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MEINER FAMILIE

The only true wisdom is in knowing you know nothing.

-Socrates

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1. INTRODUCTION

Endometritis in the mare still constitutes a great problem in the management of broodmares (Bain, 1966; Causey, 2006; LeBlanc and Causey, 2009). It is ranked as the third most common clinical condition in horses (Traub-Dargatz et al., 1991). Such inflammatory conditions of the equine uterus can be classified as acute, chronic or subclinical endometritis (LeBlanc and Causey, 2009; Hurtgen, 2006). All forms of endomeritis may cause substantial reductions of a mare’s fertility. Therefore, endometritis has developed as one of the major problems in the horse breeding industry (Hurtgen, 2006; Liu and Troedsson, 2008).

During the breeding season, a timely diagnosis and an efficient treatment is essential for a successful breeding outcome (LeBlanc, 2010; Witte et al., 2010). Prebreeding diagnosis of endometritis should include clinical examination, transrectal palpation, ultrasonography of the reproductive tract, vaginal examination, uterine culture, cytology and endometrial biopsy (Couto and Hughes, 1984; Reiswig et al., 1993; Bourke et al., 1997; Dascanio et al., 1997; Card, 2005; Aguilar et al., 2006). Although there is a wide spectrum of diagnostic aids, identifying the reason for infertility still challenges practitioners in the field. Uterine cultures have been known to lead to false positive and false negative results (Waelchli et al., 1988; Pycock and Newcombe, 1997; Nielsen, 2005; Aguilar, 2006). Therefore, they have been regarded as poor predictors of fertility (Digby and Ricketts, 1982; Shideler et al., 1982). While taking a single sample, the uterine swab touches only a small area of the endometrium and thus, focal infections may be missed (Brook, 1984; Ball et al., 1988). For practical reasons, endometrial culture, however, is still the most common method for diagnosing infectious endometritis in mares in the field (Blanchard et al., 1981; Mackintosh, 1981; Dascanio et al., 1997; Card, 2005). In the last several years it has been repeatedly demonstrated that uterine cytology diagnoses significantly higher percentages of mares with endometritis compared to uterine culture (Ball et al., 1988; Nielsen, 2005; Riddle et al., 2007). Nevertheless, these studies also showed that the cytological examination is still not able to reach the accuracy of the histological examination commonly known as the 'best standard' method (Reiswig et al., 1993; Bourke et al., 1997; Nielsen, 2005; LeBlanc et al., 2007). Therefore it has been recommended to combine a bacteriological with either a cytological or histological examination to get a more reliable diagnosis of inflammatory processes (Digby and Ricketts, 1982).

Additionally, in recent years the problem of subclinical inflammatory processes became more obvious. Subclinical endometritis has currently been described as an endometritis with absent clinical signs, such as intrauterine fluid accumulation in ultrasonography (LeBlanc and Causey, 2009). Concerning subclinical endometritis, ultrasonography and uterine culture may cause false negative examination results (LeBlanc and Causey, 2009). In contrast, the exsudative types of endometritis, which show fluid accumulation within the uterus, are more easy to detect with the currently used diagnostic methods (Reiswig et al., 1993; Bourke et al., 1997; Dascanio et al., 1997; Card, 2005; LeBlanc and Causey, 2009). Thus, recently it has become more and more obvious that identifying the cause for sub- or infertility might require more than swabbing (LeBlanc, 2010).

The objective of the first study of my thesis was to validate uterine cytology as a diagnostic aid in identifying mares with subclinical endometritis. Specifically, the hypothesis was tested that the cytological examination reaches higher sensitivity and specificity to detect subclinical endometritis in mares compared to the uterine swab as the currently known routine method. Therefore, three diagnostic methods, uterine swab, cytological brush and endometrial biopsy were compared with regard to their ability to detect subclinical endometritis.

The paper describing the results of this research was published in Theriogenology in 2011:

W. Overbeck, T.S. Witte, W. Heuwieser: Comparison of three diagnostic methods to identify subclinical endometritis in mares. Theriogenology 2011; 75 (7): 1311-8.

The histological presence of polymorphonuclear neutrophils (PMN) in the stratum compactum of the endometrium indicative for inflammation is commonly used as 'best standard' to diagnose endometritis in the mare (Reiswig et al., 1993; Bourke et al., 1997; Nielsen, 2005; LeBlanc et al., 2007). Endometrial biopsy and histopathology has been used for more than 40 years for the evaluation of the health status of the equine uterus (Brandt and Manning, 1969). After it has been demonstrated that fertility outcome is correlated with the presence of specific microscopic lesions such as endometrosis, angiosclerosis, endometrial maldiffentiation, endometrial atrophy, endometritis and lymphatic lacunae (Kenney and Doig, 1986; Schlafer, 2007), endometrial histopathology has been used to predict foaling probabilities. Histopathologically, the diagnosis of both, degenerative and inflammatory lesions, as well as combinations of these findings is one important aspect for the assessment of a mare´s fertility (Kenney and Doig, 1986). In the last decades, several studies have shown

that a histological examination provides a reliable prognosis of a mare's fertility (Doig et al., 1981; Shideler et al., 1982; Van Camp, 1988; Waelchli et al., 1988; Nielsen, 2005). Therefore, endometrial biopsy has become an integral part of breeding soundness evaluation in the problem broodmare and has been established as 'best standard' to diagnose endometritis in the mare (Reiswig et al., 1993; Bourke et al., 1997; Nielsen, 2005; LeBlanc et al., 2007). However, endometrial biopsy is an invasive technique which requires special equipment and specific laboratories. Additionally, it is often not approved by the owner and therefore not the first choice routine method in the field. The validity of endometrial biopsy has been constantly investigated in the research of equine reproduction. Several studies investigated the representativeness of a single endometrial biopsy sample, resulting in varying conclusions. Some older studies considered one histological sample as representative for the health status of the whole uterus (Bergmann and Kenney, 1975; Blanchard et al., 1987; Waelchli and Winder, 1989; Runge, 1995). In contrast, recent studies identified one single sample not enough for a generalized diagnosis (Dybdal et al., 1991; Fiala et al., 2010).

The cytological examination is supposed to represent the health status of the whole uterus as well. But science-based information on the comparability of cytological examinations between different sampling locations within the equine uterus is not available. Therefore, the objective of the second study was to determine the representativeness of a single cytological and a single histological sample for the health status of the whole uterus. Furthermore, PMN found by the two techniques at a specific location of an equine uterus in vitro were compared to evaluate if PMN numbers correlate between these two methods.

The paper demonstrating the results of the second study was published in Theriogenology in 2013:

W.Overbeck, K.Jäger, H.-A. Schoon, T.S. Witte: Comparison of cytologigal and histological examinations in different locations of the equine uterus – an in vitro study. Theriogenology 2013; 79(9): 1262-8.

The two papers are presented in the format outlined in the guide for authors of the respective journal.

2. RESEARCH PAPERS

2.1 Comparison of three diagnostic methods to identify subclinical endometritis in mares

W. Overbeck, T.S. Witte, W. Heuwieser (Theriogenology 2011;75(7): 1311-8)

Comparison of three diagnostic methods to identify subclinical endometritis in mares

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2.1.1 Summary

The objective of this study was to compare the accuracy of a uterine swab (US), a cytological brush (CB) and an endometrial biopsy (EB) to detect subclinical endometritis in mares. Cytological and bacteriological results of all three techniques were related to histological occurrence of polymorphonuclear neutrophils (PMNs) in the stratum compactum, commonly known as 'best standard' to diagnose endometritis.

Samples were taken from 55 mares of different breeds without clinical signs of endometritis. Samples for US, CB and EB were collected, smeared on a microscopic slide and cultured for bacterial growth. Endometrial biopsy samples were additionally stored in 4% formaldehyde for histological analysis. Bacteriological cultures and cytological samples of all techniques were classified as negative (no uterine pathogens in monoculture; < 2% PMNs) or positive (uterine pathogens in > 90% of the grown colonies; > 2% PMNs) for endometritis. Uterine pathogens were diagnosed in 20.0% of the mares. Isolation of pathogens was not associated with positive cytological findings (r = -0.23; P = 0.87). None of the six mares with an *Escherichia coli* infection (10.9%) showed a positive cytological result. In contrast, two of five mares infected with *Streptococcus zooepidemicus* had a positive cytological result.

Histologically, the presence of PMNs in the stratum compactum was regarded as positive for endometritis when the mare was in diestrus at time of sampling. Compared to the 'best standard', sensitivity for cytology of CB, US and EB was 0.17, 0.00 and 0.25, respectively. Specificity for cytology of CB, US and EB was 0.83, 0.93 and 0.85, respectively. Sensitivity of uterine culture was 0.25, 0.33 and 0.25 for CB, US and EB, respectively. Specificity for culture of CB, US and EB was 0.80, 0.83 and 0.95, respectively. In conclusion, cytological or bacteriological examinations alone provide a high incidence of false negative results. Sensitivity of cytology combined with bacteriology of CB was 0.42. A combination of a bacteriological and a cytological examination of a CB sample improved the diagnostic performance in subfertile mares. Based on these results, we can recommend the CB to improve the diagnosis of subclinical endometritis in the mare compared to the US alone as currently used routine method.

2.1.2 Keywords

Subclinical endometritis; Endometrial biopsy; Endometrial cytology; Mare

2.1.3 Introduction

Endometritis in the mare is known to inflict major losses in the broodmare practice, as its prevalence ranges from 25 to 60% in barren mares [1–5]. During the breeding season, a timely diagnosis and an efficacious treatment is therefore essential for a successful breeding outcome [6]. It is well known that various factors contribute to the pathogenesis of endometritis. Microbial pathogenesis, such as induction of inflammation, epithelial adherence, resistance to phagocytosis and viscosity of secretions vary greatly between pathogens [2] and are playing an important role in influencing fertility problems. Furthermore, subclinical endometritis has become a more important reason for subfertility in the mare. This has currently been described by LeBlanc and Causey [7] as an endometritis with hidden clinical signs, e.g. absence of intrauterine fluid in ultrasonography, that is difficult to diagnose.

Prebreeding diagnosis of endometritis should include clinical examination, transrectal palpation, ultrasonography of the reproductive tract, vaginal examination, uterine culture, cytology and endometrial biopsy [8 –13]. In the last decades, the lack of a reliable diagnostic technique challenged the accurate diagnosis of endometritis in the mare. Although uterine culture is known to lead to false positive or false negative results [12,14–16] and has been regarded as a poor predictor of fertility [17,18], aerobic endometrial culture is still the most common method for diagnosing infectious endometritis [11,13,19,20]. False positive cultures are caused by contamination of the sampling instrument from the environment, external genitalia or vagina [18,21]. Therefore, double-guarded swab systems and an aseptic sampling technique are essential when taking the uterine swab (US). False negative results can be associated with inadequate sampling. The US touches only a small area of the endometrium and focal infections might be missed [22,23]. LeBlanc et al. [24] demonstrated that cultures of

uterine flushes double the ability to detect chronic infectious endometritis in mares compared to cultures of swabs alone. In cases of subclinical infection, routine diagnostic techniques such as ultrasonography and uterine culture may create more easily false negative results [7]. Thus, the problem of identifying the underlying cause of subfertility emphasizes that more than swabbing is required [25].

