of this study was to investigate effects of the intrauterine presence of *E. coli*, *T. pyogenes*, α -hemolytic streptococci, or CNS at 10 ± 1 d in milk (DIM) on the type of bacteria 2 wk later and their influence on uterine infections and subsequent reproductive performance. Furthermore, we set out to quantify 2 relevant methodological factors (i.e., laboratory and sampling instrument). Bacteriological samples were collected at 10 ± 1 and 24 ± 1 DIM from the uterine lumen using a cytobrush (CB). Vaginal mucus was classified by vaginoscopy. In a subsample, bacteriological results of 3 different laboratories and of CB and cotton swabs (CS) were compared. Samples of uterine discharge were collected at 10 ± 1 DIM and bacteriological samples were taken using CB and CS. Bacteria were identified and bacterial growth quantified on a 4-point scale. Animals infected with *E. coli* or *T. pyogenes* at 10 ± 1 DIM had a higher risk for an infection with the same bacterial species at 24 ± 1 DIM [*E. coli* relative risk (RR) = 3.7 and *T. pyogenes* RR = 2.9]. Moreover, the risk of being diagnosed with abnormal vaginal discharge at 24 ± 1 DIM increased in cows with *E. coli* (RR = 1.7) or *T. pyogenes* (RR = 1.7) at 10 ± 1 DIM. Uterine infection with α -hemolytic streptococci or CNS did not increase the risk of an infection with *T. pyogenes* or *E. coli* or abnormal vaginal discharge 2 wk later. Cows with *E. coli* at 10 ± 1 DIM or *T. pyogenes* at 24 ± 1 DIM had greater days to first artificial insemination than cows positive for the 3 remaining bacterial species. Cows with *T. pyogenes* at 10 ± 1 DIM

had more days to pregnancy and more cows were culled when positive for *E. coli* at 10 ± 1 DIM. Agreement of bacteriological results of 3 different laboratories were significant for laboratory A + B and A + C for the CB and CS. The highest agreement considering the bacterial species was for *E. coli*. All results for laboratory A + B and A + C for the CB and CS were significant. The results generated from samples collected with CB agreed nicely with those from CS from each laboratory (laboratory A: 250/272; laboratory B: 264/272; laboratory C: 253/272).

Key words: dairy cow, bacteriology, uterine infection, cytobrush

INTRODUCTION

The uterine lumen in early postpartum dairy cows is commonly contaminated with different bacteria (Bondurant, 1999; Földi et al., 2006; Sheldon et al., 2008). Calving difficulties, retained fetal membranes, and manual manipulations increase the risk of uterine infection (Lewis, 1997; Sheldon and Dobson, 2004; Potter et al., 2010). Most cows eliminate the bacterial contamination in the first 2 wk after calving (Bondurant, 1999; LeBlanc et al., 2002). Persisting infections can lead to puerperal metritis (PM) or clinical endometritis (CE; Földi et al., 2006). Puerperal metritis is defined by an abnormally enlarged uterus and a fetid, watery, red-brown uterine discharge and, in the acute form, is associated with signs of systemic illness and fever $>39.5^{\circ}\text{C}$ within 21 DIM (Sheldon et al., 2006). Clinical endometritis can be defined as purulent uterine discharge detectable in the vagina after 21 DIM or mucopurulent discharge detectable in the vagina after 26 DIM (Sheldon et al., 2006). Bacterial uterine infections have been shown to negatively affect uterine and ovarian function and fertility (Miller et al., 2007; Williams et al., 2007; Kaneko and Kawakami, 2008). Bacterial species that cause PM or CE (Elliott et al., 1968; Bonnett et al., 1991; Petit et al., 2009) were classified according their expected pathogenic potential and arranged in a table based on recent studies by Williams et

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al. (2005) into recognized uterine pathogens associated with uterine endometrial lesions (*Trueperella pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum*, and *Prevotella melaninogenica*); potential pathogens frequently isolated from the bovine uterine lumen and cases of endometritis, but not commonly associated with uterine lesions; and opportunist contaminants transiently isolated from the uterine lumen and not associated with endometritis.

Although *E. coli* is widespread in the environment, specific *E. coli* strains from cows with uterine disease have been isolated (Sheldon et al., 2010). These endometrial pathogenic *E. coli* have been shown to be more adherent and invasive for endometrial epithelial and stromal cells compared with *E. coli* isolated from the uterus of clinically unaffected animals (Sheldon et al., 2010). Such pathogenic strains cause diseases of mucosal surfaces such as metritis or clinical endometritis in the bovine genital tract.

