Aus der
Tierklinik für Fortpflanzung
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Relationship between previous and concurrent aerobic bacteriological findings in the uterus and the prevalence of puerperal diseases in dairy cows

Inaugural-Dissertation
zur Erlangung des Grades eines
Doktors der Veterinärmedizin
an der
Freien Universität Berlin

vorgelegt von
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Berlin 2013

Journal-Nr.: 3672

Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

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Zweiter Gutachter:	Univ.-Prof. Dr. Johannes Handler
Dritter Gutachter:	Prof. Dr. Martina Hoedemaker, PhD

Deskriptoren (nach CAB-Thesaurus):
dairy cows, puerperal disorders, endometritis, Bacteria, Escherichia coli, neutrophils, Arcanobacterium pyogenes, reproductive performance, cytology, laboratory diagnosis

Tag der Promotion: 06.03.2014

Bibliografische Information der *Deutschen Nationalbibliothek*
Die Deutsche Nationalbibliothek verzeichnet diese Publikation in der Deutschen Nationalbibliografie; detaillierte bibliografische Daten sind im Internet über <http://dnb.ddb.de> abrufbar.

ISBN: 978-3-86387-487-2
Zugl.: Berlin, Freie Univ., Diss., 2013
Dissertation, Freie Universität Berlin
D 188

Choriner Str. 85 - 10119 Berlin
verlag@menschundbuch.de – www.menschundbuch.de

Meinen Eltern und meinem Mann

Arbeite ruhig und gediegen,
was nicht fertig wird, bleibt liegen.
Halte stets die Ruhe heilig;
nur Verrückte haben's eilig.
(Anonymus)

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1. INTRODUCTION

Puerperal diseases are highly prevalent in dairy cows. Due to the negative effects on subsequent reproductive performance and the depression of milk yield (LeBlanc et al., 2011) the risk factors and interrelationships with other diseases were examined in a multitude of studies. The uterine lumen in early postpartum dairy cows is commonly contaminated with different bacteria (LeBlanc et al., 2002, Sheldon et al., 2008). Depending on the immune status of the cow these infections can be eliminated or lead on to puerperal metritis (**PM**) or clinical endometritis (**CE;** Foldi et al., 2006). There are different factors which have a negative effect on immune defense of the cow around parturition. A low dry matter intake around parturition can lead to increased non-esterified fatty acids (**NEFA**; Huzzey et al., 2007) which reduce the myeloperoxidase activity of neutrophils (Hammon et al., 2006). Additionally, high NEFA concentrations increase the risk for fatty liver syndrome which is associated with impaired neutrophil function (Zerbe et al., 2000).

Bacterial species of the uterus can be categorized into 3 different groups according to their pathogenic potential. Some of the recognized uterine pathogens were not only associated with PM or CE but had also negative effects on the immune system. *Escherichia* (***E.***) *coli* is the most prevalent bacteria in the first week postpartum (Williams et al., 2005) and specific *E. coli* strains have been isolated from cows with uterine disease (Sheldon et al., 2010). These strains were shown to be more adherent and invasive for endometrial, epithelial and stromal cells and cause diseases of mucosal surface such as PM or CE (Sheldon et al., 2010). Additionally, Zerbe et al. (2001) found a functional depression of neutrophils by *E. coli*. Other pathogens like *Trueperella* (***T.***) *pyogenes* were found later in the puerperium potentially due to the impairment of the endometrial surface (Williams et al., 2007) and the negative effect on neutrophils by *E. coli* (Zerbe et al., 2001). *T. pyogenes* supports an infection of the uterus with gram-negative pathogenic anaerobes such as *Fusobacterium* (***F.***) *necrophorum* or *Prevotella* (***P.***) *melaninogenica* (Williams et al., 2005, Foldi et al., 2006) and is correlated with an increased risk for abnormal vaginal discharge or fetid odor (Williams et al., 2005). Alpha-hemolytic *Streptococci* (***AHS***) and coagulase negative *Staphylococci* (***CNS***) belong to the opportunistic contaminants of the uterus without associations with endometritis (Williams et al., 2005) and were often isolated. So far these bacteria attached little importance in literature whether they can affect other species. However, *AHS* infected cows were found to be negatively correlated with CE (Williams et al., 2005).

Subclinical endometritis (**SCE**) is also a uterine disease in the postpartum period and beyond which has negative effects on reproductive performance outcomes of dairy cows

(Galvao et al., 2009). The parameters to diagnose cows with SCE differ from study to study in respect to times of examination and cut points for percent of polymorph nuclear neutrophil leucocytes (**PMNL**). However, studies coincide in the essential points of increased PMNL (at least > 4%), the absence of abnormal vaginal discharge and no clinical signs of illness (Kasimanickam et al., 2004, Sheldon et al., 2006). Dubuc et al. (2010) investigated risk factors for different puerperal disorders and made out that cows with a low body condition score (BCS) at parturition (≤ 2.75), hyperketonemia (≥ 1.1 µmol/L beta-hydroxybutyrate acid) or increased haptoglobin concentrations (≥ 0.8 g/L) had a higher risk to be diagnosed with SCE at 35 DIM.

Another area of interest was the usability of the cytobrush (**CB**) for the collection of cells and bacteria in the uterus of dairy cows. Usually, the CB is used for cervical and endometrial cell collection in women and was modified for the collection of uterine cells in cows (Kasimanickam et al., 2004). Moreover, in most of the studies investigating bacterial flora of the uterus cotton swabs (**CS**) were used (Williams et al., 2005, Azawi et al., 2008, Petit et al., 2009). In this context the bacteriological outcomes of different laboratories were compared. The objective was to test the agreement between 3 laboratories concerning the occurrence of *E. coli*, *T. pyogenes*, *AHS* and *CNS* of given samples.