Subclinical endometritis has been demonstrated to cause poor reproductive performance in dairy cattle [26,27]. A substantial proportion of clinically healthy cows were identified as having subclinical endometritis diagnosed by uterine cytology (i.e. uterine lavage, cytological brush) [26–29]. Whereas, harvesting of bovine endometrial cells with the cytological brush (CB) is a more consistent, rapid and easier method than the lavage method [30]. These cytological samples were superior to samples from uterine flushes in respect to the amount and preservation of cells, and the time needed to collect the sample [30]. Recently, it has been reported that microbiological findings were not sufficient to determine the status of subclinical endometritis in dairy cows [31]. Since 1980, the CB technique has been used successfully in human medicine to study endocervical and uterine cytology [32–34]. Equally, examining uterine cells harvested through uterine lavage or cytological brushes, gained importance in diagnosing endometritis in mares [10,35]. It has been demonstrated repeatedly that significantly higher percentages of mares with endometritis are diagnosed by uterine cytology compared to uterine culture [16,22,36]. Endometrial biopsy (EB) however, provides the most reliable statement of changes within the endometrium. Endometrial biopsy is not only used for the detection of degenerative changes according to the classification of Kenney and Doig [37], but also to determine distribution and severity of inflammatory processes [38,39]. The histological presence of polymorphonuclear neutrophils (PMNs) in the stratum compactum of the endometrium is commonly used as 'best standard' to indicate endometritis [9,10,16,24]. Nevertheless, EB is an invasive technique which requires special equipment and laboratories. Additionally, examination results are only available with a certain time lag, due to further processing (shipping, examining and transmitting of results). Therefore, its practical relevance is restricted to special cases.

The objective of the present study was to compare the accuracy of three diagnostic methods (US, CB and EB) to detect subclinical endometritis in mares. Specifically, we tested the hypothesis that endometrial cytology, based on smears obtained from a CB, achieves higher sensitivity and specificity to detect subclinical endometritis under field conditions, compared to the US currently being recognized as the routine method.

2.1.4 Materials and methods

2.1.4.1 Animals

The study was conducted between July and November 2009 at different stud farms in Brandenburg, Germany. Sixty-six mares, regardless of the stage of estrus cycle, from different breeds were presented and examined for the study. The age of the mares ranged from two to 29 years (11.4 ± 6.1). They had different breeding histories such as maiden mares, mares with endometritis, early embryonic death or abortion. The majority of the mares were barren because of failure to conceive or exclusion from breeding for other reasons, like use as riding horses. All mares were examined by transrectal palpation and ultrasonography (Picker CS 9100, Physia GmbH, Neu-Isenburg, Germany) for genital health, determination of cycle stage and to exclude pregnancy before sampling. If intraluminal uterine fluid was detected, mares were diagnosed with clinical endometritis and excluded from the evaluation.

2.1.4.2 Experimental design

Before collecting samples from the uterus, the tail was bandaged and the perineum and vulva were cleaned three times with paper towels and an iodine solution to reduce contamination of the internal genital tract. A double-guarded US (Minitüb, Tiefenbach, Germany), a single-guarded CB (CytologyBrush, Minitüb, Tiefenbach, Germany) and an EB (Kevorkian biopsy forceps, Eickemeyer, Tuttlingen, Germany) were taken consecutively under manual control. This order was maintained in each mare. With a sleeved arm, the instruments were introduced through the vagina and cervix into the uterus. Specimens of US and CB were collected by rotating them alternately to the right and to the left to obtain cellular material from the adjacent endometrium in the uterine corpus. Endometrial biopsies were taken from the base of either uterine horn with a Kevorkian biopsy forceps as previously described [38,40,41]. In brief, the biopsy forceps was introduced through the cervix into the uterus by a sleeved arm. After the forceps was placed in the uterine lumen, the sleeved arm was withdrawn from the vagina and inserted into the rectum to guide the forceps to the desired place and to put endometrial mucosa into the forceps.

Samples of US and CB were immediately smeared on a sterile glass slide (Mediware, Servoprax, Wesel, Germany) to obtain a cytological sample and afterwards stored in a culture

medium (Meus s.r.l., Piove di Sacco, Italy) until bacteriological examination. All EB samples were swabbed for bacteriological examination and then smeared on a sterile glass slide for cytological examination. Afterwards, EB samples were stored in 4% formaldehyde until further histological examination. The EB samples were consequently evaluated in two different ways; to obtain a cytological and bacteriological sample as well as to obtain a histological result used as 'best standard' to assess the sensitivity and specificity data of the cytological and bacteriological examinations. In order to compare the results of the three diagnostic methods, three cytological samples (US, CB, EB), three bacteriological samples (US, CB, EB) were obtained from each mare. Results were correlated to the presence of PMNs in the tissue sample as 'best standard' to diagnose subclinical endometritis.

2.1.4.3 Bacteriology

Within two hours after sampling, bacteriological swabs from each of the three techniques were smeared on a sheep blood agar petri dish (Sarstedt, Nümbrecht, Germany) and enriched in brain heart infusion (BHI) medium (Oxoid Ltd., Basingstoke, Hampshire, England) and a standard nutrition I bouillon (Merck, Darm- stadt, Germany) at the laboratory of the Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universität, Berlin. Blood plates were incubated at 37°C and examined two times every 24 h. After 24 h a sample of both, BHI and nutrition I bouillon was also smeared on sheep blood agar and examined 24 h later. After 48 h culture samples were categorized either as substantial growth of uterine pathogenic bacteria in monoculture or as no bacterial growth or contamination. If > 90% of the grown colonies on the blood agar were of one species, it was considered as monoculture. Mixed cultures of more than three pathogens were considered as contamination. The common bacteria, *β-hemolytic Streptococcus*, *Escherichia coli* (*E. coli*) and *Pseudomonas spp*. were considered as equine uterine pathogens [1,18,42].

2.1.4.4 Cytology

After sampling and preparing the smears, the cytological specimens were fixed immediately (Haema-Fixierlösung, LT-SYS, Berlin, Germany) and stained within two hours (Haema-Schnellfärbung, LT-SYS, Berlin, Germany) at the laboratory of the Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universität, Berlin. The presence of

PMNs and epithelial cells was examined with light microscopy (BHS, Olympus, Hamburg, Germany) under oil immersion (x 1000). A total of 300 cells were counted and the required visual fields (high power fields; HPF) were documented. Smears were regarded as indicative for inflammation if the amount of PMNs was > 2% [12,22].

2.1.4.5 Histology

Endometrial biopsies were fixed in 4% formaldehyde and sent to a commercial pathological laboratory (Institute for Veterinary Pathology, University of Leipzig, Germany). Biopsy specimens were processed and evaluated as previously described [38,39]. Additionally, they were routinely classified using the grading system of Kenney and Doig [37], modified by Schoon et al. [39]. If PMNs occurred in the stratum compactum and the mare did not show signs of estrus at time of sampling, the sample was considered as positive for endometritis.

2.1.4.6 Statistical analysis

All clinical findings and results from the laboratories were documented on data capture forms and transferred into a spreadsheet (Excel 2003, Microsoft, Munich, Germany). Descriptive statistical analyses were carried out with the SPSS statistic package (SPSS for Windows 16.0, SPSS Inc., Munich, Germany). Comparisons were made between culture results, cytological results, histological results, microorganisms recovered and clinical signs of endometritis. Variables were compared using contingency tables and Pearson Chi-square tests.

Level of significance was set at a P value < 0.05. The number of HPF required to count 300 cells in cytological samples were compared using the paired t-test. Bacteriological and cytological results were related to the histological presence of PMNs in the stratum compactum of the endometrium as ‘best standard’ to diagnose endometritis [5,15,37,38,41]. Sensitivity and specificity of the different diagnostic tests were calculated.

2.1.5 Results

The majority of the mares did not show signs of clinical endometritis as determined by presence of intrauterine fluid in ultrasonography. The prevalence of clinical endometritis was 16.7%. These 11 mares were excluded from further evaluation.

In 26.8% of the mares samples were collected during estrus and in 73.2% in diestrus. In total, 165 cytological samples (US, CB, EB), 165 bacteriological samples (US, CB, EB) and 55 tissue samples were evaluated from the remaining 55 mares. However, three histological samples (5.5%) were not adequate for evaluation. Consequently, cytological and bacteriological specimens were examined and compared with 52 EB results.

2.1.5.1 Bacteriological findings

In five and six of the 55 mares, *Streptococcus zooepidemicus* (*Strep. zooepidemicus*) (9.1%) and *E. coli* (10.9%) were detected in monoculture, respectively. These findings were not associated with positive cytological findings (r = -0.23; P = 0.87). The proportion of positive and negative cytological results differed in mares infected with *Strep. zooepidemicus* compared to mares infected with *E. coli* (Fig. 1). Sensitivity and specificity of uterine culture from US, CB and EB compared to the presence of PMNs in the stratum compactum as 'best standard' is shown in Table 1.

2.1.5.2 Cytological findings

In 11 of 55 (20.0%) cytological smears from CB, percentage of PMNs was > 2%. Presence of PMNs > 2% were lowest in cytological smears of US (5.5%) compared to smears of CB (20.0%) and EB (18.5%). The number of HPF (x 1000) needed to count 300 cells in cytological smears of CB (48.4 ± 40.8) was significantly (P < 0.01) lower than in samples of US (113.1 ± 87.7), demonstrating that cytological smears of CB had higher cell yields compared to cytological smears of US. Sensitivity and specificity of cytology from CB, US and EB compared to the presence of PMNs in the stratum compactum as 'best standard' and sensitivity and specificity of an examination combining CB and US is shown in Table 1.

Sensitivity and specificity of uterine culture from CB, US and EB compared to the presence of PMNs in the stratum compactum as "best standard" is shown in Table 1.

2.1.5.3 Histological findings

In three mares, a histological evaluation of the EB specimen was not possible. Endometrial alterations were found in 90.4% of 52 mares, with the majority having category IIA or IIB (80.8%). Cellular infiltration with PMNs in the stratum compactum ranged from '-' to '+'. Histological samples found positive for PMNs in diestrus mares, were observed in 12 mares (23.1%).

2.1.6 Discussion

An accurate diagnosis of subclinical endometritis is important to predict the breeding performance of broodmares. When clinical signs of inflammation are absent, routine diagnostic methods like ultrasonography or uterine culture might cause false negative results, biasing assessment of the reasons for poor reproductive performances of those mares [7]. Therefore, this study was designed to compare the diagnostic performance of a US, CB and EB to diagnose subclinical endometritis in mares. In this study, specimens were harvested under field conditions and the samples of every technique were examined bacteriologically and cytologically. Additionally, histological evaluations of samples from EB were regarded as 'best standard' and compared with the results of the cytological and bacteriological examinations.

