Comparison of different cytological evaluation techniques

for the diagnosis of subclinical endometritis

and their reproducibility

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Index of contents

List of abbreviations ...................................................................................................................4

1. Introduction .........................................................................................................................5

2. Materials and methods .........................................................................................................9

  2.1 Study design ....................................................................................................................9

  2.2 Statistical analysis ........................................................................................................11

3. Results .................................................................................................................................12

  3.1 Descriptive statistics ........................................................................................................12

  3.2 Correlation and agreement between the different counting methods .................15

  3.3 Prevalence of SE determined by different evaluation methods .........................17

  3.4 Comparison of two different examiners .................................................................17

4. Discussion ............................................................................................................................19

5. Summary .............................................................................................................................23

References ................................................................................................................................24

List of tables ................................................................................................................................28

List of figures ............................................................................................................................29
List of abbreviations

e.g. for example
CE clinical endometritis
SE subclinical endometritis
dpp days postpartum
PMN polymorphonuclear neutrophils
Ex1 original examiner
Ex2 second examiner
1. Introduction

A regular uterine function is an important factor for economic feasibility in dairy cattle. The inflammation of the postpartum endometrium is a risk factor for subfertility in the subsequent breeding period. In most cows, pathogenic bacteria can be isolated from the uterus (SHELDON et al., 2006). Uterine infection causes, e.g., delayed uterine involution, histological lesions of the endometrium, severe inflammatory responses and perturbed embryo survival (GILBERT et al., 2005; SHELDON et al., 2006). These effects result in prolonged intervals from calving to first service, lower conception rates, prolonged days open and more cows culled because of infertility (DRILLICH et al., 2003; GILBERT et al., 2005; SHELDON et al., 2006).

Within the first three weeks after calving, uterine diseases can be defined as postpartum metritis and clinical metritis. Later, the terms postpartum or clinical endometritis (CE) and subclinical endometritis (SE) are established. Based on a generally accepted definition by Sheldon et al (SHELDON et al., 2006), vaginal discharge is a criterion for the diagnosis of CE. Clinical endometritis manifest either a purulent (≥ 50%) uterine discharge in the vagina ≥ 21 days postpartum (dpp) or a mucopurulent (50% pus, 50% mucus) discharge in the vagina after 26 dpp (SHELDON et al., 2006). Recent research, however, showed a lack of specificity of this criterion and suggested to use results of endometrial cytology for the diagnosis of endometritis (BARLUND et al., 2008). Subclinical endometritis is characterized by the absence of purulent material in the vagina but an endometrial inflammation determined by cytology (GILBERT et al., 1998; KASIMANICKAM et al., 2004).

Lymphocytes, macrophages, eosinophil leukocytes and neutrophil leukocytes are the most important cells related to inflammatory processes (BONDURANT et al., 1999; RAAB et al., 2004). Polymorphonuclear neutrophils (PMN) represent the major proportion of cells for an elimination of infection in the uterus based on their ability for phagocytosis (HUSSAIN et al., 1991; MATEUS et al., 2002). The criterion for an inflammation of the endometrium is the proportion of PMN determined in cytological samples from the uterus (GILBERT et al.,...
Although an infiltration of PMN can be regarded as physiological during estrous (BUTT et al., 1991; GRIFFIN et al., 1974), Madoz et al (MADOZ et al., 2013) found that there is no significant influence of different stages of the estrous cycle on the variation of PMN percentage for the diagnosis of SE.

Different methods can be used to collect endometrial and inflammatory cells from the uterus, including biopsy, low volume uterine lavage and the cytobrush technique (BARLUND et al., 2008; RODENBUSCH et al., 2007; HOEDEMAKER et al., 1992; KASIMANICKAM et al., 2005; SHELDON et al., 2006). The cytobrush technique has been described as a reliable and easy to perform method that provides results quickly (KASIMANICKAM et al., 2005). The cytobrush was described in the late 1970s for the retrieval of cells, to detect malignant degeneration of the cervical canal of women (TSUBOTA et al., 1990; RAAB et al., 2004). Almost 10 years ago, Kasimanickam et al (KASIMANICKAM et al., 2005) described the cytobrush technique as a useful method for the evaluation of bovine endometrial cells.