The overall objective of my thesis was to study dynamics of relevant pathogens in the postpartum period of dairy cows. In the first study I investigated the relationship between aerobic bacterial species (*E. coli*, *T. pyogenes*, *AHS* and *CNS)* at 10 ± 1 DIM and their presence and effect on each other and uterine infections 2 weeks later. Additionally, the cytobrush was evaluated for the collection of endometrial cells and bacteria in combination and the agreement between 3 laboratories concerning bacteriological results of uterine samples. The objective of the second study was to determine the relationship between bacterial findings in the uterus and percent PMNL in the early postpartum period and to test different cut points for the diagnosis of SCE.

The manuscripts of the papers are publicated in the Journal of Dairy Science and formatted according to its guidelines for authors.

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

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

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Journal of Dairy Science - Volume 95, Issue 12, Pages 7105-7114, December 2012

You can read the text here.

http://dx.doi.org/10.3168/jds.2012-5627

2.2 Presence of Escherichia coli, Trueperella pyogenes, alpha-hemolytic Streptococci, and coagulase negative Staphylococci and prevalence of subclinical endometritis

Presence of Escherichia coli, Trueperella pyogenes, alpha-hemolytic Streptococci and coagulase negative Staphylococci and prevalence of subclinical endometritis

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Journal of Dairy Science - Volume 96, Issue 10, Pages 6347-6354, October 2013

http://dx.doi.org/10.3168/jds.2013-6646

3. DISCUSSION

The objectives of the studies for this thesis were to examine the bacterial dynamics of the uterus of postpartum dairy cows at 10 ± 1 DIM and 24 ± 1 DIM and their relationship with PM, CE, SCE and subsequent reproductive performance. Furthermore, different cut points for SCE, agreement of different laboratories and 2 uterine collection tools (i.e., CB and CS) were investigated.

Bacteriological samples were only cultured for aerobic uterine pathogens and thus an effect of anaerobic bacteria, such as *F. necrophorum or P. melaninogenica* , on puerperal disorders or PMNL cannot be excluded.

The results of the two trials show different associations between isolated uterine bacteria and puerperal disorders. Isolated bacterial species of the first study corresponded to earlier studies (Williams et al., 2005, Foldi et al., 2006). Particularly, the decrease in bacterial contamination of the uterus with increasing days postpartum (Elliott et al., 1968) could be confirmed for *E. coli* and *AHS*. The effects of known uterine pathogens at current diseases shown in other studies (Williams et al., 2005, Westermann et al., 2010) could be repeated. Cows in which *T. pyogenes* could be isolated from the uterus had higher risks to be diagnosed with abnormal vaginal discharge at 10 ± 1 DIM and 24 ± 1 DIM compared to cows not infected. However, *E. coli* did not affect abnormal vaginal discharge at current infection.

Interestingly, cows positive for *E. coli* showed a high risk for persistence of this pathogen despite the observation that 80% of cows cleared their infection. *E. coli* occurred in 59% at 10 ± 1 DIM with high growth density (> 100 colonies per plate; Williams et al., 2005). These examinations, the functional depression of neutrophils (Zerbe et al., 2001) and the specific pathogenic strains for the endometrial cells (Sheldon et al., 2010) explain the persistence of *E. coli*. Furthermore, my findings provide evidence for speculations of previous studies (Zerbe et al., 2001, Williams et al., 2007) that *E. coli* increased the risk for subsequent *T. pyogenes* infections. The coherence between *E. coli* at 10 ± 1 and abnormal vaginal discharge at 24 ± 1 DIM is attributable to the appearance of *T. pyogenes* as direct trigger for mucopurulent discharge or worse, as it was seen in the results. The lack of a direct effect on abnormal vaginal discharge may confirm an association of *E. coli* with SCE. However, there was no effect on existence of PMNL at 24 ± 1 DIM due to an infection with *E. coli* 2 weeks earlier. The high number of cows culled and greater days to first artificial insemination (**AI**) in the first trial of cows infected with *E. coli* compared to cows not infected with this pathogen or positive for *T. pyogenes* can be explained by the damage of the endometrium (Zerbe et al., 2001). In the second trial *E. coli* had no effect on reproductive

performance outcomes due to the exclusion criteria (i.e. no abnormal vaginal discharge for cows at 24 ± 1 DIM) which assured that most cows had cured their infection.