Both, endometrial culture obtained with US and the cytological examination of a CB sample identified the same number of mares (11/55) having an infection or inflammation of the endometrium. Despite this numerical agreement of the two diagnostic techniques, however, a correlation between the bacteriological and cytological results did not exist (r = -0.23; P = 0.87). In only 18.2% of the cytologically positive mares, culture was also positive. The challenge of false negative culture results in diagnosing equine endometritis [7,16,22,23] is obvious when PMNs are present in a sample without uterine pathogens in uterine culture

[43,44]. This can be explained by the observation that the uterine swab may miss focal infections [22,23]. Digby [45] assumed that this phenomenon could also be evoked by the presence of antimicrobial preparations in the uterus, deep-seated infection or non-infectious irritation. In contrast, a positive cytological result is assumed to be a reliable indication for acute endometritis [9,23,45]. However, false negative cytological results are possible when PMNs are artificially washed away from the surface of the endometrium. With a combination of bacteriological and cytological examination of the CB sample, the number of mares identified to have endometritis in our study was significantly higher (19/55, $P < 0.01$) compared to the CB or US technique alone. Therefore, the combination of cytological and bacteriological examination of a sample obtained with the CB leads to a higher sensitivity in diagnosing endometritis (Table 1). This combined evaluation of a CB in order to improve endometritis diagnosis has not been described yet. Earlier studies showed that cytology detects twice as many mares with endometritis as did endometrial culture [18,36]. The PMN threshold used by these authors (PMN > 0.5%) to classify samples as positive for endometritis, however, differed compared to our study. Our threshold (PMN > 2%) was chosen because of the small, but constant amount of PMNs in the uterus of healthy mares during proestrus and estrus that should be considered physiologically [46]. Unfortunately, standardized methods for interpretation of endometrial cytology are still not available [13]. The prevalence of relevant pathogens isolated from the endometrium in this study (45.5% *Strep. zooepidemicus*, 54.5% *E. coli*) was similar to those reported in earlier studies [1,18,42]. The percentage of cytological samples indicative of inflammation varied between *Strep. zooepidemicus* (2/5) and *E. coli* (0/6) (Fig.1). These results agree with findings of Riddle et al. [36], who found that *Pseudomonas*, *Proteus* and coliforms like *E. coli* were more unlikely to have cytological evidence of inflammation. These bacteria produce mucus of high viscosity and slip through the immune response by circumventing the cellular defense system and hindering cilia function [7]. Another study demonstrated that in only 12.0% of uterine culture samples positive for anaerobes, cytological evidence of inflammation existed [47]. Thus, it is reasonable to assume that subfertility in the mare without considerable presence of PMNs in cytology may be caused by gram-negative bacteria like *E. coli*. If in a clinically healthy mare, however, cytological results are negative and bacteriological samples show uterine pathogens, a false-positive bacteriological result is possible. This underlines again the need of an aseptic sampling method to obtain reliable culture results. While a gold standard diagnostic technique with 100% specificity is currently not available, at least a double-guarded culture system

should be used to minimize contamination. Therefore, today all commercially available endometrial swabs are sterile, double-guarded swabs [16].

The detection of PMNs in the stratum compactum by an experienced examiner in a histological examination has been assumed as the 'best standard' to diagnose any form of endometritis in the mare [5,15,37,38,41]. In the last decades, several studies have shown that one biopsy, examined for inflammatory and degenerative processes, is representative for the status of the whole uterus [48–50]. Other studies, however, showed considerable variations within different sites of the uterus of a given mare [51,52]. Obviously, this area of research is controversial and needs further investigation. Kenney [38] hypothesized that in cases of inflammation, PMNs are always present in the endometrium. A recent study of Palm et al. [53] confirmed this statement. The authors demonstrated that PMN numbers, determined in the stratum compactum, increased after uterine infusion of semen extenders, saline or seminal plasma as inflammatory agents. In contrast, PMN numbers of uterine flushes did not change after instillation of these agents. In healthy sows infiltrations with PMNs were regularly present in the subepithelial connective tissue during proestrus and estrus, while in the surface and glandular epithelia PMNs were observed only occasionally [54]. Therefore, the occurrence of PMNs in the stratum compactum, in a cycle stage other than proestrus and estrus was used in the present study as 'best standard' to diagnose endometritis and to determine sensitivity and specificity of the bacteriological and cytological examination.

No test regarded as 'best standard' can be 100% accurate [16]. Therefore, when comparing a standard with a new method it is important to recognize that neither approach may be ideal. Compared to histological detection of PMNs in the stratum compactum as 'best standard' sensitivity of uterine culture was highest in samples of US (0.33) in our study. Results of uterine culture harvested by EB (0.25) and CB (0.25) were lower, probably because of the unguarded or single-guarded instruments. A comparable sensitivity of uterine culture (0.34) was reported by Nielsen [16]. Specificity of uterine culture was highest by EB (0.95) and comparable for CB (0.80) and US (0.83, Table 1). Kenney [38] reported that the agreement between histological and cultural findings was 75%. His calculation was based on 47% negative histological results which corresponded with negative culture results and 28% positive histological results associated with uterine pathogens in culture. Our study showed a similar agreement between histological and cultural results of 69.8%. Our data, previous studies [18,36] and a recent study [55] clearly demonstrate the poor performance of the endometrial swab and underline that uterine culture is insufficient for a precise diagnosis.

LeBlanc et al. [24] reported that culture from uterine flushes improves clinical detection of infection. The sensitivity of bacterial examination from uterine flushes was 0.71 and twice as sensitive as swab culture (0.34) calculated by Nielsen [16]. The uterine flush, however, is difficult to perform in the field and requires special equipment [56]. It has been assumed that uterine swabs are the commonly used routine method because of low costs, ease of collection and safety of use [15]. Endometrial cytology is easy to perform in the field. Additionally, cells can be harvested not only by cytological brush, but also by preparing a smear from an endometrial biopsy specimen or centrifuged flushes. The evaluation of the CB smears in our study was faster than the evaluation of cytological smears of US, due to the lower number of HPF (1000x) needed to count 300 cells in cytological smears of CB (48.4 ± 40.8) compared to the samples of US (113.1 ± 87.7) ($P < 0.01$). In our study, cytological results were achieved by CB, US and EB, and compared with each other. The technique to obtain the different cytological smears was similar, only the method of harvesting differed. Compared to the 'best standard', sensitivity of cytology obtained by US was 0. Due to this insensitivity and the high amount of HPF to count 300 cells (113.1 ± 87.7), the uterine cotton swab is clearly inappropriate for uterine cytology. Sensitivity of cytology obtained by CB and EB was 0.17 and 0.25, respectively. Specificity of cytological examination of the three techniques was comparable with 0.83, 0.93 and 0.85 for cytology of CB, US and EB, respectively (Table 1). These sensitivity and specificity data emphasize that a cytological or bacteriological examination alone remains insufficient for diagnosing equine subclinical endometritis. All methods yield high rates of false negative results. With a combination of cytology and bacteriology, sensitivity data rose to 0.42 (Table 1). If a double-guarded CB would provide more reliable results in a combination of cytological and bacteriological examination needs further investigation.

In conclusion, our data emphasize that, regardless of the diagnostic technique, the results are insufficient for a sensitive and specific diagnosis of subclinical endometritis. In diagnosing subclinical endometritis, CB or US alone do not provide reliable results of inflammatory processes within the endometrium. Our data showed that the combination of a cytological and a bacteriological examination of one endometrial sample taken with the CB might be the most promising approach. The CB is a quick and safe technique, easy to achieve under field conditions and improves the diagnosis of subclinical endometritis in the mare compared to the US alone as currently used routine method.

2.1.7 Figures and tables

Figure 1: Isolated bacteria and their evidence of inflammation in cytology.

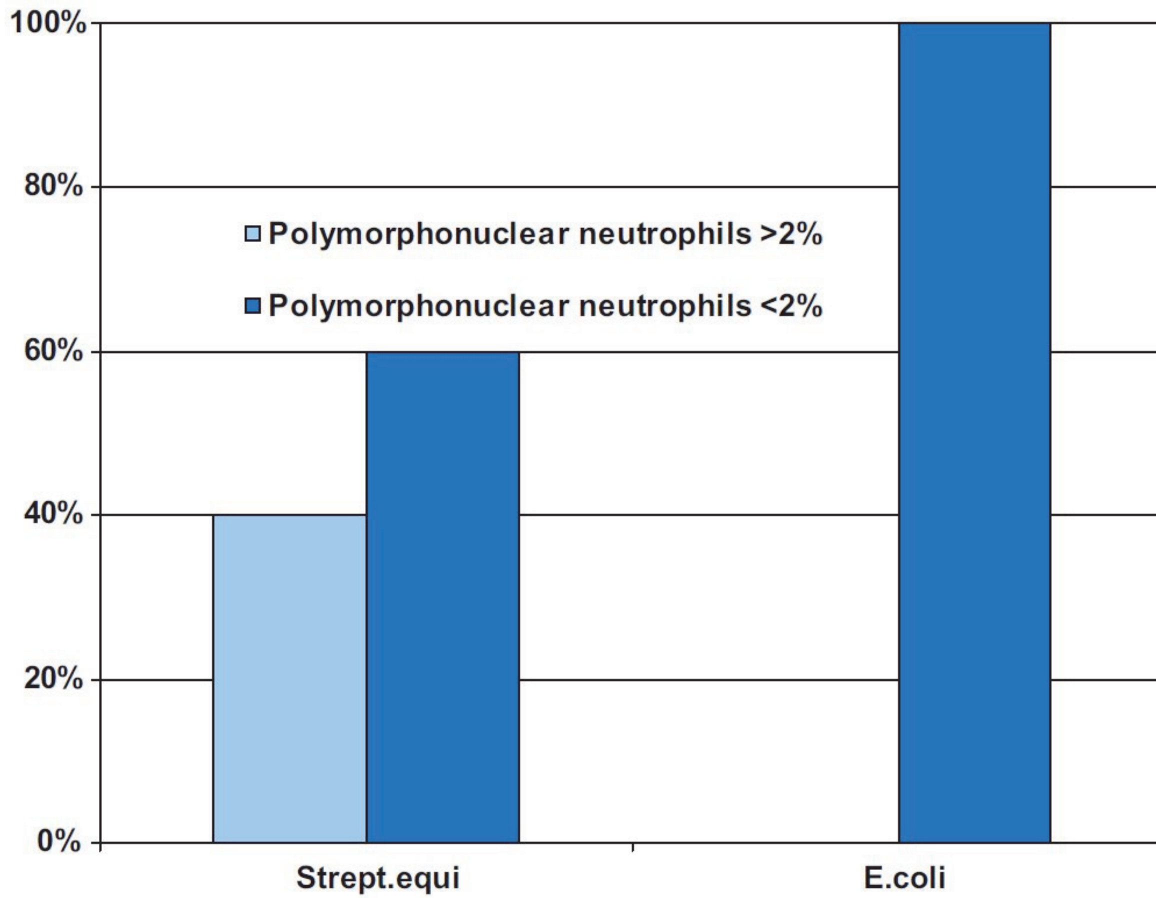

Table 1: Sensitivity and specificity data of cytological and bacteriological examinations.

Method	Sensitivity		Specificity	
	Cytology	Bacteriology	Cytology	Bacteriology
Cytological brush	0.17	0.25	0.83	0.80
Swab	0.00	0.33	0.93	0.83
Biopsy	0.25	0.25	0.85	0.95
CB Cytology + Bacteriology	0.42		0.70	

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2.2 Comparison of cytological and histological examinations in different locations of the equine uterus - an in vitro study

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Comparison of cytological and histological examinations in different locations of the equine uterus - an in vitro study

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2.2.1 Summary

Endometritis constitutes a major problem in managing broodmares. The histological occurrence of polymorphonuclear neutrophils (PMN) in the stratum compactum of the endometrium is accepted as 'best standard' to diagnose endometritis in mares. The objective of this study was to determine the distribution of PMN within different sampling locations of the uterus by cytological examinations and to compare it with PMN numbers in endometrial biopsies of the corresponding location. Cytological and endometrial samples were obtained from 37 uteri within 2 ± 1 h after slaughter through small incisions from five different, pre-defined locations of each uterus. Cytological samples were smeared on microscopic slides, stained and classified as negative (< 2% PMN) or positive (≥ 2% PMN) for endometritis. Histologically, the number of PMN were counted in three high power fields (HPF) by an experienced pathologist and afterwards classified as positive for this type of endometritis when a total of ≥ 5 PMN occurred in 3 HPF (X 40 magnification). Biopsies were additionally evaluated considering lymphoplasmacellular endometritis, periglandular fibrosis (endometrosis) and angiosclerosis. The prevalence of positive cytological and histological samples was 14.6% and 17.8%, respectively. A fair agreement between the two diagnostic methods could be detected (k = 0.29; P < 0.01). Numbers of PMN differed between the sampling locations resulting in positive and negative locations within a positive scored uterus, both in cytologically positive scored uteri (8/10) and in histologically positive scored uteri (13/14). There was no significant difference in PMN numbers within the different locations, neither in cytological (P = 0.78) nor in histological examination (P = 0.79). Additionally, no significant differences in assessment of endometrosis (P = 0.96) and angiosclerosis (P = 0.67) within the locations could be observed.