Trueperella pyogenes is often isolated from the uterine lumen of cows affected with PM or CE (Bonnett et al., 1991; Huszenicza et al., 1999) and synergistically affects the infection with gram-negative anaerobes such as *F. necrophorum* and *P. melaninogenica* (Williams et al., 2005; Földi et al., 2006; Sheldon et al., 2009b). The presence of *T. pyogenes* correlates with an increased risk for abnormal vaginal discharge or fetid odor (Williams et al., 2005) and elevated amount of PMNL in the epithelium and stratum compactum (Bonnett et al., 1991; Azawi et al., 2008).

Coagulase-negative staphylococci and α -hemolytic streptococci were frequently isolated in the first weeks postpartum from the uterine lumen of dairy cows (Williams et al., 2005). The presence of α -hemolytic streptococci was negatively correlated with the risk of abnormal vaginal mucus (Huszenicza et al., 1999; Williams et al., 2005) and both bacteria are considered to be opportunists. No information is available, however, about whether they have the potential to pave the way for infections with pathogenic bacteria later in the postpartum period.

In studies investigating uterine infections, cotton swabs (CS) were used for bacterial examinations of the vagina, cervix, or uterine lumen of cows (Williams et al., 2005; Azawi et al., 2008; Petit et al., 2009), bitches, or mares (Kasimanickam et al., 2005). A technique utilizing a cytobrush (CB) has been evaluated for the collection of cervical and endometrial cells in human medicine (Koonings et al., 1992) and was modified to collect endometrial cells in cows (Kasimanickam et al., 2005; Kaufmann et al., 2009; Plöntzke et al., 2011). This method has provided relevant insights on the association of the number of PMNL and reproductive

performance but was never validated for collection of bacteriological samples.

The overall objective of this study was to investigate relationships between *E. coli*, *T. pyogenes*, α -hemolytic streptococci, and CNS at 10 ± 1 DIM and their presence and effect on each other or uterine infections 2 wk later. Furthermore, we set out to evaluate 2 relevant methodological factors (i.e., laboratory and sampling instrument).

Specifically, we tested the following hypotheses: (1) an infection of the uterus with *T. pyogenes* or *E. coli* at 10 ± 1 DIM increases the risk of infection with the same pathogens and increases the risk for CE at 24 ± 1 DIM, (2) an infection of the uterus with α -hemolytic streptococci or CNS at 10 ± 1 DIM increases the risk of uterine infection and CE at 24 ± 1 DIM, (3) agreement of bacteriological findings of uterine samples is high between different laboratories, and (4) agreement of bacteriological findings is high between samples collected with CB and CS.

MATERIALS AND METHODS

Study Farm

The study was conducted between September 2009 and August 2010 on a commercial dairy farm in Brandenburg, Germany. The herd consisted of 1,162 Holstein dairy cows with an average milk production of 9,831 kg per lactation (4.04% fat and 3.31% protein). Cows were managed according to the guidelines set by the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (Hellmann and Radeloff, 2000). The main barn was positioned in a northeast-southwest orientation with open ventilation and mechanical fans. Lactating cows were housed in freestall facilities with cubicles, rubber mats, and slotted floors, whereas dry cows were straw bedded. Early postpartum cows were fed a TMR consisting of 43.2% concentrate and mineral mix, 35.9% grass silage, and 20.9% corn silage ($NE_L = 1.65$ Mcal/kg). Cows were milked 3 times per day (approximately at 0600, 1500, and 2200 h). After calving, cows were assigned to the postpartum group until 18 to 20 DIM and examined for clinical endometritis by vaginal inspection between 21 and 27 DIM. Cows were first inseminated by an AI technician after a voluntary waiting period of 40 DIM.

Study Design

Experiment 1. The farm was visited weekly to examine all cows at 10 ± 1 (exam 1) and 24 ± 1 DIM

(exam 2). Cows were excluded from the study if they were treated with antibiotic drugs or nonsteroidal or steroidal antiinflammatory drugs after parturition. A total of 269 cows were enrolled at exam 1.

At exam 1 and 2, body temperature was measured with a digital thermometer (Burfeind et al., 2010). Body condition score was assessed on a scale from 1 to 5 (Edmonson et al., 1989). Bacteriological samples of the endometrial surface were collected using the CB technique as described before (Kasimanickam et al., 2005). The CB (Cytobrush Plus GT; 20-mm length and 6-mm diameter; Leisegang Feinmechanik-Optik GmbH, Berlin, Germany) was covered by a one-way catheter, enveloped with a sanitary plastic sleeve, to protect it from contamination. After cleaning the vulva with dry cellulose wipes, the instrument was inserted via the cervix into the uterine body. Inside the uterus, the sleeve was retracted and the brush pushed gently into the lumen. The endometrial sample was taken by rolling the brush while in contact with the uterine wall. The brush was retracted into the catheter to protect it during the passage through the genital tract. Finally, the brush was stored in a transport medium (Uni-Ter Amies CLR; Meus S.r.l., Piove di Sacco, Italy) for bacteriological analysis. On every fourth visit, a CB was unwrapped, dipped into sterile NaCl, and stored in a transport medium as a negative control sample to analyze if the flora of the environment could influence the bacteriological results.