Trueperella pyogenes is most frequently isolated later in the puerperium in these trials as well as in other studies (Williams et al., 2005). However, *T. pyogenes* was isolated at 10 ± 1 DIM with a higher risk for persistent infection 2 weeks later. For this well known uterine pathogen severe endometrial lesions and a substantial amount of PMNL in the epithelium and stratum compactum (Bonnett 1991, Azawi 2008) have been described. These injuries of the endometrium and potential previous infections, e.g. *E. coli*, which impaired endometrial immunity, increase the risk for persistent infections. Due to the high risk of persistence for *T. pyogenes* and the association with abnormal vaginal discharge at concurrent infections these bacteria increased the risk for abnormal vaginal discharge or CE, respectively, 2 weeks later in the first trial. Also anaerobic pathogens which were not identified in these trials could have increased the risk for CE. The presence of *F. necrophorum* or *P. melaninogenica* is synergistically affected by *T. pyogenes* (Williams et al., 2005, Foldi et al., 2006, Sheldon et al., 2009) and *F. necrophorum* were identified to be associated with mucopurulent discharge (Williams et al., 2005) as well. These characteristics of *T. pyogenes* and effects on gram-negative anaerobes could be an explanation for the effect on PMNL in the second trial. Despite the finding that cows at 24 ± 1 DIM had normal vaginal discharge, they had significant higher odds to be diagnosed with SCE (> 18% PMNL) when identified with *T. pyogenes* 2 weeks before. Most of the bacteria have been eliminated by the cows but the stimulus for the immune system determined the persisting high counts of PMNL%. The long-term effect of these infections could be seen in the first trial were cows needed more days to first AI and more days to pregnancy compared to cows infected with *E. coli* or without infection of these 2 bacterial species.

So far, *AHS* have been considered as opportunistic bacteria in the uterus they showed some pathogenic characteristics in both trials. The higher risk for abnormal vaginal discharge at 10 ± 1 DIM in cows infected with *AHS* was described for the first time. These findings could not be repeated 2 weeks later and no other synergistic effects on the same or other bacterial species at 24 ± 1 DIM were found. So, the association of *AHS* with abnormal vaginal discharge was potentially attributed to anaerobic pathogens. However, cows of the second trial tended to have a higher risk for SCE at 24 ± 1 DIM when positive for *AHS* 2 weeks earlier and needed more days to pregnancy compared to cows without *AHS*. An infection with *AHS* also decreased the percentage of pregnant cows after 250 DIM. The effect of anaerobic pathogens should be considered for these findings, as well. Nevertheless, another study could not demonstrate any concurrent associations of known uterine pathogens including anaerobes (i.e. *E. coli, T. pyogenes, F. necrophorum, Proteus spp. and P. melaninogenica*) and PMNL% at 28 or at 42 DIM. These results are novel and confirm the

negative effects on reproductive performance and a pathogenic character of *AHS* which warrants further research.

CNS were isolated only in small numbers as single species in both trials. There were neither protective effects concerning vaginal discharge as described by Williams et al. (2005) nor any pathogenic effects of previous infections on subsequent bacterial incidences or reproductive performance parameters.

Cut points for SCE are important to diagnose cows as sick or not and have large influence on clinical ramification (i.e. treatment with hormonal or antibiotic drugs or not). The variation in cut points and examination days in literature impede a general definition for this disease. Studies concerning SCE showed negative effects on reproductive performance e.g. increased time to pregnancy (Kasimanickam et al., 2004, Gilbert et al., 2005, Galvao et al., 2009) and decreased conception rates (Kasimanickam et al., 2004, Kaufmann et al., 2009, McDougall et al., 2011). These observations were used in the second trial to compare animals with different number of PMNL in the uterus to find a cut point for cows between 20 and 30 DIM. Cows of the groups 5 – 18% or 10 – 18% PMNL, respectively, showed the best reproductive performance parameters, especially days to pregnancy. Significant differences due to the PMNL groups were only found in primiparous cows but not in multiparous cows. Similar associations for primiparous cows were shown in the study of Kaufmann et al. (2009). The lower reproductive performance outcomes of cows < 5 or < 10%, respectively, and > 18% PMNL can be explained by the different immune status of the cows. High or low uterine PMNL levels could be influenced by the functionality of blood PMNL which decreased around parturition (Kehrli et al., 1989) and is negatively affected by elevated NEFA (Hammon et al., 2006). Even the treatment with dexamethasone can result in a high increase of uterine PMNL in response to a stimulus.

Bacteriological results of vaginal or other samples vary between different laboratories despite they were taken from the same animal. Therefore, in the first trial we examined if bacteriological results of 3 laboratories and between collection method (i.e. cotton swab and cytobrush) differ or yield similar results. The cytobrush and cotton swab results from the laboratories concerning the outcomes for *E. coli*, *T. pyogenes*, *AHS* or *CNS* were in good agreement both in positive or negative findings (> 80%). Despite similar initial situations of the sampling material, small differences in storing conditions, cooling time or cultivation could have influenced the bacteriological results. The agreement was the best for *E. coli* regardless of laboratory or sampling method. The storage at -80°C for 40 days or 1h at room temparature had no significant effect on growth scores of *E. coli* (Musser and Gonzalez, 2011). These colonies were described to be stable at 4°C up to 96h, but concentrations increased at room temperature (24°C) at 24h (Rishmawi et al., 2007, Stoner et al., 2008). These findings 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). The consistency of its characteristics and frequency in cultivation may have also influenced the high agreement.

Recent studies used guarded cotton swabs to describe the bacterial content of the vagina or the uterus (Sheldon et al., 2002, Williams et al., 2005, Petit et al., 2009). The cytobrush technique was validated for the collection of cells of the genital tract (Kasimanickam et al., 2004) and has been used more recently to harvest endometrial cells and bacteria simultaneously (Westermann et al., 2010). Due to the different use and structure the bacteriological outcomes of each laboratory were compared. The agreement between both sampling methods was high for all laboratories (> 90%) and demonstrated that the cytobrush is a valuable technique to obtain relevant information of endometrial inflammation and infection.