In conclusion, PMN numbers of a cytological examination of the endometrium showed fair agreement to the occurrence of PMN in the stratum compactum of the histological examination at the same sampling location. Although there was a variation in number of PMN in both methods (cytology and histology), statistically significant differences could not be detected within the different locations (P = 0.78; P = 0.79). Implying that, the decision about taking more than one sample should be critically considered and further research is warranted to determine the number of sampling locations to get a reliable examination result.

2.2.2 Keywords

In vitro; Endometrial cytology; Endometrial biopsy; Endometritis; Mare

2.2.3 Introduction

Subclinical endometritis is a major challenge in reproductive management of broodmares. Currently defined as uterine inflammation without fluid accumulation within the uterus [1], subclinical endometritis is a problem for most practitioners because a reliable and practicable diagnostic technique is not available. In contrast, the exsudative types of endometritis which go conform with fluid accumulation within the uterus are more easy to detect with currently used diagnostic methods [1-5]. Although several diagnostic tools such as transrectal palpation, ultrasonography of the reproductive tract, vaginal examination, uterine culture and cytology are available, the correct diagnosis of subclinical endometritis in the mare still constitutes a challenge under field conditions. It has been shown that bacteriological examination from a uterine swab alone is insufficient to obtain reproducible findings of repeated samplings [6-8]. Uterine culture alone might lead to either false positive or false negative results [6,9-11] without any information of the incidence of inflammatory processes. Recently, subclinical endometritis has been discussed as a reason for subfertility and assumed to be connected with false negative culture results [1,6]. Therefore, collecting endometrial cells with a cytological brush in order to evaluate the presence of polymorphonuclear neutrophils (PMN) within the uterine epithelium has been recommended to enhance diagnostic performance [8,12-14]. The presence of PMN in the stratum compactum of the endometrium has been accepted as 'best standard' to indicate exsudative endometritis [2,3,6,12]. In the last years the cytological brush has been demonstrated to be an efficient technique to obtain well-preserved endometrial cells [3,7,15-18]. However, sensitivity and specificity of both bacteriological and cytological examination was relatively low compared to the 'best standard' of PMN in the stratum compactum collected by endometrial biopsy [6,7,9]. Endometrial biopsy has been used for more than 40 years for the evaluation of the

health status of the uterus [19]. Histopathologically, the diagnosis of both degenerative and inflammatory lesions as well as combinations of these findings is one important aspect for the assessment of a mare´s fertility [20,21]. It has been demonstrated that fertility outcome is correlated with the presence of e.g. endometrosis (defined as age-associated, degenerative alterations of the uterine glands and their surrounding stroma), angiosclerosis (i.e. an age and parity related, degenerative lesion of the uterine blood vessels), endometrial maldifferentiation, endometrial atrophy, endometritis and lymphatic lacunae [20-24]. Therefore, endometrial histopathology has been used to predict foaling probabilities. Several studies have shown that a histological examination provides a reliable prognosis of a mare's fertility [6,9,25-27]. Therefore, endometrial biopsy has become an integral part of breeding soundness evaluation in the problem broodmare [26]. The results, however, need to be evaluated in the context of a complete reproductive examination, with regard to seasonal changes and the reproductive history of a mare [26-28]. It is unclear if these additional scores might cause bias in the evaluation. Although the procedure is invasive, requires special equipment and laboratories and time for evaluation, different studies underline the reliability of this diagnostic method [21,23,29].

Both techniques (i.e. cytological and histological examination) are supposed to represent the health status of the whole uterus. Several studies have investigated the representativeness of a single endometrial biopsy sample, demonstrating varying results [30-34]. Some older studies consider one histological sample representative for the health status of the whole uterus [30,31,34]. In contrast, more recent studies identified one biopsy sample not adequate to support a precise diagnosis [32,33]. These studies concentrated on degenerative changes and biopsy grades according to the categorization of Kenney and Doig [20] at different locations of the reproductive tract. To our knowledge, science-based information on the comparability of cytological examinations between different sampling locations within the equine uterus is not available.

Therefore, the objective of this study was to determine the agreement of PMN numbers from cytological and histological samples considering five different locations within a given uterus. We hypothesized 1) that one sample of either method would represent the actual health status of the whole uterus and 2) that PMN numbers correlate between the methods.

2.2.4 Materials and methods

The study was conducted between May and December 2010 and included 37 mares with unknown age and breeding history. The mares were slaughtered at an EU accredited abattoir and their reproductive tracts were removed immediately after death. Stage of cycle was determined by examination of the ovaries. The mare was regarded to be in diestrus or estrus when a corpus luteum or a follicle of ≥ 3.0 cm in diameter without a corpus luteum on either ovary was present, respectively. All samples were taken within 2 ± 1 h after slaughter. Cytological samples and endometrial biopsies were obtained from five defined locations of each uterus (A - E; Figure 1) always in the same order of sampling. Each uterus was cut open at location A, followed by locations B, C, D, and E for approximately 2 cm with a sterile scalpel. A cytological sample was collected with a cytological brush (Cytobrush Plus GT, Medscand Medical, Malmö, Sweden) by rotating the brush alternately to the right and to the left to obtain cellular material from the adjacent endometrium. The brush was introduced into the uterine lumen through the 2 cm incisions without touching the environment. Cells were collected from the dorsal uterine wall to avoid contamination with intrauterine fluid. Immediately after that, an endometrial biopsy was taken from the same location with a Kevorkian biopsy forceps (Eickemeyer, Tuttlingen, Germany). The forceps was introduced to the desired location and endometrial mucosa was guided into the forceps with a gloved hand from the outside. This order was maintained for the five locations and in each uterus, respectively. In total, 185 cytological and 185 histological samples were obtained and numbers of PMN were compared between cytological and histological examinations.

2.2.4.1 Cytological evaluation

Cytological brushes were smeared on glass slides (Mediware, Servoprax, Wesel, Germany) within 1 ± 1 min after harvesting. Smears were fixed (Haema-Fixierlösung, LT-SYS, Berlin, Germany) and stained (Haema-Schnellfärbung, LT-SYS, Berlin, Germany) within 1 h at the laboratory of the Clinic for Animal Reproduction, Faculty of Veterinary Medicine, Freie Universität, Berlin. The presence of PMN and epithelial cells was examined using light microscopy (BHS, Olympus, Hamburg, Germany) under oil immersion (X 1000). A total of 300 cells per smear were counted. Smears were regarded as indicative for inflammation if

PMN numbers were ≥ 2% [10,35]. A whole uterus was defined as positive for endometritis when ≥ 1 location was scored positive for inflammation according to the cytological examination.

2.2.4.2 Histological evaluation

After sampling, endometrial biopsies were fixed in 4% formaldehyde and sent to the Institute for Veterinary Pathology, University of Leipzig, Germany for histopathological evaluations. The biopsies were routinely processed in the Hypercenter XP (Co. Shandon, Frankfurt/Main), embedded in paraplast (Co. Vogel, Gießen), and histological slides with a thickness of 3 - 4 µm were cut with a microtome (Co. Reichert-Jung, Vienna, Austria). Every biopsy was examined by means of two slides using different methods: The first slide was routinely stained with Hemalaun-Eosin (H.-E.), the second one underwent an enzymehistochemical preparation for the demonstration of PMN by means of Naphtol-AS-D-Chlorazetat-Esterase (ClAE), in which PMN appear bright red (Figure 2) [36]. For the latter method, the production of the substrate solution started with the dissolution of 3.58 mg Naphtol-AS-D-Chloracetate (Co. Sigma, Bioscience, Nr. 0758, Munich) in 0.9 mL Dimethylsulfoxide (Co. Merk, Nr. 1.02952, Darmstadt) and 0.1 mL Triton X (Co. SERVA, Nr. 37238, Heidelberg) until the clear yellow colour has vanished. The process was continued with the preparation of a Hexazonium solution, i.e. the dissolution of 50 mg Pararosanilin (Co. Sigma, Bioscience, Nr. P-3750, Munich) in 3 mL 1 N HCl (Co. Merck, Nr. 1.09057, Darmstadt) + 0.5 mL 1 M Natriumnitrite (Co. Roth, Nr. 8604.1, Karlsruhe) solution; usable after 5 min when the colour changed from brown to yellow. The preparation of the incubation fluid included the addition of 1 mL substrate solution under constant agitating in 100 mL Phosphat-buffered saline (pH = 7.2) + 0.5 mL Hexazonium solution until a pink colour change appeared. Incubation of the slides was done for 1.5 h at 37°C in the incubation fluid. Afterwards, the slides were rinsed in tap water and Aqua destillata. For the counterstaining, the slides remained in Mayer's Haemalaun-solution for 5 min. They were blued in tap water for 10 min and in Aqua destillata for 5 min. Slides were dried and automatically covered with Xylol-coated film (Tissue-Tek©, Co. Sakura, Staufen). A skin biopsy from a horse with purulent dermatitis served as positive control.

Evaluating the biopsies was done as a blank test with a standard light microscope (Co. Olympus, Hamburg), considering inflammatory alterations, endometrosis, angiosclerosis and

lymphatic lacunas. Special regard was paid to the documentation of the localization, cell type, degree and distribution of inflammatory cells. For a quantitative analysis of PMN, these cells were counted in slides prepared with ClAE (Figure 2). In each slide, three randomly selected high power fields (HPF) in the stratum compactum including luminal epithelium and in the stratum spongiosum were examined, using a standard light microscope (X 40 magnification; Co. Olympus, Hamburg).

A sample was scored as positive for exsudative inflammation when ≥ 5 PMN were detected in 3 HPF [37-39]. The whole uterus was regarded as positive for endometritis if ≥ 1 location was scored positive for inflammation according to the histological results. Furthermore, each biopsy sample was classified regarding the degree of endometrosis and angiosclerosis on a 4 - point scale (0 = none, 1 = mild, 2 = medium, 3 = severe). These results are shown in table 1. Parameters were compared between the five different locations within one uterus (Table 2).