Vaginal examination was performed by vaginoscopy and assigned a vaginal discharge score (VDS) according to its distribution of clear mucus and pus. Depending on the time of examination postpartum, 2 different vaginal discharge scores were used. At the first examination, vaginal discharge was scored from 0 to 4 according to Sheldon et al. (2006): 0 = clear mucus; 1 = red-brown/yellowish, non-smelling discharge; 2 = mucus with pus <50%; 3 = mucus with pus ≥50%; and 4 = watery, reddish-brown, fetid discharge. Cows were considered as affected with PM if diagnosed with VDS 2 to 4. At the second examination, vaginal discharge was scored from 0 to 3 as described by LeBlanc et al. (2002): 0 = clear mucus; 1 = predominantly clear mucus containing pus flecks; 2 = mucopurulent mucus containing ≤50% pus; and 3 = exudate containing purulent or red-brown material or foul smelling, or both. Cows were regarded as affected with CE when diagnosed with VDS 1 to 3.

Bacteriological samples were cultured for aerobic bacteria on sheep blood agar and enriched in brain heart infusion (BHI) medium (Oxoid Ltd., Basingstoke, Hampshire, UK) and a standard nutrition I bouillon (Merck KGaA, Darmstadt, Germany) for 48 h at 37°C. The broths were streaked onto sheep blood agar plates

and cultured along with the other plates for a second time 24 h at 37°C. Bacteria were identified on the basis of the characteristics of the colony, Gram stain, morphology, hemolysis, biochemical profile, and other standard tests such as oxidase, catalase, and coagulase reaction and growth on MacConkey agar. Plates containing 1 or more colony-forming units were regarded as positive bacterial growth. Plates with more than 3 species or with mixed culture of moderate degree or higher were considered contaminated.

Experiment 2. Between April and August 2009, a subset of 68 cows was used to collect approximately 5 mL of uterine discharge at 10 ± 1 DIM. A disposable catheter enveloped with a sanitary plastic sleeve was inserted via the cervix into the uterus. After withdrawing the plastic sleeve, a sterile 20-mL syringe was placed on the catheter end and fluid aspirated into the catheter. The content was transferred in a sterile plastic tube to the laboratory of the Clinic for Animal Reproduction (Freie Universität Berlin, Germany). There, tubes were shaken using a vortex for 10 s and 4 CS and 3 CB were immersed into the uterine discharge and stored in transport medium (Uni-Ter Amies CLR; Meus S.r.l.). One CS and 1 CB each were sent to 3 different laboratories (laboratory A, B, and C) for bacteriological analysis. Laboratory A got 2 CS to test the intraassay repeatability of the results. All samples were labeled with consecutive numbers. Per-sample positive or negative findings of *E. coli*, *T. pyogenes*, α -hemolytic streptococci, and CNS were compared; consequently, 272 results per laboratory and sampling method and 68 results per bacterial species were compared.

Statistical Analysis

Results of the vaginal and cytological examination, body temperature, and BCS were recorded on case report forms on farm and transferred into a spreadsheet (Excel 2003; Microsoft Corp., Redmond, WA). Animal-specific data (parity and date of parturition) and treatment and reproductive-performance data (first insemination, last insemination, number of inseminations, outcome of pregnancy diagnosis, and date of culling) were obtained from the herd management software and electronically transferred into the spreadsheet.

Reproductive performance was characterized by median days to first AI (DFAI), pregnancy rate (number of cows pregnant after first AI divided by number of cows inseminated $\times 100$), days to pregnancy, proportion of cows pregnant within 250 DIM (number of cows documented to be pregnant at 250 DIM divided by number of cows enrolled $\times 100$), mean services per pregnancy, and proportion of cows culled within 250

DIM. Data were analyzed using SPSS for Windows (PASW Statistics 18.0; SPSS Inc., Munich, Germany).