In conclusion, the results of the two trials showed that previous infections with *E. coli* or *T. pyogenes* are essential risk factors for CE, whereas both negatively affected reproductive performance parameters and *E. coli* seemed to pave the way for *T. pyogenes*. The SCE was shown to be affected by previous infections with *T. pyogenes* or *AHS*. Additionally, cows infected with *AHS* had longer days to pregnancy intervals and showed a decrease in the number of cows pregnant. Hence, more research is needed to prove the pathogenic potential of *AHS* including anaerobic bacteria. The bacteriological results of different laboratories showed high agreements between CB and CS. The CB validated for the collection of cells from the uterus is also useful for bacterial analysis.

4. SUMMARY

Relationship between previous and concurrent aerobic bacteriological findings in the uterus and the prevalence of puerperal diseases in dairy cows

Puerperal metritis, clinical endometritis and subclinical endometritis are highly present in dairy cows. To prevent or treat those diseases in an appropriate way the knowledge of risk factors and coherences for their appearance is important. Various metabolic parameters were identified to be associated with puerperal diseases and bacteria could be isolated from the uterus of cows with clinical signs of illness.

The uterine lumen of postpartum dairy cows is commonly contaminated with different bacterial species after parturition. Cows who fail to eliminate the pathogenic species often develop puerperal metritis or later in the puerperium clinical endometritis. Bacterial uterine infections have been shown to negatively affect ovarian function and fertility. The objective of my first study was to investigate relationships between *Escherichia coli* , *Trueperella pyogenes,* alpha-hemolytic *Streptococci*, and coagulase negative *Staphylococci* and their presence and effect on each other and uterine infections 2 weeks later. Therefore bacteriological samples were collected at 10 ± 1 and 24 ± 1 DIM from the uterine lumen using a cytobrush. Vaginal examination was performed by vaginoscopy. The vaginal discharge was assigned according to its distribution of pus to identify cows with puerperal metritis (10 ± 1 DIM) or clinical endometritis (24 ± 1 DIM). Bacteria were identified on the basis of the characteristics of the colony and standard tests (e.g. Gram stain, morphology, hemolysis, biochemical profile) and bacterial growth quantified on a 4-point scale. Cows 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; *T. pyogenes* RR = 2.9]. Cows infected with *T. pyogenes* also had a greater risk to be diagnosed with abnormal vaginal discharge at first (RR = 2.4, $P < 0.001$) and second (RR = 2.4, $P < 0.001$) examination. In terms of risk factor for clinical endometritis, the risk of being diagnosed with abnormal vaginal discharge at 24 ± 1 DIM increased in cows positive for *E. coli* (RR = 1.7) or *T. pyogenes* (RR = 1.7) at 10 ± 1 DIM. These cows also had more days to pregnancy (*T. pyogenes* 139 d, $P < 0.05$) and more cows were culled (*E. coli* 15.8%, $P < 0.05$) or had significant more days to first artificial insemination (*E. coli* 70 d, $P < 0.001$). These results demonstrate that cows infected with *E. coli* or *T. pyogenes* are prone to persistent infections 2 weeks later and also had higher risks to develop clinical endometritis. Infections with *AHS* only increased the risk for abnormal vaginal discharge which warrants further research of

their role in the uterus. *CNS* did not affect the infection with the same or other bacterial species 2 weeks later, as well as *AHS*, or influenced the vaginal discharge.

Recent studies describing uterine bacterial contents have used guarded cotton swabs or biopsy method. The cytobrush technique I used to collect bacteriological samples was originally evaluated in human medicine to collect cell material from the vagina or the uterus to diagnose inflammations by means of polymorph nuclear neutrophil leucocytes. The technique has been validated for cows to diagnose the SCE. Several recent studies have utilized the CB to harvest endometrial cells and bacteria from uterine lumen simultaneously, however, it never was validated for it. Therefore my second objectives of the first study were to evaluate the cytobrush for the collection of bacteria from the uterus and to test the agreement of bacteriological findings between 3 different laboratories. Uterine discharge was aspirated with a disposable catheter from 68 cows at 10 ± 1 DIM and transferred into a sterile plastic tube. From these tubes 1 CS and 1 CB each were sent to 3 different laboratories and the positive or negative findings of *E. coli, T. pyogenes, AHS* and *CNS* were compared. One laboratory received a second CS to test the intraassay repeatability. The agreement of bacteriological results of the laboratories were significant for laboratory A + B and A + C for each sampling method, CB and CS (A + B: CB = 86%, CS = 92%; A + C: CB = 82%, CS = 86%). The highest agreement considering the bacterial species was found for *E. coli* (A + B: CB = 89.7%, CS = 98.5%; A + C: CB = 82.4%, CS = 88.2%). The interlaboratory agreements between CB and CS were significant for each of the 3 laboratories (A = 92%; B = 97%; C = 93%) as was intralaboratory repeatability for laboratory A. Due to a missing gold standard it is impossible to prove which results are more accurate. But these results consider the cytobrush a valuable technique to generate information about infectious and inflammatory properties in the endometrium with a single sample.