2.2.4.3 Statistical analysis

All clinical findings and results from the laboratories were documented on data capture forms and transferred into a spreadsheet (Excel 2003, Microsoft, Munich, Germany). Descriptive statistical analyses were carried out with the SPSS statistic package (SPSS for Windows 16.0, SPSS Inc., Munich, Germany). Categorical variables (i.e. endometrosis and angiosclerosis) were compared using Pearson Chi-square test in cross tab function, whereas continuous variables (i.e. PMN numbers of histological and cytological examination) were compared using the Kruskal-Wallis test. Cohen's k coefficient was calculated to evaluate the agreement between PMN numbers of cytological and histological examination. The agreement was considered: poor if $k \leq 0.2$, fair if $0.21 < k < 0.4$, moderate if $0.41 < k < 0.6$, substantial if $0.61 < k < 0.8$, good if it exceeds $k > 0.8$ [17]. Level of significance was set at a P value ≤ 0.05.

2.2.5 Results

2.2.5.1 Clinical findings

Imminent signs of estrus (i.e. follicles ≥ 3 cm) were observed in 10 of 37 genital tracts. Corpora lutea indicative for diestrus were detected in 27 genital tracts. Three of 37 uteri contained 50 to 200 mL of white to light yellow cloudy fluid and were classified as macroscopically positive for exsudative endometritis. These three uteri were also scored positive for endometritis in cytological and histological examination.

2.2.5.2 Cytological findings

Overall, 10 of 37 uteri were cytologically positive for endometritis (27.0%). The remaining 27 uteri (73.0%) were scored negative for endometritis. In total, 27 of 185 single samples were positive for inflammation (14.6%) (Table 1). Eight of the 10 positive scored uteri had both, endometritis positive and endometritis negative locations. Variations of numbers of PMN within the five locations, however, were not significant ($P = 0.78$). Five of the 10 cytologically positive scored uteri were also diagnosed positive in histological examination. Twelve out of 27 positive single samples were scored positive both by cytological and histological examination.

2.2.5.3 Histological findings

Based on the histological presence of PMN in the stratum compactum, 14 of 37 uteri were scored positive for exsudative inflammation. Thirteen of the 14 positive scored uteri (92.9%) had both endometritis positive and endometritis negative locations. In one uterus all five sampling locations were scored positive for inflammation. In total, 33 of 185 single samples were found positive for inflammation (17.8%) (Table 1). Additionally, the histological examination found 30 locations positive for lymphoplasmacellular inflammation (16.2%). The agreement between the two diagnostic methods regarding positive or negative exsudative endometritis scores at a chosen sampling location was $k = 0.29$ ($P < 0.01$). Variation of PMN numbers within the five locations, however, was not significant ($P = 0.79$). Agreement of

grades of endometrosis and angiosclerosis between the different locations within one uterus is shown in table 2.

2.2.6 Discussion

Subclinical endometritis is well known to constitute one of the major problems in the management of broodmares. The commonly used diagnostic technique of uterine culture to diagnose endometritis in the field has been demonstrated to produce false negative as well as false positive diagnoses [6-8]. Several studies have shown that cytology is a more reliable diagnostic method for clinical [6,35,40] and subclinical endometritis [7] than uterine culture. In this context it is crucial to considerate that the detection of bacteria by means of uterine culture must not be equalized with the presence of an endometritis. The interpretation of the results of complementary examination methods is the basis for a diagnosis as comprehensive as possible. Besides, information on variation of inflammatory processes between different sampling sites within a given uterus, however, is not available. Therefore, the objective of the present study was to determine if one cytological or one histological sample is representative for the health status of the whole uterus while sampling five different, pre-defined locations. Additionally, we compared results of cytological examinations in regard to PMN numbers with those of endometrial biopsies at the corresponding locations.

In this study, five locations per uterus (n = 37) were sampled by means of cytobrush and biopsy. Overall 27 of 185 cytological samples were indicative for inflammation (Table 1). Our threshold for cytologically positive smears (PMN ≥ 2%) was chosen because of the small number of PMN in the uterus of healthy mares that should be considered physiologically, depending on the stage of the sexual cycle (i.e. estrus) [35,41,42]. A uterus was defined as positive for endometritis when at least one of the five sampled locations was scored positive for the counts of PMN defined above, resulting in 10 of 37 uteri positive for exsudative endometritis by cytological examination. By means of histological examination 33 of 185 samples were found positive for inflammation (Table 1), resulting in 14 of 37 uteri scored positive for endometritis. Altogether, only 12 of the 33 histologically positive samples were detected as positive by cytological examination as well. The agreement of PMN numbers

between the cytological and histological examinations at a chosen sampling location was fair ($k = 0.29$; $P < 0.01$). This observation helps to explain the low sensitivity and specificity of cytology compared to PMN numbers in histological examination in previous studies [6,7]. The sampled locations in vivo of a cytological specimen and a biopsy sample are with high probability of a not comparable location. Additionally, a recent study showed a similar agreement of $k \leq 0.3$ between three cytological collection techniques [17]. Furthermore, studies of Schoon et al. showed that especially non-purulent endometritis and degenerative changes (i.e. endometrosis, angiopathies, endometrial atrophy, etc.) as crucial factors for fertility disturbances can only be assessed by means of microscopically examination of an endometrial biopsy [21,23,24]. In eight of the 10 cytologically positive uteri and in 13 of the 14 histologically positive uteri both, positive and negative endometritis scores could be detected abreast. These results indicate that an inflammatory process is not spread uniformly within the endometrium. The inconsistent PMN numbers within one uterus are in agreement with an earlier study by Blanchard et al. [31], that also showed variability in the occurrence and severity of inflammation between different sampling locations. In their study the uteri of five mares were sampled at 12 locations and examined for degenerative parameters and inflammatory cells. The authors conclude that histological categorization according to Kenney and Doig [20] might change within one uterus if only inflammation would be considered into categorization. The repeatability of the categorization of endometrial biopsy samples has been discussed controversially. In contrast to more recent studies, that consider a single biopsy sample as not representative for the health status of the whole uterus [32,33], some older studies hold the oppositional view [30,31,34]. In the genital tracts of 49 mares with 13 biopsies taken from each uterus, Runge [43] showed that the samples of one uterus can be classified relatively uniform in one or two categories. Only mares with none or high-grade pathological changes were assigned to the same category, while the graduation of endometria with moderate lesions differed considerably [43]. In our study, histological samples could not be classified into the four categories according to Kenney and Doig [20] due to the unknown breeding history (particularly time of barrenness) of the mares. Therefore, we compared the criteria endometrosis and angiosclerosis (Table 1). The agreement within the five locations concerning the scores for endometrosis and angiosclerosis is shown in table 2. The percentages of identically scored locations within one uterus were 13.5% for endometrosis and 24.3% for angiosclerosis, respectively. Differences between the five locations were not significant ($P = 0.96$; $P = 0.67$). Gross and LeBlanc (1984) sampled mares in monthly intervals and demonstrated that seasonal changes influenced the degree of

endometrosis in the endometrium resulting in a change of the prognostic category [44]. A variation in endometrosis was also observed in another study after experimental intrauterine infection with *Streptococcus equi subsp. zooepidemicus* and appropriate treatment [45]. Therefore, it has been postulated that diagnosing endometritis needs the context of a complete reproductive examination, with regard to the reproductive history of a mare and seasonal changes [26-28]. Additionally, a study of Ricketts and Alonso [46] showed that the assessment of biopsy categories might change after a specific uterine treatment or after sexual rest [20,47]. They recommend a second biopsy examination after an indicated treatment to generate a sound breeding prognosis [46].

As demonstrated in the present study, the examination of the endometrial surface with the cytological brush to determine PMN numbers shows only fair agreement with the occurrence of PMN in the stratum compactum of endometrial biopsy specimens of the same sampling location ($k = 0.29$; $P < 0.01$). While it has been demonstrated that the cytological examination as a non-invasive diagnostic method is useful to collect well-preserved cells [3,7,15-17], previous studies show that cytology is not able to reach the accuracy of the endometrial biopsy results even for identical locations within the uterus. Furthermore, it is important to consider that the histopathological evaluation is able to detect any type of inflammation, purulent forms as well as non-purulent forms [6]. Especially concerning the differentiation of variable types of inflammation, endometrial biopsy is founded 'best standard' to diagnose endometrial inflammation [6,20,21]. Nevertheless, when comparing a standard with a new method, it is important to recognize that neither approach might be ideal. Regardless, we have to reject the hypothesis that PMN numbers correlate between the two diagnostic methods at a given location.

In conclusion, PMN numbers of a cytological examination of the endometrium showed fair agreement to the occurrence of PMN in the stratum compactum of the histological examination at the same sampling location. However, there were no significant differences in PMN numbers of cytological as well as histological examinations within the different locations of the uteri. Additionally, degenerative changes (i.e. endometrosis, and angiosclerosis) did not show significant differences within the different locations. Implying that, under clinical conditions, the biopsy is taken from the dorsal aspects of the junction between corpus and cornua uteri or one of the horns as proposed by Runge [43], the decision about further sampling of biopsies should be critically considered regarding quality and distribution of the lesions diagnosed by means of cytological and microscopical examination.

Nevertheless, further research is warranted to determine the number of sampling locations in cases of different types of endometritis because the results of this study show an inhomogeneous distribution pattern of inflammatory alterations in the uterus. It is desirable to optimize the interaction of all diagnostic performances while maintaining practical feasibility.

2.2.7 Figures and tables

Figure 1: Sampling locations: (A) uterine body; (B) tip of left horn; (C) basis of left horn; (D) basis of right horn; (E) tip of right horn.

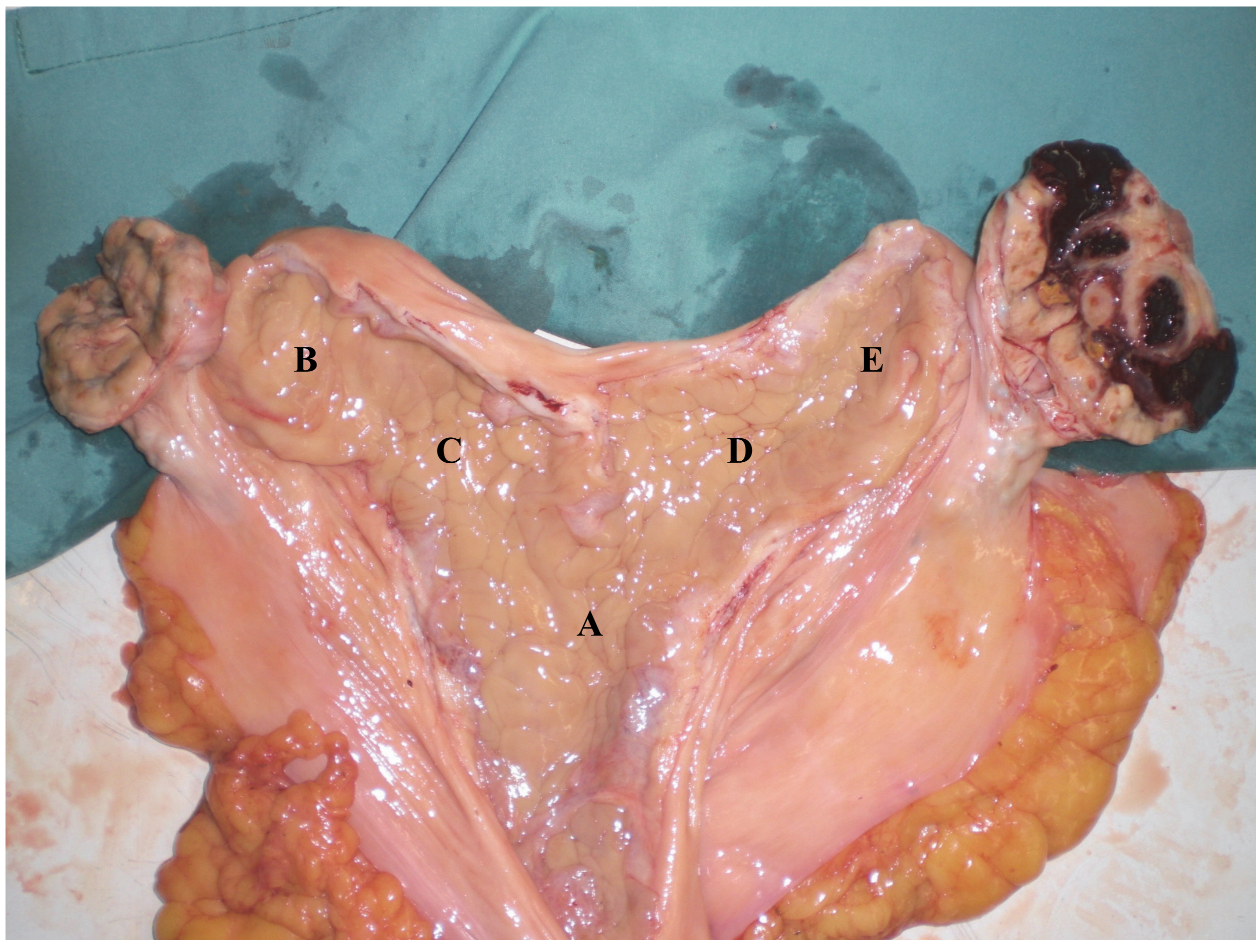

Figure 2: Histological evaluation of PMN with ClAE staining. PMN appear bright red.