The cytobrush is screwed on a metal rod, protected by a disposable plastic catheter, inserted into the uterus and rolled alongside the uterine wall (PRUNNER et al., 2012). Afterwards the samples are rolled onto a microscopic slide, fixed, stained, and evaluated under the microscope (PRUNNER et al., 2012). The number and type of cells that are counted, the magnification of the microscope, the number of high power fields and other criteria for the evaluation of the samples are not standardized and vary between studies. Whereas, e.g. Westermann et al (WESTERMANN et al., 2010) and Kaufmann et al (KAUFMANN et al., 2009) counted a total of 300 endometrial cells and PMN, Kasimanickam et al (KASIMANICKAM et al., 2004) and Barlund et al (BARLUND et al., 2008) counted a minimum of 100 cells (endometrial cells, PMN) (BARLUND et al., 2008; KASIMANICKAM et al., 2004), squamous cells (BARLUND et al., 2008). Others counted PMN in high power fields and used different magnifications (PRIETO et al., 2012). Not only the criteria for the evaluation of the cytological smears but also the threshold of PMN for the definition of SE is still under discussion. Frequently used thresholds range between 5% and 10% of PMN (Table 1).
Table 1
Different thresholds of PMN for the definition of SE used by several researchers

<table>
<thead>
<tr>
<th>Author / Year of publication</th>
<th>PMN threshold</th>
<th>dpp</th>
<th>Justification for threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barlund et al 2008</td>
<td>&gt; 8%</td>
<td>28 to 41</td>
<td>Correlated with literature</td>
</tr>
<tr>
<td></td>
<td>&gt; 18%</td>
<td>21 to 28</td>
<td></td>
</tr>
<tr>
<td>Baranski et al 2012</td>
<td>&gt; 8%</td>
<td>21 to 28</td>
<td>Correlated with literature</td>
</tr>
<tr>
<td></td>
<td>&gt; 5%</td>
<td>21 to 28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt; 10%</td>
<td>35 to 42</td>
<td></td>
</tr>
<tr>
<td>Gilbert et al 2005</td>
<td>&gt; 5%</td>
<td>40 to 60</td>
<td>Correlated with uterine involution</td>
</tr>
<tr>
<td>Hammon et al 2006</td>
<td>&gt; 25%</td>
<td>28 (± 3)</td>
<td>Correlated with uterine health and energy status</td>
</tr>
<tr>
<td>Kasimanickam et al 2004</td>
<td>&gt; 18%</td>
<td>20 to 33</td>
<td>Correlated with an impairment of reproductive performance</td>
</tr>
<tr>
<td></td>
<td>&gt; 10%</td>
<td>34 to 47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8%</td>
<td>21 to 33</td>
<td></td>
</tr>
<tr>
<td>Madoz et al 2013</td>
<td>6%</td>
<td>34 to 47</td>
<td>Correlated with literature and the stage of estrous</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>21 to 62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4%</td>
<td>48 to 62</td>
<td></td>
</tr>
<tr>
<td>Plöntzke et al 2010</td>
<td>&gt; 5%</td>
<td>18 to 38</td>
<td>Correlated with literature</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>32 to 52</td>
<td></td>
</tr>
<tr>
<td>Sens et al 2013</td>
<td>10%, 18%</td>
<td>21 to 27, 24±1</td>
<td>Correlated with literature</td>
</tr>
</tbody>
</table>
There is no generally accepted gold standard for the evaluation of cytological smears for the diagnosis of SE. Thus, the objective of this study was to evaluate deviations related to different methods for estimating the proportion of PMN in a cytological endometrial sample. The results should help to define a generally accepted method for the determination of PMN for the diagnosis of SE in cows.
2. Materials and methods

2.1 Study design

The study was conducted on 10 commercial dairy farms in Lower Austria. A total of 100 cows, 99 Holstein cows and one Brown Mountain cow, housed in free-stall facilities with cubicles interspersed with straw, except of one farm which had a sloped floor system with deep litter, were examined. A slotted floor was detected on 8 of these farms whereas the other farms used a solid floor system. The cows were fed a ration of grass silage, corn silage, hay and concentrates. The herd average milk yield was 8.568 kg per lactation. A voluntary withdrawal period was defined for some of these farms between 40 to 50 days and the cows were artificially inseminated, generally.

