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The prevalence of subclinical endometritis and intrauterine infections in repeat breeder cows

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ABSTRACT

The objectives of this study were to assess the prevalence of subclinical endometritis and the presence of common uterine pathogens in repeat breeder cows. A total of 121 cows with three or more consecutive artificial inseminations without conception and no clinical signs of disease were defined as repeat breeder cows and were enrolled in this trial. Intrauterine samples were collected with the cytobrush technique to determine the prevalence of subclinical endometritis and bacteriologic infections. Blood samples were analyzed for concentrations of progesterone and estradiol in plasma to assess ovarian activity. Furthermore, breed, parity, history of calving and postpartum uterine infection, clinical findings of transrectal palpation, and backfat thickness were analyzed as potential factors for the prevalence of subclinical endometritis in repeat breeder cows. The prevalence of subclinical endometritis in repeat breeder cows was 12.7%; but common uterine pathogens, *Escherichia coli* and *Trueperella pyogenes*, were found in only one and three cows, respectively. Ovarian activity was determined in 95.0% of all cows. Recorded variables had no effect on the prevalence of subclinical endometritis in repeat breeder cows. In conclusion, subclinical endometritis and uterine infections linked to common pathogens were playing a minor role as a cause for repeat breeder cows in this study. Alternative reasons for failure to conceive in these cows are discussed.

1. Introduction

Good fertility of dairy cows is the key to economically successful dairy farming. It is generally accepted that uterine disorders in the postpartum period have a negative impact on reproductive performance. In the past decades, the knowledge of the pathophysiology of clinical disorders, e.g., metritis, endometritis, and subclinical endometritis (SE) has increased significantly [1–3]. Short- and long-term effects of these diseases on fertility have been described [4,5]. After the postpartum period, repeat breeding is considered one of the most important reproductive disorders in cattle [6]. Repeat breeder cows (RBCs) are defined as cows with regular cycles of 17 to 25 days and with three or more artificial inseminations (AIs) without conception [7,8]. Recently reported mean prevalence of repeat breeding ranges from 10% to 14% in dairy cows [6,9].

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Although several causes of repeat breeding have been described, e.g., inadequate estrus detection [10], prolonged estrus, delayed LH peak, and late postovulatory rise in plasma progesterone [11–13], infections [14], and genetic factors [11], the particular reason often remains speculative. With a new diagnostic technique in endometrial cytology, more information can be obtained about these cows. The cytobrush technique has been established as a diagnostic method to detect SE in cows with no signs of clinical endometritis [15,16]. The proportion of polymorphonuclear neutrophils (PMN) in the total number of endometrial cells is indicative for SE. Different thresholds of PMN for defining SE have been used, ranging from ≥5% [17] to greater than 18% [15]. Some studies have reported the negative impact of SE diagnosed in the postpartum period on fertility [4], whereas others did not confirm these results [17].

In RBCs, one study reported a prevalence of 52.7% SE, and it has been hypothesized that SE is one of the main reasons for cows to become a repeat breeder [18]. Information about intrauterine pathogens in RBCs is rare. It has been assumed that bacteriologic findings are coincidental and not responsible for the failure of cows to conceive [19].

Beside an affected endometrium as a possible reason for failure to conceive, RBCs may also be associated with impaired function of the ovaries [11–13]. The different aspects of anestrus cows have been described by [20]. Although RBCs are defined as cyclic cows [8], ovarian activity of these cows at the time of insemination is often unknown.

Therefore, the objectives of this study were to describe the prevalence of SE and the presence of common uterine pathogens in the RBC to assess whether RBCs are affected by SE, uterine pathogens or both. Such information would be valuable for discussing prevention and treatment strategies for RBC.

2. Materials and methods

2.1. Animals, examinations, and recorded data

This study was approved by the institutional ethics committee and the national authority according to §8 of Law for Animal Experiments, Tierversuchsgesetz-TVG (BMWF-68.205/0137-II/3b/2011).

During the study period from October 2011 to February 2012, RBCs from 40 commercial dairy farms in Austria were enrolled. Herd size ranged from 20 to 218 cows and average 305-day milk production was 5875 to 11,900 kg. Cows were housed in free stalls (n = 38) and tie stalls (n = 2). Rations on all farms were based on corn silage, grass silage, hay and supplemented with concentrates and minerals. All cows enrolled in this study were bred by AI; no bulls were used in the herds. Repeat breeder cows were defined as cows with three or more consecutive AIs without conception. Cows were selected during regular herd visits from the farmers' herd records (≥3 inseminations). Exclusion criteria were a history of hysterotomy, fetotomy, or severe injuries at parturition. All cows eligible were examined by vaginal examination using a metal speculum and a torch, and by transrectal ultrasonographic assessment of the ovaries (5-MHz linear-array transducer; Easi-Scan, BCF Technology Ltd., Bellshill, Scotland). Ultrasonography was used to identify ovarian activity by the presence of a CL, a follicle, or both. Only cows with no clinical signs of uterine disorders, i.e., endometritis and no abnormalities of the ovaries, i.e., ovarian cysts, were enrolled. Furthermore, blood samples were collected from the coccygeal vein and analyzed for concentrations of progesterone and estrogens.