Samples were considered bacteriologically positive when at least *T. pyogenes*, *E. coli*, α -hemolytic streptococci, or CNS were found. Relative risks (**RR**) with 95% confidence intervals were calculated by RR analysis and binary logistic regression for vaginal discharge at 10 ± 1 and 24 ± 1 DIM, respectively and detection of bacteria at 24 ± 1 DIM. Bacterial isolates from the same cow at first and second examination were compared. *Escherichia coli* was seen as the main pathogen if *T. pyogenes* was not isolated. For RR analysis between *E. coli* and vaginal discharge or bacterial isolates at 24 ± 1 DIM, samples positive for *T. pyogenes* were excluded as described previously (Westermann et al., 2010). For analysis of RR between findings of CNS or α -hemolytic streptococci and vaginal discharge or bacterial findings, samples positive for *T. pyogenes* or *E. coli* were also excluded. The effect of an intrauterine infection with the different bacterial species on days to first service and days to pregnancy were calculated with ANOVA.

Fisher's exact test was used to calculate different agreements between the sampling methods and laboratories: (1) agreements of bacteriological findings between CB and CS, (2) agreements of positive and negative findings overall and considering each bacterial species individually between the 3 laboratories, and (3) between both swab samples analyzed by laboratory A.

RESULTS

Experiment 1

Two hundred sixty-nine Holstein-Friesian cows were included in the study. A second sample could not be collected from 15 cows (5.6%) because the cows were culled or sold. Hence, a total of 254 cows were eligible for final analyses.

At the first examination *T. pyogenes*, *E. coli*, α -hemolytic streptococci, and CNS were isolated in 152 of 269 samples (56.5%). In 39.4% of the samples ($n = 106$) a mixed bacterial content or other species were isolated and 4.1% ($n = 11$) of the samples were bacteriologically negative. At the second examination, the number of bacteriologically negative findings increased to 30 (11.9%). *Trueperella pyogenes*, *E. coli*, α -hemolytic streptococci, and CNS were isolated in 72 of 254 (28.3%) samples. *Trueperella pyogenes*, *E. coli*, α -hemolytic streptococci, and CNS were found in 27 (10.7%), 22 (8.7%), 18 (7.1%), and 11 (4.3%) samples, respectively. Mixed cultures or other species were found in 59.7% ($n = 151$) samples (Table 1).

Escherichia coli at the first examination was isolated 61 times as the main pathogen: in 23 samples in monoculture, in 19 samples in combination with α -hemolytic streptococci, and in 19 samples in combination with mixed cultures (Table 2). Fifty-seven of those cows were reexamined at the second examination. *Escherichia coli* in monoculture was repeatedly isolated in 8 cows and

Table 1. Intrauterine bacteriological findings in Holstein dairy cows considering 2 sampling intervals

Item	Examination at DIM			
	10 ± 1 DIM (total no. = 269)		24 ± 1 DIM (total no. = 254)	
	n	%	n	%
Bacteriological findings	152	56.5	72	28.5
<i>Trueperella pyogenes</i> in monoculture	4		13	
With <i>Escherichia coli</i>	5		3	
With CNS or α -hemolytic streptococci	7		1	
With mixed or other flora	9		10	
Total	25	9.3	27	10.6
<i>E. coli</i> in monoculture	23		1	
With CNS or α -hemolytic streptococci	20		2	
With mixed or other flora	18		15	
Total ¹	66	24.5	22	8.7
α -Hemolytic streptococci in monoculture	32		11	
With mixed or other flora	26		5	
Total ¹	84	31.2	18	7.1
CNS in monoculture	3		4	
With mixed or other flora	5		6	
Total ¹	13	4.8	11	4.3

¹Combinations with bacteria that are listed before are not shown. Therefore, numbers of isolates do not sum up to the total number of positive samples for this species.

Table 2. Relationships of bacteriological findings between 10 ± 1 and 24 ± 1 DIM

Bacteriological findings at 10 ± 1 DIM	Bacteriological findings at 24 ± 1 DIM									
	<i>T. p.</i> ¹	<i>T. p.</i> + <i>E. coli</i> ²	<i>T. p.</i> + MO ³	<i>E. coli</i>	<i>E. coli</i> + α -h. s. ⁴	α -h. s.	CNS	MO	Clear	No second sample
<i>T. p.</i>	1							2		1
With <i>E. coli</i>	1		1			1		2		
With α -h. s. or CNS			1			1		3	1	1
With MO			2				1	4	1	1
<i>E. coli</i>	3	1		3		1		10	2	3
With α -h. s. or CNS	2	2		2	1	1		11	1	
With MO	1			3			2	9	2	1
α -h. s.	4		1			2		15	7	3
With MO	1			3		3	1	14	2	2
CNS			1			1			1	
With MO							2	2	1	

¹*T. p.* = *Trueperella pyogenes*.²*E. coli* = *Escherichia coli*.³MO = mixed flora or other bacteria.⁴ α -h. s. = α -hemolytic streptococci.

in 1 cow in combination with α -hemolytic streptococci. A new infection with *T. pyogenes* in monoculture or in combination with *E. coli* occurred in 9 cows.