The subclinical endometritis has a high prevalence in dairy cows but the approaches to diagnose the disease showed a wide variety of cut points for the amount of percent PMNL and sampling time. SCE was proved to have a severe negative impact on fertility. Recent studies identified a low body condition score, hyperketonemia or increased haptoglobin concentrations as risk factors for their development. My objective of the second study was first, to determine the relationship between aerobic bacteria in the uterus of healthy animals and PMNL in the early postpartum period. Second I compared 2 classification schemes for the diagnosis of SCE. Cytological and bacteriological samples were taken from 149 cows at 10 ± 1 and 24 ± 1 DIM. Cows were included into the study it they were diagnosed with no or clear vaginal mucus at the second examination. Additionally, 131 cows at 21 to 27 DIM without signs of clinical endometritis were sampled. The cytobrush technique was used for sample collection and vaginal examination was performed by vaginoscopy. The cut points for PMNL in the uterus were set at 5, 10 and 18%. Cows infected with *AHS* at 10 ± 1 DIM had

significant (P = 0.006) higher percent PMNL, more days to pregnancy (Hazard Ratio = 1.7, P = 0.038) and decreased odds of pregnancy by 250 DIM (Odds Ratio = 0.4, P = 0.022) compared to cows not infected with *AHS*. Anaerobic bacteria were not isolated in this study so their effect cannot be excluded. However, in another study *Fusobacterium necrophorum* and *Prevotella melaninogenica* , 2 well known uterine pathogens, did not show any association with percent PMNL at concurrent infection in which enhances the results for *AHS* as risk factor for SCE. Also *T. pyogenes* increased the odds about 5.1 to diagnose the same cow with more than 18% PMNL 2 weeks later but without abnormal vaginal discharge. The impairment of the endometrium induced by *T. pyogenes* and stimulus for the immune system could explain the long period cows need to cure the infection and the persistence of PMNL in the uterus. *AHS*, *T. pyogenes*, *E. coli* or *CNS* isolated at 24 ± 1 DIM did not affect the distribution of percent PMNL at the same examination day. For the comparison of different cut points the reproductive performance outcomes were compared when the same cows (24 ± 1 and 21 to 27 DIM) were arranged in 2 different schemes: PMNL1 (< 5, 5 – 18, > 18% PMNL) and PMNL2 (< 10, 10 – 18, > 18% PMNL). Reproductive performance outcomes were numerically better in the intermediate groups (5 – 18 and 10 – 18%) compared to cows with low (< 5/ < 10%) or high (> 18%) PMNL. Considering parity reproductive performance parameters differed significantly. In primiparous cows days to pregnancy were lower in cows of each intermediate group (5 -18: 68 d, P = 0.017; 10 – 18: 70 d, P = 0.039) compared to cows with > 18% PMNL (144.5 d). In multiparous cows the reproductive performance parameters did not differ significantly neither in PMNL1 nor in PMNL2. These results demonstrate that the cut point of 18% PMNL had a high diagnostic value, at least for primiparous cows, at 21 to 27 DIM to identify cows with SCE.

In conclusion, the results of the two trials showed that previous infections with *E. coli* or *T. pyogenes* are essential risk factors for CE, whereas both negatively affected reproductive performance parameters and *E. coli* seemed to pave the way for *T. pyogenes*. The SCE was shown to be affected by previous infections with *T. pyogenes* or *AHS*. Additionally, cows infected with *AHS* had longer days to pregnancy intervals and showed a decrease in the number of cows pregnant. Hence, more research is needed to prove the pathogenic potential of *AHS* including anaerobic bacteria. The bacteriological results of different laboratories showed high agreements between CB and CS. The CB validated for the collection of cells from the uterus is also useful for bacterial analysis.

5. ZUSAMMENFASSUNG

Zusammenhänge zwischen vorhergehenden und aktuellen bakteriellen Infektionen des Uterus von Milchrindern und ihr Einfluss auf die Prävalenz puerperaler Erkrankungen

Puerperale Erkrankungen beim Rind wie die puerperale Metritis, klinische Endometritis und subklinische Endometritis treten sehr häufig in deutschen Milchleistungsbetrieben auf. Um diese Erkrankungen zu vermeiden oder zielgerichteter behandeln zu können ist die Erforschung ihrer Risikofaktoren und Zusammenhänge ihres Auftretens essentiell. Verschiedene metabolische Parameter konnten bereits als Risikofaktoren für puerperale Erkrankungen identifiziert werden. Auch wurden verschiedene Bakterien aus dem Uterus von klinisch erkrankten Kühen isoliert und untersucht.

Der Uterus von Milchrindern ist nach der Abkalbung üblicherweise mit Bakterien besiedelt. Wenn das Immunsystem nicht in der Lage ist pathogene Keime zu eliminieren, kann eine puerperale Metritis oder später im Puerperium eine klinische Endometritis entstehen. Bakterielle Infektionen haben einen negativen Einfluss auf die Ovarfunktion sowie die Fertilität. Daraus ergab sich als Ziel der ersten Studie, den Zusammenhang zwischen der Präsenz verschiedener Keime des Uterus (bekannt pathogen: *Escherichia coli*, *Trueperella pyogenes;* Opportunisten: alpha-haemolysierende *Streptokokken*, coagulase negative *Staphylokokken*) und bakteriellen Funden 2 Wochen später herzustellen und den Einfluss auf die Entstehung von Uteruserkrankungen zu untersuchen. Für die Entnahme bakterieller Proben aus dem Uterus wurden die Studientiere an Tag 10 ± 1 und 24 ± 1 postpartum mittels Cytobrush beprobt. Der vaginale Ausfluss wurde mit einem Vaginoskop untersucht, um Tiere mit einer puerperalen Metritis (10 ± 1) oder klinischen Endometritis (24 ± 1) zu identifizieren. Die Anzahl der Koloniebildenden Einheiten auf den Blutagarplatten wurde mittels einer 4 Punkte Skala bewertet. Die Studie ergab, dass Kühe bei denen *E. coli* oder *T. pyogenes* an Tag 10 ± 1 postpartum (**pp**) isoliert werden konnte ein erhöhtes Risiko aufwiesen, dieselben Bakterien 2 Wochen später wieder im Uterus zu finden (*E. coli* Relatives Risiko (**RR**) = 3,7; *T. pyogenes* RR = 2,9). Kühe bei denen *T. pyogenes* isoliert wurde hatten zum entsprechenden Untersuchungstag ebenfalls ein erhöhtes Risiko mit mucopurulentem oder purulentem Ausfluss aufzufallen (Tag 10 ± 1: RR = 2,4, $P < 0,001$; Tag 24 ± 1: RR = 2,4, $P < 0,001$). Weiterhin wurden erhöhte Risiken für die Entstehung einer klinischen Endometritis an Tag 24 ± 1 pp gefunden, bei Kühen die 2 Wochen zuvor mit *E. coli* (RR = 1.7) oder *T. pyogenes* (RR = 1,7) infiziert waren. Selbige Kühe mit *T. pyogenes* hatten eine signifikant verlängerte Güstzeit (139 d, $P < 0,05$) im Vergleich zu Kühen ohne