The endometrial samples of the cows were collected 20 to 30 days after parturition with the cytobrush technique. Samples were taken from cows with \( n = 24 \) and without \( n = 76 \) clinical signs of endometritis. The cytobrush (Gynobrush, Heinz Herenz, Hamburg, Germany) with a diameter of 7 mm was screwed on a stainless steel rod, guarded with a disposable plastic catheter (Minitüb GmbH & Co., Tiefenbach, Germany) and covered with a plastic sleeve to protect the brush from contamination. The instrument was inserted under transrectal palpation into the body of the uterus, the plastic sleeve was retracted and the brush was rotated on the endometrium. Subsequently, the cytobrush was retracted into the plastic catheter and removed. The brush was rolled onto a microscopic slide, fixed and stained (Hemacolor Merck, Darmstadt, Germany). One hundred slides were examined under the microscope with 6 different methods by using \( \times400 \) magnification. In every sample the percentage of PMN was determined by evaluation of endometrial cells and PMN; other cells, e.g. lymphocytes, macrophages, were not included.

For all applied methods, the slides were examined in a meandering pattern. Methods C100, C300 and C500 counted 100, 300 and 500 cells (endometrial cells and PMN), respectively. For method HPF100 and HPF300, 100 and 300 cells were counted in 10 high power fields per slide. Therefore the examiner selected 10 fields in each slide with a large amount of cells in every field by random. For HPF100, 100 cells in 10 high power fields were counted in each
slide, whereas for HPF300, 300 cells in 10 high power fields were counted in each slide. With method EST, the PMN percentage was estimated by viewing the slide under the microscope in a meandering pattern but without counting cells. The different methods were performed in the described order with all slides, starting with 100 slides counted with C100, afterwards all 100 slides with C300 etc. Finally, a second examiner evaluated all slides according to method C300 to analyse inter-observer repeatability.
2.2 Statistical analysis

The statistical analyses were performed by using MS Excel (Excel 2007, Microsoft Office 2007, Microsoft Deutschland GmbH) and SPSS (PASW statistics 17.0, SPSS Inc.).

First, a descriptive analysis for every method was performed and the results were illustrated in a Boxplot diagram. Because there is no reference method (gold standard) for the cytological examination, the arithmetic mean of the percentage of PMN determined by the 6 different methods was calculated and used as reference to identify deviations of the methods and the single samples from this mean.

The correlation between the proportions of PMN determined with different methods was calculated by Spearman-Rho test and presented as scatterplot.

A weighted Cohen’s Kappa coefficient for the identification of the overall agreement between the methods was calculated, and a Kappa coefficient for the general agreement between the methods was determined.

Moreover the impact of the different methods on the prevalence of SE was calculated. For this, all samples from cows with signs of CE (vaginal discharge) were excluded (n = 24), resulting in 76 included samples. A proportion of 5% PMN was set as threshold for the diagnosis of SE. The mean prevalence of SE was calculated and correlations and kappa statistics were determined.

The reproducibility between two examiners was analysed for method C300. For this, in addition to the original examiner (Ex1) a second examiner (Ex2) counted the same slides. The agreement between Ex1 and Ex2 was determined by Spearman-Rho-correlation and Kappa statistics.
3. Results

3.1 Descriptive statistics

The descriptive results revealed a wide spread of proportion of PMN (Table 2).

Table 2
Descriptive results of 100 evaluated slides

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C100</td>
</tr>
<tr>
<td>Median</td>
<td>4.00</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.00</td>
</tr>
<tr>
<td>Maximum</td>
<td>46.00</td>
</tr>
<tr>
<td>IR(^2)</td>
<td>1.00 - 18.75</td>
</tr>
<tr>
<td>Prevalence of SE(^3)</td>
<td>33</td>
</tr>
</tbody>
</table>

\(^1\)Method:
- C100 = counting 100 cells
- C300 = counting 300 cells
- C500 = counting 500 cells
- HPF100 = counting 10 high power fields on 100 cells
- HPF300 = counting 10 high power fields on 300 cells
- EST = estimation of the PMN percentage

\(^2\)IR = Interquartile Range

\(^3\)Subclinical endometritis (SE) was determined in cows with no signs of clinical endometritis (n = 76)

The maximum proportion of PMN in a single sample ranged between the different methods from 46.0% (C100) to 98.0% (HPF300). The interquartile range was greatest in C100 (IR =
1.00 to 18.75%) and smallest in HPF300 (IR = 0.00 to 1.92%) and EST (IR = 0.00 to 2.00%). The results of all methods are shown in Figure 1.