Trueperella pyogenes was isolated in monoculture, in combination with *E. coli*, and in combination with α -hemolytic streptococci in 4, 5, and 7 of the 25 samples, respectively. At the second examination, 22 cows positive for *T. pyogenes* at exam 1 were reexamined and *T. pyogenes* was found in 6 cows.

Coagulase-negative staphylococci and α -hemolytic streptococci in monoculture were isolated in 58 and 8 cows, respectively. At the second examination *T. pyogenes* and *E. coli* were found in 7 and 3 cows, respectively (Table 2).

Cows infected with *T. pyogenes* had greater risk to be diagnosed with abnormal vaginal discharge at first (RR = 2.4, $P < 0.001$) and second examination (RR = 2.4, $P < 0.001$) than cows without an infection of *T. pyogenes*. The presence of α -hemolytic streptococci increased the risk of abnormal vaginal discharge at the first examination (RR = 2.0; $P < 0.001$). Cows infected

with *T. pyogenes* or *E. coli* at the first examination had higher risks ($P < 0.05$) to be diagnosed with VDS 1 to 3 two weeks later (Table 3). For those cows, the relative risk to be diagnosed with VDS 2 or 3 at the second examination was also higher (*T. pyogenes* RR = 2.7, $P < 0.001$; *E. coli* RR = 1.9, $P < 0.01$). Cows with an intrauterine infection of *T. pyogenes* or *E. coli* at first examination were at higher risk to be rediagnosed with the same bacterial species at the second examination (*T. pyogenes* RR = 2.9, *E. coli* RR = 3.7; Table 3). When α -hemolytic streptococci were present at the first examination, RR for an infection with identical bacterial species, *T. pyogenes* or *E. coli* (Table 3) at the second examination did not increase. Positive findings of CNS at exam 1 only increased the risk to be rediagnosed with the same bacterial species at exam 2.

Median DFAI for cows without *E. coli* or *T. pyogenes*, with *E. coli*, and with *T. pyogenes* were 59, 70 and 69, respectively (Table 4). Cows with *E. coli* at first or *T. pyogenes* at second examination had greater DFAI than cows positive for *T. pyogenes*, α -hemolytic strep-

Table 3. Relative risks with 95% CI of abnormal vaginal discharge and presence of identical bacterial species at 24 ± 1 DIM if the cow was positive for 1 of the 4 bacterial species at 10 ± 1 DIM

Pathogen identified at 10 ± 1 DIM	24 $\pm$ 1 DIM			
	Abnormal vaginal discharge		Identical bacterial species	
	Relative risk	95% CI	Relative risk	95% CI
<i>Escherichia coli</i>	1.7**	1.28–2.25	3.7**	1.67–8.18
<i>Trueperella pyogenes</i>	1.7*	1.20–2.37	2.9*	1.29–6.38
α -Hemolytic streptococci	1.4	1.02–1.84	1.8	0.74–4.40
CNS	0.3	0.05–1.87	10.3*	2.44–43.14

* $P < 0.05$; ** $P < 0.01$.

Table 4. Descriptive reproductive performance traits of 254 cows examined at 10 ± 1 DIM

Trait	Bacteriological findings at 10 ± 1 DIM		
	Negative ¹	<i>Escherichia coli</i> ²	<i>Trueperella pyogenes</i> ³
Number of cows	174	57	23
Cows inseminated (%)	92.5	91.2	91.3
Mean services per pregnancy	2.7	2.5	3.4
Median days to first AI	59	70 ^a	69
Pregnancy rate (%)	19.5	19.3	13.0
Median days to pregnancy	96	108	139 ^b
Cows pregnant (%)	71.8	64.9	65.2
Cows culled (%)	5.7	15.8 ^b	4.3

^{a,b}Values differ significantly (ANOVA) within a row (^a*P* < 0.001; ^b*P* < 0.05).
¹Cows without *T. pyogenes* or *E. coli*.
²Cows with *E. coli*.
³Cows with *T. pyogenes*.

tococci, or CNS at 10 ± 1 DIM (*P* < 0.001) or *E. coli*, α-hemolytic streptococci, or CNS at 24 ± 1 DIM (*P* < 0.001). Median days to pregnancy were significantly higher for cows with *T. pyogenes* at first examination compared with cows with *E. coli*, α-hemolytic streptococci, or CNS at 10 ± 1 DIM (*P* < 0.05). More cows were culled when identified with *E. coli* at 10 ± 1 DIM (Table 4) compared with cows with *T. pyogenes* or cows without *E. coli* or *T. pyogenes*.