diese beiden Keime oder ohne *E. coli*. Tiere mit *E. coli* hingegen wiesen eine höhere Rastzeit auf (70 d, P < 0,001) und wurden zu einem größeren Teil ausgemerzt (15,8%, P < 0,05). Diese Ergebnisse zeigen, dass Kühe mit *E. coli* oder *T. pyogenes* im Uterus zu persistierenden Infektionen mit demselben Keim neigen und erhöhte Risiken für die Entstehung von klinischen Endometritiden bestehen. Infektionen mit alpha-hämolysierenden *Streptokokken* (***AHS***) zeigten nur an Tag 10 ± 1 pp ein erhöhtes Risiko für veränderten vaginalen Ausfluss. Weitere Untersuchungen zu seinen potentiell pathogenen Eigenschaften im Uterus sind nötig. Das Vorhandensein koagulase negativer *Staphylokokken* (*KNS*) hatte weder Einfluss auf den vaginalen Ausfluss noch auf Infektionen mit denselben oder anderen Bakterien 2 Wochen später.

In den meisten Studien, die sich mit der Bakterienflora des Uterus beschäftigen, werden Wattetupfer (**Tu**) zur Probenentnahme verwendet. Die Cytobrush (**Cy**) ist eine Technik aus der Humanmedizin, mit der Zellen aus dem Genitaltrakt entnommen werden, um Entzündungen Anhand der Anzahl von polymorph kernigen neutrophilen Leukozyten (**PMNL**) festzustellen. Diese Technik wurde für die Veterinärmedizin modifiziert und speziell für Rinder zur Diagnose der subklinischen Endometritis genutzt. Einige neuere Studien nutzten die Cytobrush zur Entnahme von Zellen und bakteriellen Proben, jedoch ohne Validierung. Daher war eine weitere Zielstellung meiner ersten Studie die Validierung der Cytobrush für die Entnahme von Bakterien und Zellen aus dem Uterus. In diesem Zusammenhang sollte auch die Übereinstimmung der bakteriellen Ergebnisse von 3 unterschiedlichen Laboren getestet werden. Hierfür wurde mittels einem doppelt umhüllten Einwegkatheter jeweils 5 ml Ausfluss von 68 Kühen an Tag 10 ± 1 pp aus dem Uterus entnommen und in sterile Plastikröhrchen übertragen. Von diesen Röhrchen wurden jeweils 1 Cytobrush und 1 Tupfer an die 3 Labore gesendet und die positiven oder negativen Ergebnisse von *E. coli, T. pyogenes, AHS* und *CNS* miteinander verglichen. Ein Labor erhielt immer eine zweite Tupferprobe der gleichen Kuh, um die Wiederholbarkeit innerhalb eines Labors zu untersuchen. Die Übereinstimmung der bakteriellen Ergebnisse war signifikant für Labor A + B und A + C für jede der beiden Probenentnahmemethoden (Labor A + B: Cy = 86%, Tu = 92%; Labor A + C: Cy = 82%, Tu = 86%). Die höchsten Übereinstimmungen hinsichtlich der Bakterienspezies wurden bei der Anzucht von *E. coli* erreicht (Labor A + B: Cy = 89.7%, Tu = 98.5%; Labor A + C: Cy = 82.4%, Tu = 88.2%). Die Übereinstimmungen innerhalb der Labore, zwischen den Cytobrush und Tupferproben waren ebenfalls signifikant (Labor A = 92%; Labor B = 97%; Labor C = 93%) genauso wie die Wiederholbarkeit der 2 Tupferproben von Labor A. Da es in dieser Studie keinen Goldstandard für den tatsächlichen Bakteriengehalt im Uterus gab, mit denen die Ergebnisse verglichen werden konnten, ist es nicht möglich eine Aussage darüber zu machen, welche Ergebnisse wirklich zutreffen. Jedoch belegen diese Ergebnisse, dass die Cytobrush sowohl für die Entnahme von Zellen

als auch für bakterielle Proben verwendbar ist und somit gleichzeitig Informationen über den Entzündungs- und Infektionsstatus des Uterus gewonnen werden können.