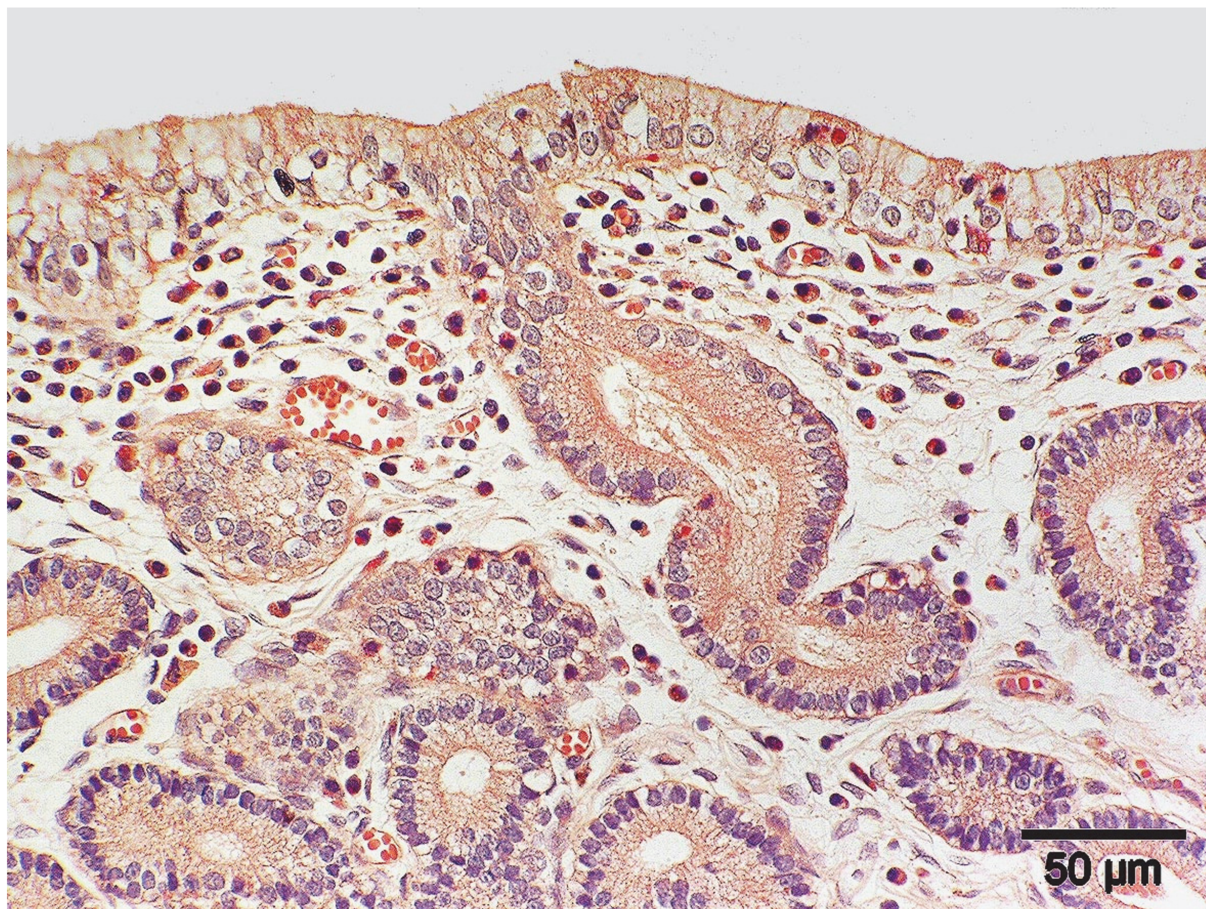

Table 1: Numbers of PMN counted in cytological samples and ClAE staining, as well as scores of endometrosis and angiosclerosis at the different locations.

	Location																			
Uterus	A				B				C				D				E			
	PMN in C.	PMN in H.	E. score	A. Score	PMN in C.	PMN in H.	E. Score	A. Score	PMN in C.	PMN in H.	E. Score	A. Score	PMN in C.	PMN in H.	E. Score	A. Score	PMN in C.	PMN in H.	E. Score	A.Score
1	4	0	2	0	0	0	1	1	0	0	2	1	0	0	2	1	1	1	2	1
2	0	0	2	2	0	0	1	2	1	1	2	2	0	0	1	2	0	0	2	2
3	0	6[2]	1	0	3	5[2]	1	0	0	8[2]	2	0	1	4	1	1	1	3	2	0
4	0	0	0	3	0	0	0	2	0	0	1	2	0	0	1	3	0	0	0	3
5	2	5[2]	2	2	3	2	2	2	1	2	2	3	2	2	1	2	4	2	2	3
6	2	3	2	1	2	0	2	1	0	0	3	1	0	0	2	1	2	1	2	0
7	0	0	0	1	0	0	2	0	1	0	0	0	0	1	0	1	2	0	0	1
8	3	1	1	0	0	2	1	0	3	0	1	0	1	1	1	0	0	2	0	0
9	7[2]	5[2]	2	1	2	5[2]	2	1	5	1	3	2	4	2	3	2	2	11[2]	3	0
10	0	5[2]	2	0	7[2]	7[2]	2	1	8[2]	6[2]	2	0	4	10[2]	1	1	2	4	2	0
11	4	0	2	2	4	1	2	2	1	0	2	0	n.e.[1]	0	2	2	0	3	2	1
12	4	1	1	0	1	3	0	1	2	2	0	0	0	24[2]	0	0	3	3	0	1
13	0	22[2]	1	1	1	0	1	1	0	0	1	2	3	1	0	0	2	5[2]	1	0
14	4	0	2	2	1	1	2	1	0	0	1	1	0	0	1	2	0	1	2	1
15	10[2]	0	2	3	12[2]	0	2	3	4	0	3	3	25[2]	0	2	3	7[2]	0	3	3
16	0	1	2	2	0	0	2	0	0	0	1	0	0	0	2	2	0	1	2	0
17	10[2]	3	2	3	0	3	2	3	0	6[2]	2	3	13[2]	5[2]	2	2	7[2]	8[2]	2	2
18	0	0	2	2	0	0	1	2	0	0	2	2	0	0	3	2	0	0	2	2
19	1	0	1	0	0	0	0	2	0	0	1	2	0	0	0	1	0	0	0	0
20	1	0	2	2	7[2]	0	3	2	3	0	2	1	1	0	2	2	0	0	2	0
21	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2	0
22	1	1	0	2	0	4	2	2	0	2	1	3	5	7[2]	1	2	0	7[2]	2	3

23	1	5[2]	3	1	0	0	2	0	1	0	2	2	0	2	3	0	0	3	3	1
24	6[2]	2	0	2	0	3	0	0	48[2]	1	0	2	5	2	1	2	7[2]	0	0	2
25	35[2]	9[2]	0	0	76[2]	5[2]	1	1	81[2]	31[2]	0	2	115[2]	11[2]	2	0	63[2]	7[2]	1	1
26	0	8[2]	1	0	0	16[2]	1	0	0	11[2]	1	0	0	13[2]	2	0	0	3	1	0
27	0	4	1	0	0	3	2	0	0	5[2]	2	0	0	1	2	0	0	2	1	0
28	0	2	2	0	0	4	2	0	0	2	2	0	0	0	2	0	0	1	2	0
29	0	3	1	3	0	1	2	3	1	0	1	3	0	3	2	2	1	4	2	3
30	5	0	1	2	2	3	0	2	4	2	0	2	0	2	0	2	0	1	0	3
31	0	3	0	0	0	5[2]	1	0	0	1	0	0	0	2	0	0	0	3	0	1
32	2	0	1	2	2	0	2	2	0	3	2	3	0	3	1	2	0	2	2	2
33	4	0	1	1	2	2	1	2	0	0	2	2	2	0	1	2	3	2	1	2
34	5	0	2	1	6[2]	4	2	1	7[2]	0	2	0	4	1	2	0	5	1	2	1
35	2	0	2	0	0	0	2	0	0	1	2	0	6[2]	1	1	1	4	0	1	0
36	0	3	2	0	3	1	2	0	1	0	2	0	1	2	2	0	0	0	1	0
37	300[2]	4	2	2	46[2]	3	2	2	158[2]	22[2]	1	3	260[2]	14[2]	2	0	212[2]	2	2	2

PMN in C. = PMN numbers in cytology: [1)] n.e. = not evaluated; [2)] positive for endometritis (≥ 2%)

PMN in H. = PMN numbers in histology: [2)] positive for endometritis (≥ 5 PMN/3HPF)

E. Score = Endometrosis score: 0 = none; 1 = mild; 2 = moderate; 3 = severe

A. Score = Angiosclerosis score: 0 = none; 1 = mild; 2 = moderate; 3 = severe

Table 2: Agreement of grades of endometrosis and angiosclerosis between the five locations within the uteri of 37 mares.

	Number of uteri graded as positive for			
Type of assessment	Endometrosis		Angiosclerosis	
	Absolute	%	Absolute	%
Identical score of all 5 locations	5	13.5	9	24.3
Identical score (± 1) of all 5 locations	33	89.2	27	73.0

2.2.8 References

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3. DISCUSSION

The overall objective of this study was to test and compare the currently available diagnostic methods for the diagnosis of subclinical endometritis in broodmares. The first study was conducted to compare the ability and reliability of the commonly used diagnostic techniques, i.e. endometrial swab and endometrial biopsy, with the new diagnostic method cytological brush to diagnose subclinical endometritis in mares. In the second study the representativeness of cytology and histology was evaluated in identifying inflammatory processes within different locations of the equine uterus.