Experiment 2

Agreements of bacteriological results of laboratory A + B and A + C were highly significant for CB and CS. Discrepancies between laboratory A + B and A + C were found when distinct bacterial species were considered. Agreement for *E. coli* was high (Table 5). Agreements for *T. pyogenes* and α-hemolytic streptococci were not significant of laboratory A + C from CS and CB, respectively (Table 5). Interlaboratory agreements between CB and CS were significant for all 3 laboratories as was intralaboratory repeatability for laboratory A.

DISCUSSION

Experiment 1

The objective of the present study was to examine the bacterial content in the uterus of postpartum dairy cows at 10 ± 1 DIM and 24 ± 1 DIM and their relationship with PM, CE, and subsequent reproductive performance. Furthermore, agreement of different laboratories and collection tools (i.e., CB and CS) were studied.

Overall, bacterial species isolated in experiment 1 were similar to those of previous reports (Williams et al., 2005; Földi et al., 2006). Samples were not cultured for anaerobe uterine pathogens such as *F. necrophorum* and *P. melaninogenica*. Hence, their effects cannot be discussed.

It has been shown that bacterial contamination of the uterus decreases with increasing days postpartum (Elliott et al., 1968). In our study, this observation could be confirmed for *E. coli* and α-hemolytic streptococci. *Escherichia coli* and *T. pyogenes* are relevant uterine pathogens and most frequently isolated (Huszenicza et al., 1999; Sheldon and Dobson, 2004). Their pathogenic

Table 5. Number of positive (++) and negative (–) agreements from 68 samples and disagreements (+–) of each bacterial species for cytobrushes and cotton swabs from laboratory A + B and A + C

Bacterial species	Cytobrush						Cotton swab					
	Laboratory A + B			Laboratory A + C			Laboratory A + B			Laboratory A + C		
	++	–	+–	++	–	+–	++	–	+–	++	–	+–
<i>Escherichia coli</i>	21	40	7	17	39	12	20	47	1	8	52	8
<i>Trueperella pyogenes</i>	4	51	13 ^a	2	62	4 ^a	7	53	8 ^a	0	59	9 ^b
α-Hemolytic streptococci	23	28	17 ^a	12	25	31 ^b	10	48	10 ^a	20	28	20 ^a

^{a,b}Values within a column with different superscripts differ (*P* < 0.01).

potential was the focus of various studies, describing their pathomechanism, as well as effect on mucus character, ovarian activity, and fertility (Huszenicza et al., 1999; Williams et al., 2005; Sheldon et al., 2010).

Cows enrolled in this study did not show any signs of systemic illness and remained untreated throughout the whole observation period. Prevalence rates for *E. coli* were 24.5 and 8.7% at first and second examination, respectively. Fifty-nine percent of samples (36/61) positive with *E. coli* showed high growth density (**HGD**; i.e., >100 colonies per plate) according to Williams et al. (2005). Infections with *E. coli* at 10 ± 1 DIM increased considerably the risk for persistent infections, CE, or new infections with *T. pyogenes*, despite the observation that 49 of 61 cows cleared their infection with *E. coli* between both examinations. The high risk for persistent infections can be explained by *E. coli* strains specifically pathogenic for the uterus (Sheldon et al., 2010). It has also been assumed that an infection with *E. coli* increases the susceptibility of the endometrium to an infection with *T. pyogenes* (Williams et al., 2007), induced by a functional depression of neutrophils (Zerbe et al., 2001). Our data support this assumption, as *T. pyogenes* appeared as a consequence of an infection with *E. coli* (RR = 1.8, $P > 0.1$) but not vice versa.

It is well documented that the presence and character of vaginal discharge has diagnostic value to identify cows with clinical endometritis (LeBlanc et al., 2002; Williams et al., 2005; Sheldon et al., 2006). We examined the relationship between bacteriological findings in the uterus and vaginal discharge at the same day and 2 wk later to provide further evidence for the relevance of VDS. We did not find an effect of *E. coli* on vaginal discharge on the same examination day as described previously (Williams et al., 2005; Westermann et al., 2010). However, the higher risk for abnormal vaginal discharge 2 wk after an infection with *E. coli* has not been described yet. We speculate that this was due to the pathogenic characteristics of *E. coli* to predispose the endometrium to other bacteria (Williams et al., 2007). This assumption was confirmed by the prevalence of *T. pyogenes*, which was higher in cows that had been infected with *E. coli* at 10 ± 1 DIM. To describe relationships between bacteria and vaginal discharge, we considered the definition of LeBlanc et al. (2002) for CE. They did not find an association between mucus with flecks of pus and reduced pregnancy rate and, therefore, excluded cows with only slight alterations from the case definition. Clinical endometritis was defined as the presence of purulent or foul discharge between 20 and 33 DIM or mucopurulent discharge after 26 DIM. In our study, we found a higher risk for

VDS 1 to 3 in cows infected with *E. coli* at 10 ± 1 DIM compared with cows not infected with *E. coli*. When we excluded VDS 1, as suggested, the risk for VDS 2 or 3 and, consequently, the risk for CE at exam 2 increased. The higher numbers of cows culled and greater DFAI for animals infected with *E. coli* compared with cows infected with *T. pyogenes* or without infection of *E. coli* or *T. pyogenes* can be explained by the damage of the endometrium as postulated previously (Zerbe et al., 2001).