Die subklinische Endometritis kommt sehr häufig bei Milchrindern vor wobei die Ansätze, diese Erkrankung zu diagnostizieren sehr unterschiedlich sind. Alle Definitionen enthalten die Kriterien von unverändertem vaginalem Ausfluss und einer erhöhten prozentualen Anzahl von PMNL im Uterus. Jedoch unterscheiden sich die Angaben in den Grenzwerten für PMNL und dem Untersuchungszeitpunkt. Da diese Erkrankung einen nachgewiesenen negativen Einfluss auf die Fruchtbarkeit betroffener Kühe hat, war sie Teil verschiedener Studien in denen ein geringer BCS um den Abkalbezeitraum herum, Hyperketonaemie oder erhöhte Haptoglobinkonzentrationen im Blut als Risikofaktoren für die subklinische Endometritis identifiziert werden konnten. Das Ziel der zweiten Studie war die Untersuchung der Zusammenhänge zwischen bakteriellen Vorkommen von *E. coli, T. pyogenes, AHS* und *CNS* im Uterus von Milchrindern und der Anzahl an PMNL zum gleichen Zeitpunkt und 2 Wochen später. Zudem wurden 2 verschiedene Einteilungsschemata für unterschiedliche Prozente an PMNL zur Diagnose der subklinischen Endometritis miteinander verglichen. Von 149 Kühen wurden an Tag 10 ± 1 und 24 ± 1 pp bakteriologische und zytologische Proben mit der Cytobrush aus dem Uterus entnommen, wobei Tiere nur in die Studie aufgenommen wurden, wenn sie zur 2. Untersuchung keinen oder nur klaren vaginalen Ausfluss zeigten. Zusätzlich wurden weitere 131 Kühe zwischen 21 und 27 Tagen pp beprobt, welche auch keinen oder nur klaren vaginalen Ausfluss hatten. Der vaginale Ausfluss wurde mittels Vaginoskop bestimmt. Die Grenzwerte für PMNL im Uterus wurden bei 5, 10 und 18% gesetzt. Die Auswertung der Bakteriologie zeigte, dass Kühe mit *AHS* bei der ersten Untersuchung signifikant (P = 0,006) höhere Prozente an PMNL aufwiesen im Vergleich zu Kühen ohne diesen Keim. Zudem zeigten diese Tiere eine verlängerte Güstzeit (Hazard Ratio = 1,7; P = 0,038) und eine geringere Wahrscheinlichkeit für eine erfolgreiche Trächtigkeit nach 250 Tagen pp (Odds Ratio = 0,4; P = 0,022). Der Einfluss von anaeroben Bakterien kann hierbei nicht komplett ausgeschlossen werden, da diese nicht analysiert wurden. In vorhergehenden Studien konnte jedoch kein Zusammenhang zwischen anaeroben pathogenen Keimen (*Fusobacterium necrophorum* und *Prevotella melaninogenica)* im Uterus und dem prozentualen Anstieg von PMNL gefunden werden. Dies bestärkt den Verdacht von *AHS* als Risikofaktor für die subklinische Endometritis. Auch *T. pyogenes* erhöhte die Wahrscheinlichkeit dieselbe Kuh 2 Wochen später mit über 18% PMNL zu diagnostizieren. Da dieser Keim eine starke Schädigung des Endometriums hervorruft und als Wegbereiter für andere Bakterien das Immunsystem zusätzlich stimuliert, brauchen infizierte Kühe ohne Behandlung anscheinend eine längere Zeit, ehe sich das Endometrium wieder regeneriert hat. Keine der an Tag 24 ± 1 pp isolierten Bakterien (*AHS, T. pyogenes, E. coli* oder *CNS*) hatten zur gleichen Zeit einen Effekt auf die

Anzahl an PMNL im Uterus. Um die verschiedenen Grenzwerte für die subklinische Endometritis zu vergleichen, wurden die Fruchtbarkeitsdaten aller Kühe von Tag 24 ± 1 und 21 – 27 pp analysiert. Dieselben Kühe wurden jeweils in den 2 festgelegten Schemata miteinander verglichen: PMNL1 (< 5, 5 – 18, > 18% PMNL) und PMNL2 (< 10, 10 – 18, > 18% PMNL). Die Fruchtbarkeitsdaten fielen jeweils in den mittleren Gruppen der 2 Schemata am besten aus, unterschieden sich aber in keinem Wert signifikant zu den Kühen mit < 5 bzw. < 10% oder > 18% PMNL. Bei der getrennten Betrachtung von primiparen und multiparen Kühen zeigten sich signifikante Unterschiede bei den Erstgenannten. Primipare Kühe der mittleren Gruppen wurden schneller wieder tragend (5 -18%: 68 d, $P = 0,017$; 10 – 18%: 70 d, $P = 0,039$) im Vergleich zu Kühen mit > 18% PMNL (144.5 d) und verhältnismäßig eher im Vergleich zu den Tieren mit < 5 bzw. < 10% PMNL (< 5%: 80d; < 10%: 77d). Bei den multiparen Kühen unterschieden sich die Fruchtbarkeitsdaten weder in der Aufteilung nach PMNL1 noch nach PMNL2. Diese Ergebnisse zeigen, dass der Grenzwert von 18% PMNL einen hohen diagnostischen Wert, zumindest für primipare Kühe, besitzt, um sie zwischen 21 und 27 Tagen pp mit einer subklinischen Endometritis zu identifizieren.