To assess the body condition of the cows, backfat thickness (BFT) was measured by ultrasound, as described by Schröder and Staufenbiel [21]. Backfat thickness was categorized into underconditioned (<15 mm), normal (16–25 mm), and overconditioned (≥26 mm). Furthermore, breed, parity, history of calving assistance and postpartum uterine disorders (metritis or endometritis), interval from calving to first service, and number of AI were recorded. Metritis was defined as inflammation of the uterus within the first 3 weeks postpartum with the presence of vaginal discharge and systemic signs of illness (rectal temperature, ≥39.5 °C), and endometritis as inflammation of the endometrium later in lactation with vaginal discharge but without systemic signs.

2.2. Uterine cytology, bacteriology, and hormone analyses

For the cytologic and bacteriologic examination, two endometrial samples from each RBC were collected with the cytobrush technique as described [15]. In brief, a cytobrush (Gynobrush; Heinz Herenz, Hamburg, Germany) screwed on a metal rod and protected by a plastic catheter, and a plastic sleeve was inserted into the uterine cavity. The plastic sleeve was drawn back, and the brush was rolled along the endometrium.

The cytologic slides were prepared by rolling the brush onto a clean glass microscope slide and dried. Slides were fixed and stained (Hemacolor Rapid staining; Merck, Darmstadt, Germany) in a laboratory as described [22,23]. The percentage of PMN was determined by counting 300 cells under a microscope at magnification × 400 (Olympus CX21; Olympus Corporation, Tokyo, Japan). A proportion of 5% PMN or greater was defined as threshold for SE [24,25]. Because the threshold for SE has been described for postpartum cows, but not for RBC, an additional category for PMN greater than 0% to less than 5% was implemented, resulting in three categories, i.e., 0%, greater than 0% to less than 5%, and 5% or greater.

The second endometrial sample for bacteriologic examination was stored in a tube with PBS buffer solution and transported to the laboratory within 12 hours. The brushes were streaked onto agar plates (Columbia Sheep Blood Agar; MacConkey, Hampshire, UK) and incubated at 37 °C for 48 hours under aerobic conditions. Bacteria were isolated and cultivated on Tryptone Soya Agar under standardized conditions (37 °C, 48 hours; for details see [26]), followed by the Fourier transform infrared (FTIR) spectroscopy classification as described previously [27,28].

Blood samples from the coccygeal vein were collected into VACUETTE tubes (Greiner bio-one, Kremsmünster, Austria) with lithium heparin as an anticoagulant. Tubes were centrifuged at 3000 rpm for 5 minutes, and plasma was stored at −18 °C until analyses. Analyses of plasma

progesterone and estradiol concentrations were performed with the validated solid-phase immunoassay method (Progesterone ELISA kit, Catalog No. ADE-900–011; 96-well kit; Enzo Life Sciences Inc., NY, USA and Estradiol ELISA Kit DE2693; Demeditec Diagnostics GmbH, Kiel, Germany). The sensitivities were 0.007 ng/mL for progesterone and 9.01 pg/mL for estradiol. The intra-assay coefficient of variation was 5.4% and 6.8%, and the interassay coefficient of variation was 8.3% and 7.3% for progesterone and estradiol, respectively. A threshold of 1.0 ng/mL of progesterone was set as indicative for the presence of an active CL [29]. Furthermore, an estradiol concentration of 20.0 pg/mL or greater was defined to indicate the presence of follicles. This threshold is higher than that in other studies [29,30] but was used to avoid false-positive findings of a follicle. The presence of a follicle or CL was additionally confirmed by transrectal ultrasonography.

2.3. Statistical analysis

Data were analyzed with a statistical software program (SPSS version 20; IBM Corporation, NY, USA). Numerical data were tested for normality of distribution by using the Kolmogorov–Smirnov test. Effects on the prevalence of SE in RBCs were analyzed by using a binary logistic regression model, including breed, parity (heifers, primiparous, multiparous), BFT (underconditioned, normal, overconditioned), calving assistance (no assistance, moderate assistance = 1–2 persons, severe assistance = >2 persons), and history of postpartum uterine disorder (yes, no) as variables. Odds ratio and 95% confidence intervals were calculated for all variables in the model. Level of significance was set at $P = 0.05$.