Huszenicza et al. (1999) compared prevalence of bacterial isolates from cows with acute puerperal (endo)metritis, which were treated after the first examination with intrauterine antibiotic drugs (i.e., oxytetracycline), with the prevalence in healthy cows. They observed higher prevalence rates of *T. pyogenes* in cows with acute puerperal (endo)metritis compared with healthy cows and a negative association between long-term presence of *T. pyogenes* and reproductive performance. In our study, prevalence of *T. pyogenes* and percentage of samples with HGD was similar at 10 ± 1 (HGD of 20/25; 80.0%) and 24 ± 1 DIM (HGD of 20/27; 74.1%). However, not all cows infected at exam 1 were rediagnosed at exam 2, but the risk for a persistent infection with *T. pyogenes* was significant. Severe endometrial lesions caused by *T. pyogenes* (Bonnett et al., 1991; Azawi et al., 2008) and increased susceptibility of the endometrium for this pathogen due to previous *E. coli* infections (Zerbe et al., 2001; Williams et al., 2007) may contribute to the persistent infection seen in our study. The association between *T. pyogenes* and abnormal vaginal discharge demonstrated in our data confirms earlier reports (Huszenicza et al., 1999; Williams et al., 2005; Westermann et al., 2010). The high risk for abnormal vaginal discharge regardless of the examination day and the high risk of persistent infection in cows infected with *T. pyogenes* 10 ± 1 DIM explain the high risk for VDS 1 to 3 at 24 ± 1 DIM. After exclusion of mild discharge, the risk increased for VDS 2 or 3, indicative for CE, at 24 ± 1 DIM after an infection with *T. pyogenes* at 10 ± 1 . Days to pregnancy in our study were greater for cows diagnosed with *T. pyogenes* at exam 1 compared with cows infected with *E. coli* or without infection of *E. coli* or *T. pyogenes*. Cows with *T. pyogenes* at exam 2 had significantly higher DFAI than cows without *T. pyogenes*. Both effects on reproductive performance have been described for *T. pyogenes* (Williams et al., 2005) and, in general, for cows with CE (LeBlanc et al., 2002). This, however, is the first report considering infections at specified DIM intervals. In recent studies, uterine samples were taken during a relatively long window (20 to 33 DIM), which might have biased the results.

Coagulase-negative staphylococci and α -hemolytic streptococci were included in our experiment because prevalence has been reported to be high in the uterus (Williams et al., 2005; Sheldon et al., 2009a). In the study of Huszenicza et al. (1999), prevalence of staphylococci and streptococci at 10 DIM was higher in cows without uterine complications compared with cows with acute puerperal (endo)metritis. These bacteria have been considered as normal uterine flora (Huszenicza et al., 1999) or opportunists (Williams et al., 2005), respectively. In studies investigating risk factors for mastitis pathogenicity of CNS varies from being protective to being the cause of subclinical and clinical mastitis (De Vliegher et al., 2012). Furthermore, a significantly lower risk for abnormal vaginal discharge or no association at all between fetid vaginal mucus odor and opportunist contaminants was reported (Williams et al., 2005). We examined the effect of α -hemolytic streptococci and CNS on vaginal mucus and on aerobic bacterial prevalence at 2 distinct times postpartum. Coagulase-negative staphylococci were isolated in only 8 of 269 samples at exam 1. Due to the low prevalence, a conclusion about their pathogenic potential is not possible. The presence of α -hemolytic streptococci was only associated with an increased risk for abnormal vaginal discharge at exam 1. We did not reconfirm this relationship at 24 ± 1 DIM as described by Williams et al. (2005). Their and our data are inconclusive regarding their role as opportunists. Overall, data do not provide consistent evidence for a preventive effect of α -hemolytic streptococci.

Our first hypothesis, that an infection of the uterus with *E. coli* or *T. pyogenes* increases the risk for a persistent infection with identical bacterial species and CE can be confirmed. As described earlier, the terminology CE may not be fully appropriate, as vaginal discharge is not an accurate descriptor for the location (vagina, cervix, or uterus) of the inflammation (Dubuc et al., 2010a,b). The second hypothesis can neither be proven nor rejected because of insufficient animals with CNS and no significant risk for *E. coli* or *T. pyogenes* resulting from an infection with α -hemolytic streptococci.