To evaluate the sensitivity and specificity of the three diagnostic techniques in the first study, histological examination of endometrial biopsy specimens were used as 'best standard'. Although the presence of polymorphonuclear neutrophils (PMN) in the stratum compactum of endometrial biopsy specimen is commonly accepted as 'best standard' to diagnose endometritis in the mare (Reiswig et al., 1993; Bourke et al., 1997; Nielsen, 2005; LeBlanc et al., 2007), endometrial biopsy is used less frequently due to its invasiveness. We hypothesized the cytological brush also to be a reliable technique in diagnosing subclinical endometritis, which is, in contrast to endometrial biopsy, easy to perform under field conditions. Compared to the histological detection of PMN in the stratum compactum as 'best standard', sensitivity of cytological examination was 0.17, 0.00 and 0.25 for cytological brush, endometrial swab and endometrial biopsy, respectively. Sensitivity of bacteriological examination was 0.25, 0.33 and 0.25 for cytological brush, endometrial swab and endometrial biopsy, respectively. Thus, the bacteriological examination of an endometrial swab reached highest sensitivity in the first study. Nielsen (2005) reported a comparable sensitivity of uterine culture (0.34). Although earlier studies showed that cytology detects twice as many mares with endometritis as did endometrial culture (Digby and Ricketts, 1982; Riddle et al., 2007), in our study both methods reached high rates of false negative results. This underlines the data of previous studies (Digby and Ricketts, 1982; LeBlanc et al., 2007; Riddle et al., 2007; Nielsen, 2010), demonstrating that the endometrial swab might be insufficient for a precise diagnosis. The false negative results might be caused by missing the infected area while sampling. False negative cytological results might be caused by gram-negative bacteria like *Escherichia coli (E. coli)*. These bacteria are able to circumvent the cellular defence system by embedding themselves in high contents of mucus (LeBlanc and Causey, 2009). The types of bacterial pathogens isolated from the endometrium of mares in our study were similar to those reported

in earlier studies (Allen, 1979; Bain, 1966; Digby and Ricketts, 1982). *Streptococcus zooepidemicus (Strep. zooepidemicus)* was isolated in 45.5% of the samples. In 54.5% of the samples the isolated pathogen was *E. coli*. The percentage of cytological samples indicative of inflammation varies between the different bacteria species. Mares infected with *Strep. zooepidemicus* showed positive cytological results in 40.0% (2/5). In contrast, none of the six *E. coli* positive sampled mares showed evidence of inflammation in cytological examination. These results agree with findings of Riddle et al. (2007), who reported that infections with coliforms like *Pseudomonas*, *Proteus* and *E. coli* showed a significantly lower evidence of inflammation in cytological examination. Furthermore, these results coincide with a study of Ricketts and Mackintosh (1987), demonstrating that in only 12.0% of uterine culture samples positive for anaerobes, cytological evidence of inflammation was associated.

The results of the first study emphasize that in estimating subclinical endometritis, the cytological brush and the uterine swab are not able to reach the accuracy of the histological examination as 'best standard' method to assess inflammatory processes. With a combination of cytology and bacteriology the number of mares identified to have subclinical endometritis was significantly higher (0.42, $p < 0.01$) compared to one technique alone. Therefore, the combination of both diagnostic techniques evaluated out of one cytological brush sample might be the most easy and promising approach for diagnosing subclinical endometritis under field conditions.

The second study was an in vitro examination of equine uteri. It was conducted to test the representativeness of cytological and histological examinations in five different locations of the equine uterus and to compare PMN numbers of cytological examinations with those of histological examinations at the same sampling location. We hypothesized that one cytological or one histological sample alone is representative for the health status of the whole uterus.

In the past few decades the determination of the reproducibility of histopathological examinations at different locations of equine uteri resulted in controversial conclusions. Some older studies considered one histological sample as representative for the whole uterus (Bergman and Kenney, 1975; Blanchard et al., 1987; Waelchli and Winder, 1989; Runge, 1995). In contrast, recent studies identified one biopsy sample as not significant for the health status of the whole uterus (Dybdal et al., 1991; Fiala et al., 2010). Endometrial biopsy

samples are commonly classified into four grades according to the degree of endometrial degeneration (Kenney and Doig, 1986). In our study, histological samples could not be classified into the four categories due to the unknown breeding history (particularly time of barrenness) of the mares. We compared the specific criteria endometrosis and angiosclerosis between the five different locations of the uteri. The percentages of uteri with five identical scored locations were 13.5% and 24.3% for endometrosis and angiosclerosis, respectively. Nevertheless, there was no significance in the tested variation within the five locations for endometrosis ($p = 0.96$) and angiosclerosis ($p = 0.67$). Runge (1995) showed by means of 13 samples per uterus that one uterus can be classified relatively uniform in one or two categories. Means, in mares with no changes or high-grade pathological changes the different locations were assigned to the same category. In contrast, the categorization of endometria with moderate lesions, however, differed considerably (Runge, 1995). This result is underlined by our study. If the prognostic category is allowed to variate in one grade, the percentage of identically scored uteri rises to 89.2% and 73.0% for endometrosis and angiosclerosis, respectively. In cytological and histological examination a whole uterus was defined as positive for endometritis when at least one of the five sampled locations was scored as positive for the counts of PMN. In cytological examination 10 of 37 uteri revealed a positive score for endometritis with 27 samples indicative for inflammation (PMN ≥ 2%) out of 185 in total. By means of histological examination 33 of 185 samples were found positive for inflammation (≥ 5 PMN/3 high power fields in 40 x magnification), resulting in 14 of 37 uteri scored positive for endometritis. Overall, only 12 of the 33 histologically positive scored samples were also detected as positive by cytological examination. The agreement of PMN numbers between the cytological and histological examinations at a chosen sampling location was fair ($k = 0.29$; $p < 0.01$). These results emphasize that the cytological brush is not able to indicate the correct status of endometritis in the endometrium at a specific location. We have to reject the second hypothesis that PMN numbers correlate between the two diagnostic methods at a chosen location. This observation underlines the results of previous studies. It helps to explain the low sensitivity and specificity data of cytology compared to PMN numbers in histology (Nielsen, 2005; Overbeck et al., 2011). Additionally, another study showed a similar agreement of $k \leq 0.3$ between three different cytological collection techniques within one uterus (Cocchia et al., 2012).

Moreover, in eight of the 10 cytologically positive uteri and in 13 of the 14 histologically positive uteri both, positive and negative endometritis scores could be detected in our study. That shows, taking samples at different locations of one uterus might provide different

amounts of inflammatory cells resulting in different endometritis scores within one uterus. These results indicate that an inflammatory process is not spread uniformly within the endometrium. This is in agreement with an earlier study by Blanchard et al. (1987). They also showed variability in the occurrence and severity of inflammation between different sampling locations. The authors conclude that histological categorization according to Kenney and Doig (1986) might change within one uterus if only inflammation would be considered. As demonstrated by some other studies, the categorization of a mare's endometrium might change because of seasonal changes, infection or sexual rest (Gross and LeBlanc, 1984; Ricketts and Alonso, 1991; Keller et al., 2006). Therefore, it has been postulated that diagnosing endometritis needs the context of a complete reproductive examination, with regard to the reproductive history of the mare and the seasonal situation (Gordon and Sartin, 1978; Shideler et al., 1982; Van Camp, 1988). These conclusions and our findings of inhomogeneous distributions of PMN and degenerativ alterations emphazise that the results of both, cytobrush and endometrial biopsy samples, should be analyzed critically for the prediction of a mares fertility.

In conclusion, the second study shows that sampling different locations of the equine uterus results in varying PMN numbers and degenerative changes. Although there were no significant differences in PMN numbers of cytological and histological examinations as well as in degenerative changes, the prognostic category according to Kenney and Doig (1986) might change within one uterus. Endometrial biopsy is commonly accepted as 'best standard' to diagnose purulent as well as non-purulent forms of endometritis and degenerative changes (i.e. endometrosis, angiopathies, endometrial atrophy, etc.) in the mare. However, this study emphazises that the result of an endometrial biopsy examination should also be critically evaluated. This thesis shows that, alternatively, the combination of a bacteriological and a cytological examination of the cytological brush is the most easy and promising approach for diagnosing subclinical endometritis in the field. Further research is needed to optimize the interaction of the currently available diagnostic techniques in diagnosing subclinical endometritis in the mare.

4. SUMMARY

Wiebke Overbeck: Validation of three diagnostic techniques to diagnose subclinical endometritis in mares

Endometritis in the mare still constitutes a great problem in the management of broodmares. Inflammatory processes of the equine uterus can be classified as acute, chronic or subclinical endometritis. All these conditions may cause substantial reductions of a mare's fertility. In recent years the problem of subclinical inflammatory processes with hidden or subtle clinical signs became more obvious. Although several diagnostic methods such as rectal palpation, transrectal ultrasonography, vaginal examination, uterine culture, cytology or endometrial biopsy are available, the correct diagnosis of subclinical endometritis challenges practitioners in the field. Therefore, the overall objective of this study was to evaluate the ability, the reliability and the representativeness of the commonly used diagnostic techniques endometrial swab, cytological brush and endometrial biopsy to diagnose subclinical endometritis in mares.

In the first study bacteriological, cytological and histological samples were taken from 55 mares without clinical signs of endometritis. A double-guarded uterine swab (US), a single-guarded cytological brush (CB) and an endometrial biopsy (EB) were taken consecutively under manual control, smeared on microscopic slides for cytological examination and cultured for bacterial growth. Additionally, endometrial biopsy samples were evaluated for polymorphonuclear neutrophils (PMN) in the stratum compactum by a histopathological institute. Bacteriological cultures and cytological samples were classified as negative (no uterine pathogens in monoculture; < 2% PMN) or positive (uterine pathogens in > 90% of the grown colonies; > 2% PMN) for endometritis. Uterine pathogens were found in 20.0% of the mares. Isolation of pathogens was not associated with positive cytological findings ($r = -0.23$; $p = 0.87$). Two of five mares infected with *Streptococcus zooepidemicus* had a positive cytological result. In contrast, none of the six mares with an *Escherichia coli* infection showed a positive cytological result. The histological presence of PMN in the stratum compactum of the endometrium is commonly accepted as 'best standard' to diagnose endometritis in mares. Compared to the 'best standard', sensitivity of cytology of CB, US and EB was 0.17, 0.00 and 0.25, respectively. Sensitivity of uterine culture was 0.25, 0.33 and 0.25 for CB, US and EB, respectively. Cytology in combination with a bacteriological

examination of a cytological brush sample resulted in the best sensitivity of 0.42. Based on this result, a combination of a bacteriological and a cytological examination of a cytological brush sample can be recommended as an easy to perform technique to improve the diagnosis of subclinical endometritis in the field compared to the uterine culture as currently used routine method alone.

The objective of the second study was to determine the distribution of PMN within different sampling locations of the equine uterus by cytological and histological examinations and to compare PMN numbers of the two techniques at the corresponding locations. We hypothesized that one sample of either method would represent the actual health status of the whole uterus and that PMN numbers correlate between the methods. Cytological and endometrial biopsy samples were taken in vitro from 37 uteri within 2 ± 1 h after slaughter from five pre-defined locations of each uterus. Cytological samples were smeared on microscopic slides, stained and classified as negative (< 2% PMN) or positive (≥ 2% PMN) for endometritis. Histologically, the number of PMN were counted by an experienced pathologist (≥ 5 PMN/3 high power fields in 40 x magnification = positive for endometritis) and grades of endometrosis ('-' to '+++') and angiosclerosis ('-' to '+++') were evaluated. The prevalence of positive cytological and histological samples was 14.6% and 17.8%, respectively. A fair agreement between the two diagnostic methods could be detected ($k = 0.29$; $p < 0.01$). A whole uterus was defined as positive for endometritis when at least one of the five sampled locations was scored as positive for the counts of PMN. A total of 10 and 14 uteri generally positive for endometritis could be detected in cytological and histological examination, respectively. Numbers of PMN differed between the sampling locations resulting in positive and negative locations within the positive uteri, in both, cytological (8/10) and histological examination (13/14). Nevertheless, significant differences of endometritis positive or negative scores within the different locations could not be detected, neither in cytological ($p = 0.56$) nor in histological examinations ($p = 0.1$). Additionally, no significant differences in classification of endometrosis ($p = 0.96$) and angiosclerosis ($p = 0.67$) within the locations could be observed.