3. Results

3.1. Descriptive statistics

A total of 121 cows met the inclusion criteria for RBC and were enrolled in this study. Study population consisted of 77.7% dual-purpose Austrian Simmental cows, 9.8% Holstein Friesian, 8.0% Brown Swiss, and 4.5% others. Mean milk production of RBCs that finished a 305-day lactation (n = 60) was 8259 kg (range, 4456–13,495 kg). The distribution of parity was 6.1% heifers, 14.1% primiparous, and 79.8% multiparous cows. Average days from parturition to the first AI were 76 (range, 23–397), and the mean number of AI was 4.8 (range, 3–14). The mean interval from parturition to the day of sampling was 271 ± 117 (minimum 89) days in milk (DIM). The majority of RBCs was underconditioned (75%), followed by normal body condition and overconditioned cows (17% and 8%, respectively). Calving assistance was documented in 32% of the cases as moderate, in 2% as severe, and in 66%, no calving assistance was reported. A history of postpartum uterine disorders was reported in 9.9% of the RBC.

3.2. Cytology, bacteriology, and hormone analyses

A total of 110 cytologic samples were examined; 11 samples were not analyzed because of the poor quality of the smears. The proportion of cows diagnosed with SE was 12.7% (11 Austrian Simmental cows, 1 Holstein, 1 Brown Swiss, and 1 other breed). Cows diagnosed with SE were multiparous in 92.9% and primiparous in 7.1%. The percentage of samples categorized into 0%, greater than 0% to less than 5%, and 5% or greater PMN is shown in Table 1. The logistic regression model revealed no effect of breed, parity, BFT, history of calving assistance, or postpartum uterine diseases on the prevalence of SE in the RBC (Table 2).

Bacteria of the phyla actinobacteria, firmicutes, and proteobacteria were isolated. The results from bacteriologic analyses on genus level by means of FTIR spectroscopy are shown in Table 3. In 47.1% of the samples, no bacteria were detected. *Trueperella pyogenes* was found in only one sample (0.8%), *Escherichia coli* in only three of the samples (2.5%), *Corynebacterium* spp., *Streptococcus* spp., and *Staphylococcus* spp. were found in 20.7%, 19.0%, and 14.9% of the samples, respectively (Table 3).

Plasma concentrations of progesterone and estradiol averaged 2.0 ± 2.4 ng/mL and 49.6 ± 45.1 pg/mL, respectively. Using the described thresholds for progesterone and estradiol and combined with the ultrasonographic assessments of the ovaries, results indicated in 95% of the RBC ovarian activity.

4. Discussion

The objectives of this study were to describe the prevalence of SE and intrauterine infection in RBCs. Although several studies reported a prevalence of SE at the end of the postpartum period ranging from 14% to 52% [4,17,31], only little information exists about SE later in lactation or in the RBC. Salasel et al. [18] reported a proportion of 53% SE in the RBC. Although study design and the number of cows were similar, these findings did not match the results from our present study. The threshold of PMN for the diagnosis of SE, however, was 3% in the study by Salasel et al. [18], whereas we used 5%. It should be noticed that 48.2% of the samples were in the category greater than 0% to less than 5% PMN and 39.1% with 0% PMN. This study, however, was not designed to define a threshold for SE in the RBC. For that, successfully bred control cows but with similar days open as the RBC would be necessary. It is almost not possible to conduct such a trial with postponing breeding for more than 200 days on a commercial dairy farm (in the present study 271 DIM [mean] at enrollment). Mean DIM at enrollment and a lower proportion of cows with a history of uterine diseases (9.9% vs. 31%) could also explain the differences in the prevalence of SE in our study compared with the study by Salasel et al. [18]. The hypothesis of this study was that a great proportion of RBC is affected by SE,

Table 1
Proportion of categorized polymorphonuclear neutrophils (PMN; %) in cytologic samples (n = 110) of repeat breeder cows.

Category for proportion of PMN	n	%
0%	43	39.1
>0%–<5%	53	48.2
≥5%	14	12.7
Total	110	100

Table 2
Effect of variables included in a binary logistic regression model on the prevalence of subclinical endometritis in repeat breeder cows (n = 121).

Variable[a]	OR	95% CI	P value
Breed	1.37	0.49–3.79	0.549
Parity	0.93	0.69–1.26	0.643
BFT	2.02	0.35–11.63	0.433
Calving	1.11	0.24–5.17	0.897
Uterine disorders pp	3.36	0.47–23.89	0.226

Abbreviations: BFT, backfat thickness; CI, confidence interval; pp, postpartum; OR, odds ratio.