Other bacterial species or mixed cultures with more than 3 bacterial species were isolated at first and second examination in 39.4 and 59.7% of examined cows, respectively. We speculate that contamination with environmental bacteria occurred during sampling or rolling the CB on the microscopic slide. None of the 11 negative control samples, however, showed a contamination with *E. coli*, *T. pyogenes*, or CNS. Mixed cultures were isolated in 4 samples, 1 of which with 1 *Acinetobacter* species and 1 sample that was positive for α -hemolytic streptococci.

Experiment 2

To determine interlaboratory repeatability of bacteriological findings, samples (e.g., agar slants, water, and waste) with known types of bacteria and colony-forming units were used in previous studies (Wallmann et al., 2006; Kalbe et al., 2007). In our samples, the types and load of bacterial species were unknown and a gold standard did not exist. Our objective was to test the agreement of the bacteriological examinations under field conditions for uterine samples. The agreement between CB and CS between the laboratories was good. Laboratory A and B showed the highest agreement for CB (234 of 272) and CS (252 of 272). Differences between laboratory A and C might be due to different storage conditions of the samples before culture. Samples for laboratory A and B were stored for approximately 14 h at $+7^{\circ}\text{C}$ until cultivation. Samples for laboratory C were shipped unchilled for the same time. Additionally, variations in culture conditions and differentiation procedures of the laboratories may have influenced the bacteriological results. For *E. coli*, agreements of bacteriological results from 68 samples were high, regardless of laboratory or sampling method (CB: laboratory A + B: 61/68, laboratory A + C: 56/68; CS: laboratory A + B: 67/68, laboratory A + C: 60/68). The agreements of laboratory A and C for *T. pyogenes* from CB (59 of 68) and α -hemolytic streptococci from CS (37 of 68) were not significant (Table 5). We assume that the lack of cooling during the transport to laboratory C might have affected agreement. It is known that temperature, time, and type of swab (Roelofsen et al., 1999), media (Yrios et al., 1975), and transport system (Rishmawi et al., 2007; Stoner et al., 2008; Musser and Gonzalez, 2011) affect viability and stability of microorganisms. Effects of our transport medium on our 4 target pathogens have not been examined yet. *Escherichia coli* strains were stable at 4°C up to 96 h, but concentrations increased at room temperature (24°C) at 24 h (Rishmawi et al., 2007; Stoner et al., 2008). Additional storage at -80°C for 40 d or 1 h at room temperature had no significant effect on growth scores of *E. coli* (Musser and Gonzalez, 2011). These results indicate that *E. coli* can be isolated more easily from unchilled than from cooled samples but increase the risk of overgrowing fastidious organisms (Rishmawi et al., 2007).

Recent studies describing uterine bacterial contents have used guarded CS or biopsy methods (Sheldon et al., 2002; Williams et al., 2005; Petit et al., 2009). To diagnose subclinical endometritis, the CB technique has been described and validated (Kasimanickam et al., 2005). More recently this technique has been used

to harvest endometrial cells and bacteria from the uterine lumen simultaneously (Westermann et al., 2010). The advantage of this method is to obtain information about the inflammatory and bacteriological status with only one sampling procedure. Admittedly, the rate of bacteriologically positive samples from CS was higher, but the agreement between both sampling methods was high for all laboratories ($P < 0.001$). Because a gold standard does not exist, it is impossible to prove which results are more accurate. Overall, we conclude that the results from CB are meaningful for bacteriological analysis and consider using CB a valuable technique to generate information about infectious and inflammatory properties in the endometrium with 1 sample.

CONCLUSIONS

Our results demonstrate that cows infected with *E. coli* or *T. pyogenes* at 10 ± 1 DIM are prone to persistent infections with the same bacterial species and abnormal vaginal discharge 2 wk later. Accordingly, the risk to be diagnosed with clinical endometritis increases as well. Contrary to our second hypothesis, we did not find relationships between α -hemolytic streptococci or CNS and infections with the same bacterial species, *E. coli*, *T. pyogenes*, or abnormal vaginal discharge at the second examination, respectively. Cows positive for α -hemolytic streptococci had a higher risk for abnormal vaginal discharge at 10 ± 1 DIM. Further research is warranted to elucidate the role of CNS and α -hemolytic streptococci as opportunistic or pathogenic microorganisms. For pathogens examined, laboratories showed high agreements for CB and CS. The agreements between CB and CS of each laboratory and intralaboratory results were highly significant.

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