Die Ergebnisse dieser 2 Studien zeigten, dass *Escherichia coli* und *Trueperella pyogenes* wichtige Risikofaktoren für die Entstehung einer klinischen Endometritis sind. Zudem haben sich beide Keime negativ auf die Fruchtbarkeit der befallenen Kühe ausgewirkt. Ein Zusammenhang zwischen *E. coli* Infektionen und daraus folgenden Besiedelungen des Uterus mit *T. pyogenes* war auch mit meinen Daten zu belegen. Die subklinische Endometritis konnte auf die vorausgehende Infektion des Uterus *T. pyogenes* oder alpha-haemolysierenden *Streptokokken* zurück geführt werden. Da sich auch für Kühe mit *AHS* deutlich schlechtere Fruchtbarkeitskennzahlen ergaben, sollte die Pathogenität dieser Keime in anderen Studien nachgeprüft werden. Die bakteriologischen Ergebnisse der unterschiedlichen Labore stimmten sehr gut überein. Die sehr geringen Unterschiede zwischen Tupfer und Cytobrush belegen die Nutzbarkeit der Cytobrush für die Entnahme von Zell- und Bakterienproben aus dem Genitaltrakt.

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7. PUBLICATIONS

Research articles

A. Werner, V. Suthar, J. Plöntzke, W. Heuwieser:
Relationship between bacteriological findings in the second and fourth weeks postpartum and uterine infection in dairy cows considering bacteriological results
Journal of Dairy Science, 95, 2012, 1-10

A. Sens, W. Heuwieser:
Presence of *Escherichia coli* , *Trueperella pyogenes* , alpha-hemolytic *Streptococci* and coagulase negative *Staphylococci* and prevalence of subclinical endometritis
Journal of Dairy Science; 96(10), S. 6347–6354

S. Bertulat, C. Fischer-Tenhagen, **A. Werner**, W. Heuwieser:
Technical note: Validating a dynamometer for noninvasive measuring of udder firmness in dairy cows.
Journal of Dairy Science; 95, 2012, 6550-6556

Oral presentations

A. Werner, V. Suthar, J. Plöntzke, W. Heuwieser:
Relationship between bacteriological findings in the 2nd and 4th week postpartum in dairy cows and evaluation of laboratory results.
45. Jahrestagung Physiologie und Pathologie der Fortpflanzung. Berlin, 29.02.-02.03.2012
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A. Werner, V. Suthar, J. Plöntzke, W. Heuwieser:
Relationship between bacteriological findings in the 2nd and 4th week postpartum in dairy cows and evaluation of laboratory results.
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A. Sens, W. Heuwieser:
Bakterien als Risikofaktoren für die subklinische Endometritis bei Milchrindern.
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Poster

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A. Sens, W. Heuwieser:
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Reproductive biology, 13 Suppl 2, Abstracts S.55

I. Lindenberg, **A. Sens**, W. Heuwieser:
Nonsurgical castrated rams: Are they really infertile?
46th Annual Conference of Physiology and Pathology of Reproduction. Danzig/ Polen 27.02.–01.03.2013
Reproductive biology, 13 Suppl 2, Abstracts S.26

8. ACKNOWLEDGEMENTS

Zuerst möchte ich mich bei Herrn Professor Heuwieser für die Überlassung dieses sehr interessanten und wichtigen Themas bedanken. Besonders bedanken möchte ich mich für die Zeit, Problemlösungen oder neue Ansätze zu finden und die stets wertgeschätzten Ratschläge.

Ein besonderes Dankeschön gilt den Mitgliedern der Tierklinik für Fortpflanzung, bei denen ich aufgrund eines fehlenden Betreuers mehr als einmal Rat gesucht und auch gefunden habe. Und wenn nicht den, dann zumindest guten Kaffee und offene Ohren.

Hierbei speziell Vishal, du warst mir eine große Hilfe bei der praktischen Durchführung meiner Studien und eine sehr gute Unterhaltung im Stall. Auch deine Statistik-Tipps waren immer herzlich willkommen.

Liebe Lola, auch du warst mir eine wirklich große Stütze. Vielen Dank für die beste Bürogesellschaft und die vielen kleinen „Anreize“, diese Dissertation endlich fertig zu schreiben!

Mein Dank gilt auch den Mitarbeitern vom Albertshof, für ihre Geduld und gute Zusammenarbeit während meiner Datenaufnahme.

Mein größter Dank gilt meinen Eltern, die mich seit Kindesbeinen in der Berufswahl unterstützt und mir den Weg des Studiums bis hin zur Doktorarbeit geebnet haben, soweit es ihnen möglich war. Mit dieser Arbeit möchte ich euch sagen, ihr habt alles richtig gemacht!

Natürlich darf auch mein Mann hier nicht fehlen, der wohl viel Leid aber auch Freude mit mir geteilt hat. Die Nerven sind jetzt gestählt, also kann es auf gehen zu neuen Projekten…!

Zuletzt ein Dank an all die guten Freunde, die teilweise den gleichen Weg eingeschlagen haben. Bei euch konnte ich ausspannen, Kraft schöpfen und wieder von neuem beginnen. Seht was daraus geworden ist.

9. DECLERATION OF INDEPENDENCE

Table 1. Contribution of Doctorate Student

Contribution	Article 1	Article 2
Study design	+++	+++
Data collection	++	+++
Data analyses	+++	+++
Manuscript writing	+++	+++
Manuscript editing	++	++

Score: + < 50%; ++ 50 to 70% ; +++ > 70%

Hiermit erkläre ich, dass ich alle Studien selbständig durchgeführt und die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen in Anspruch genommen habe.

Potsdam den, 09.08.2013

Antje Sens, geb. Werner