In conclusion, inflammatory processes are not spread uniformly within the endometrium. Also, the hypothesis that PMN numbers correlate between the two diagnostic methods at a chosen location has to be rejected. Further research is warranted to determine the optimal number of sampling locations in cases of different types of endometritis while maintaining practical feasibility.

Overall, this thesis clearly showed that a single bacteriological or a single cytological examination of the uterus might produce false negative results to identify subclinical endometritis in mares. This might be due to the inhomogenous distribution pattern of PMN within the endometrium. And because of this, the prognostic category according to Kenney and Doig (1986) could also change within one uterus. Therefore, the results of all tested diagnostic techniques should be critically evaluated for the prediction of a mares fertility. The combination of a bacteriological and a cytological examination of the cytological brush is the most easy and promising approach for diagnosing subclinical endometritis in the field. Further research is needed to determine whether the combination of two diagnostic techniques or multiple sampling of the endometrium might improve the reliability of the diagnosis of subclinical endometritis in the mare.

5. ZUSAMMENFASSUNG

Wiebke Overbeck: Überprüfung dreier diagnostischer Methoden zur Diagnose der subklinischen Endometritis der Stute

Die Endometritis spielt im Management von Zuchtstuten eine sehr bedeutende Rolle. Entzündliche Prozesse können in akute, chronische oder subklinische Endometritiden unterteilt werden. Jede dieser Veränderungen kann die Fruchtbarkeit der betroffenen Stute erheblich negativ beeinflussen. In den letzten Jahren hat das Problem der subklinischen Endometritis mit ihren fehlenden oder nur im geringen Ausmaß vorhandenen klinischen Anzeichen mehr und mehr an Bedeutung gewonnen. Obwohl es einige diagnostische Möglichkeiten wie rektale Palpation, transrektaler Ultraschall, vaginale Untersuchung, Uterustupfer, -zytologie und -biopsie gibt, ist es für den Praktiker dennoch schwierig die subklinische Endometritis zu diagnostizieren. Ziel dieser Arbeit war es deshalb, die drei etablierten diagnostischen Methoden Uterustupfer, -zytologie und -biopsie auf ihre Fähigkeit, ihre Verlässlichkeit und ihre Wiederholbarkeit bei der Diagnose von subklinischen Endometritiden zu testen.

In der ersten Studie wurden dazu je eine Tupfer-, eine Zytologie- und eine Biopsieprobe aus dem Uterus von 55 Stuten, die keine klinischen Anzeichen einer Endometritis zeigten, genommen. Die Proben wurden mit einem doppelt behüllten Uterustupfer, einem einfach behüllten Zytologiebürstchen und einer Biopsiezange entnommen. Danach wurden sie zuerst für die zytologische Auswertung auf sterilen Objektträgern und anschließend auf entsprechenden Nährmedien ausgestrichen, um auch das bakterielle Wachstum beurteilen zu können. Zusätzlich zu den bereits genannten Untersuchungen wurden in den Biopsieproben die Anzahlen an neutrophilen Granulozyten (PMN) im Stratum compactum des Endometriums durch ein pathologisches Institut bestimmt. Die bakteriologischen und zytologischen Proben wurden entweder in 'Endometritis negativ' (keine pathogenen Keime in Monokultur; < 2% PMN) oder in 'Endometritis positiv' (pathogene Keime in > 90% des Wachtums; > 2% PMN) eingeteilt. Pathogene Keime wurden in 20% der Stuten gefunden. Positive Ergebnisse in der Zytologie waren nicht mit einem gleichzeitigen Auftreten von pathogenen Keimen verbunden ($r = -0,23$; $p = 0,87$). Zwei von fünf Stuten, die mit *Streptokokkus zooepidemicus* infiziert waren, zeigten ein positives zytologisches Ergebnis.

Die sechs Stuten, die mit *Escherichia coli* infiziert waren, wiesen hingegen allesamt kein positives zytologisches Ergebnis auf. Das Vorkommen von PMN im Stratum compactum des Endometriums ist bei der Stute als der 'best standard' zur Diagnose von Endometritiden akzeptiert. Bezogen auf den 'best standard' erreichten die Zytologieproben von Tupfer, Zytologiebürstchen und Biopsie eine Sensitivität von 0,17, 0,00 und 0,25. Bei der Auswertung der Bakteriologie erreichten Tupfer, Zytologiebürstchen und Biopsie eine Sensitivität von 0,25, 0,33 und 0,25. Die beste Sensitivität (0,42) wurde durch die Kombination von zytologischer und bakteriologischer Auswertung eines Zytologiebürstchens erreicht. Im Vergleich zu der bisherigen Routineuntersuchung des Tupfers zeigt dies, dass die kombinierte zytologische und bakteriologische Auswertung eines Zytologiebürstchens empfohlen werden kann. Die Diagnose von subklinischen Endometritiden würde dadurch in der Praxis deutlich verbessert werden.

Ziel der zweiten Studie war es, die Verteilung von PMN an verschiedenen Stellen in einem Uterus mittels Zytologiebürstchen und Biopsie zu untersuchen und die Anzahl der PMN zwischen den zwei diagnostischen Methoden an der jeweils selben Stelle zu vergleichen. Unsere Hypothesen waren, dass eine einzelne getestete Lokalisation eine verlässliche Aussage für den gesamten Uterus liefern kann, und dass die Anzahl der PMN zwischen den zwei Methoden für jeweils dieselbe Stelle miteinander korreliert. Die zytologischen Proben und Biopsien wurden von fünf vorher definierten Stellen aus 37 Uteri innerhalb von 2 ± 1 h nach der Schlachtung und Entfernung des Uterus aus dem Schlachtkörper entnommen. Anschließend wurden sie auf Objektträgern ausgestrichen, gefärbt und als 'Endometritis negativ' (< 2% PMN) oder 'Endometritis positiv' (≥ 2% PMN) beurteilt. Die Biopsien wurden von einem pathologischen Institut hinsichtlich der Anzahl an PMN (≥ 5 PMN/3 Gesichtsfelder in 40 x Vergrößerung = 'Endometritis positiv') wie auch auf das Vorkommen einer Endometrose und Angiosklerose (Einteilung in Grad '-' bis '+++') ausgewertet. Es wurden in 14,6% 'Endometritis-positive' Zytologieproben und in 17,8% 'Endometritis-positive' histologische Proben gefunden. Das ergibt eine mäßige Korrelation von $k = 0.29$ ($p < 0.01$). Ein Uterus wurde im Gesamten als 'Endometritis-positiv' bewertet, sobald einer seiner fünf beprobten Lokalisationen als positiv befunden wurde. Insgesamt wurden in der zytologischen 10 und in der histologischen Auswertung 14 Uteri als 'Endometritis-positiv' bewertet. Die Anzahl an PMN variierte, so dass es innerhalb dieser als 'Endometritis-positiv' befundeten Uteri, sowohl 'Endometritis-positive' als auch 'Endometritis-negative' Lokalisationen gab. Dies war in 8/10 Uteri bei der zytologischen und in 13/14 Uteri bei der histologischen Auswertung eingetreten. Trotzdem konnte, sowohl bei den zytologischen (p =

0,56) als auch bei den histologischen Auswertungen (p = 0,1) kein signifikanter Unterschied bei der Endometritis-Einteilung zwischen den beprobten Lokalisationen gefunden werden. Auch bei den unterschiedlichen Graden von Endometrose (p = 0,96) und Angiosklerose (p = 0,67) konnten keine signifikanten Unterschiede festgestellt werden.

Diese Studie über die Untersuchung von verschiedenen Lokalisationen zeigt, dass eine entzündliche Veränderung nicht gleichmäßig über das Endometrium verteilt ist. Auch korreliert die Anzahl an PMN bei zwei Untersuchungsmethoden an derselben Lokalisation des Uterus nicht miteinander. Daraus folgt, dass die optimale Anzahl an Proben, abhängig von der Entzündungsform und der Art der Methode, in weiteren Studien noch untersucht werden sollte.

Insgesamt zeigt diese Arbeit, dass bei der Diagnose der subklinischen Endometritis sowohl eine einzelne bakteriologische als auch eine einzelne zytologische Untersuchung zu falsch negativen Ergebnissen führen kann. Dies kann darauf zurückzuführen sein, dass bei einer vorliegenden Entzündung keine einheitliche Verteilung der PMN vorhanden ist. Dieses kann dann auch zu Veränderungen in der Einteilung in eine der Kategorien nach Kenney und Doig (1986) führen. Die Ergebnisse aller hier getesteten diagnostischen Methoden sollten aus diesem Grund immer kritisch ausgewertet werden. Die kombinierte Auswertung (bakteriologisch und zytologisch) eines Zytologiebürstchens stellt in der Praxis die einfachste und sicherste Methode für eine Diagnose der subklinischen Endometritis dar. Ob entweder eine Kombination von Untersuchungsmethoden oder eine mehrfache Beprobung die größere Verbesserung in der Sicherheit der Diagnose der subklinischen Endometritis bringen kann, sollte in weiteren Studien genauer untersucht werden.

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7. INDEX OF PUBLICATIONS

Research articles

W. Overbeck, T.S. Witte, W. Heuwieser (2011):

Comparison of three diagnostic methods to identify subclinical endometritis in mares.

Theriogenolgy 75(7): 1311-8

W. Overbeck, K. Jäger, H.-A. Schoon, T.S. Witte (2013):

Comparison of cytological and histological examinations in different locations of the equine uterus – an in vitro study.

Theriogenology 79(9): 1262-8

Poster

W. Overbeck, T.S. Witte, D. Forderung, A. Hille, W. Heuwieser (2010):

Comparison of three diagnostic techniques to detect subclinical endometritis in mares.

43. Jahrestagung "Physiologie und Pathologie der Fortpflanzung, München, 25.-26.02.2010

W. Overbeck, T.S. Witte, D. Forderung, A. Hille, W. Heuwieser (2010):

Comparison of three diagnostic techniques to detect subclinical endometritis in mares.

5. Doktorandensymposium und DRS Präsentationsseminar „Biomedical Science", Berlin, 02.07.2010

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Euch widme ich diese Arbeit!

9. DECLARATION OF INDEPENDENCE

This is to declare that I conducted all of the studies described herein myself and the manuscripts were produced independently. I confirm that I have used only the specified resources and tools to complete this thesis. My personal contributions to the research projects presented under this cumulative doctoral thesis are summarized in the following table.

Contribution	Research project 1	Research project 2
Study design	++[1]	++
Data collection	+++	++
Data analyses	+++	+++
Manuscript writing	+++	+++
Manuscript editing	++	++

[1]Score: + = < 50%; ++ = 50 to 70%; +++ = > 70%

Berlin, 15.08.2013

Wiebke Overbeck