[a] Variable: Breed (reference = Simmental, 1 = Holstein Friesian, 2 = Brown Swiss, 3 = others), parity (reference = primiparous, 1 = multiparous, 2 = heifers), BFT (reference = ≤15 mm, 1 = 16–25 mm, 2 = ≥26 mm), calving assistance (reference = no assistance, 1 = moderate assistance, 2 = severe assistance), and uterine disorders pp (reference = yes, 1 = no).

as described by Salasel et al. [18]. Therefore, risk factors for SE in RBCs were analyzed, but no significant interactions were found. A reason for this might be the low proportion of cows with SE in this study. The definition of SE in this study was based on the percentage of PMN in endometrial smears. It cannot be excluded that analyzing additional immune cells, such as macrophages, would provide more information about inflammatory reactions in the endometrium. Uterine cytology might be used in future studies also to elucidate immune status and inflammatory response of the endometrium in RBCs. Molecular biological analyses of samples obtained from the endometrium of cows with SE or subfertile cows have shown an increased messenger RNA expression of inflammatory factors, for example cytokines, interleukins, and TNFα [32–34], and changes in expression patterns of genes involved in immunomodulation [35].

Table 3
Intrauterine bacteriologic findings in repeat breeder cows (n = 121).

Phylum	Genus	n	%
Actinobacteria	*Agrococcus*	1	0.8
	Brachybacterium	1	0.8
	Brevibacterium	3	2.5
	Cellulomonas	1	0.8
	Cellulosimicrobium	1	0.8
	Corynebacterium	25	20.7
	Knoellia	1	0.8
	Microbacterium	1	0.8
	Micrococcus	5	4.1
	Trueperella	1	0.8
Firmicutes	*Aerococcus*	5	4.1
	Bacillus	19	15.7
	Enterococcus	4	3.3
	Facklamia	3	2.5
	Globicatella	7	5.8
	Lactococcus	3	2.5
	Lysinibacillus	1	0.8
	Staphylococcus	18	14.9
	Streptococcus	23	19.0
	Vagococcus	1	0.8
Proteobacteria	*Acinetobacter*	4	3.3
	Citrobacter	3	2.5
	Escherichia	3	2.5
	Moraxella	1	0.8
Total number of isolates[a]		136	
Bacteriologic negative		57	47.1

[a] Number includes multiple findings in samples.

To decipher a potential role of uterine pathogens in the RBC, bacteria were isolated from the endometrium and identified by means of FTIR spectroscopy. Fourier transform infrared spectroscopy is a vibrational spectroscopic technique with high-resolution power that is able to distinguish microbial cells at different taxonomic levels [24]. It was recently shown to be a suitable tool for the identification and discrimination of bacteria from the bovine uterus [23,26] and thus allows the comparison of the results of the present study with former ones. In cows with clinical endometritis, *T pyogenes* is regarded as one of the most important pathogens [36,37], associated with impaired reproductive performance [1]. Also in cows with SE, *T pyogenes* is a frequently isolated pathogen [38]. Furthermore, an increase in PMN is associated with the presence of *T pyogenes* but with no other uterine pathogens, as for instance *E coli* [22]. In the present study, however, *T pyogenes* was detected in only one of the enrolled RBC, but other uterine bacteria were detected, such as *Staphylococcus* spp. and *Streptococcus* spp. The latter bacterial species are frequently isolated from the bovine reproductive tract and are supposed to be opportunist contaminants [37]. In addition, there is evidence that bacteriologic findings are coincidental in the RBC [19]. Recent publications by Werner et al. [39] and Sens and Heuwieser [38], however, discussed the role of α-hemolytic streptococci as a potential uterine pathogen.

Besides diseases of the uterus, repeat breeding may also be caused by alterations of ovarian hormone profiles, resulting in prolonged duration of estrus, delayed LH peak, and late postovulatory rise in plasma progesterone [11,13]. Although concentrations of plasma progesterone and estradiol in the present study reported cyclicity in 95% of the RBC, it remains speculative if these cows ovulated in time. Further studies should focus on the increase in concentrations of estrogen, LH pulsatility, time to ovulation, and hormone profiles at the time of expected implantation of the embryo.

In this study, cows were enrolled as RBCs at regular herd visits from 40 different farms. Thus, the study population (farms and cows) was not homogenous with regard to herd size, milk yield, and management. In some cows, for example, the first AI was performed quite early after calving. Incomplete involution of the uterus might have contributed to a first insemination failure and increased the risk of becoming a repeat breeder.

In conclusion, this study found that SE, intrauterine infections, or ovarian inactivity was not strongly associated with repeat breeding. This is important information for discussing treatment strategies. On the basis of our present findings, the use of antimicrobials is not indicated and hormonal treatments [40,41] need further justifications. Future studies on the phenomena of RBCs could focus on molecular biological analyses of the endometrium, disorders of the oviduct, and hormone profiles of RBCs in estrus and at the time of expected implantation.

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