![Boxplot Diagram](image)

**Figure 1**
The Boxplot – diagram shows the median, minimum, maximum and IR for different methods: C100 = counting 100 cells, C300 = counting 300 cells, C500 = counting 500 cells, HPF100 = counting 10 high power fields on 100 cells, HPF300 = counting 10 high power fields on 300 cells, EST = estimation of the PMN percentage

The deviations of the 6 different counting techniques from the arithmetic mean of all methods are illustrated in Figure 2.
Figure 2

Deviations for determined proportion of PMN of every sample from the arithmetic mean of all methods (line). The graphic illustrate the counted results up to a value of 25 % of PMN. The different methods are indicated by color and symbols.

Method: C100 = counting 100 cells, C300 = counting 300 cells, C 500 = counting 500 cells, HPF100 = counting 10 high power fields on 100 cells, HPF300 = counting 10 high power fields on 300 cells, EST = estimation of the PMN percentage.

The figure indicates a close relation of the different counting methods in the lower range up to a proportion of 5% PMN, whereas with a greater proportion of PMN, the deviation from the mean increases. Clear differences from the arithmetic mean can be seen for methods C100 and EST. Method C100 achieved higher proportions of PMN whereas EST underestimated the proportion of PMN in comparison to all other methods. Similar deviations were not found for the other methods.
3.2 Correlation and agreement between the different counting methods

In general, the correlation between the evaluated methods was good, with a minimum correlation coefficient of $r = 0.77$ between C100 and EST (Table 3). The greatest correlation coefficient was found between C100 and C300 ($r = 0.90$, $P < 0.01$).

The calculated Kappa coefficient for the overall agreement was $\kappa = 0.67$ ($P < 0.01$). The results of the correlation between proportion of PMN and the concordance between the 6 methods are shown in Table 3.

Table 3
Correlation between proportion of PMN (Spearman–Rho–Test, $r$) and agreement of diagnosis of SE (Kappa statistics, $\kappa$) for different evaluated methods

<table>
<thead>
<tr>
<th>Method</th>
<th>C100</th>
<th>C300</th>
<th>C500</th>
<th>HPF100</th>
<th>HPF300</th>
<th>EST</th>
</tr>
</thead>
<tbody>
<tr>
<td>C100</td>
<td>$r = 0.90^*$</td>
<td>$r = 0.81^*$</td>
<td>$r = 0.83^*$</td>
<td>$r = 0.80^*$</td>
<td>$r = 0.77^*$</td>
<td></td>
</tr>
<tr>
<td>C300</td>
<td>$\kappa = 0.69$</td>
<td>$r = 0.82^*$</td>
<td>$r = 0.83^*$</td>
<td>$r = 0.81^*$</td>
<td>$r = 0.79^*$</td>
<td></td>
</tr>
<tr>
<td>C500</td>
<td>$\kappa = 0.46$</td>
<td>$\kappa = 0.71$</td>
<td>$r = 0.83^*$</td>
<td>$r = 0.79^*$</td>
<td>$r = 0.83^*$</td>
<td></td>
</tr>
<tr>
<td>HPF100</td>
<td>$\kappa = 0.55$</td>
<td>$\kappa = 0.83$</td>
<td>$\kappa = 0.88$</td>
<td>$r = 0.84^*$</td>
<td>$r = 0.81^*$</td>
<td></td>
</tr>
<tr>
<td>HPF300</td>
<td>$\kappa = 0.33$</td>
<td>$\kappa = 0.54$</td>
<td>$\kappa = 0.80$</td>
<td>$\kappa = 0.69$</td>
<td>$r = 0.80^*$</td>
<td></td>
</tr>
<tr>
<td>EST</td>
<td>$\kappa = 0.40$</td>
<td>$\kappa = 0.63$</td>
<td>$\kappa = 0.91$</td>
<td>$\kappa = 0.79$</td>
<td>$\kappa = 0.79$</td>
<td></td>
</tr>
</tbody>
</table>

*P-value < 0.01

1Method:
C100 = counting 100 cells
C300 = counting 300 cells
C500 = counting 500 cells
HPF100 = counting 10 high power fields on 100 cells
HPF300 = counting 10 high power fields on 300 cells
EST = estimation of the PMN percentage

The greatest agreement was found between HPF300 and EST (κ = 0.85, P < 0.01). The least agreement was found for C100 compared with HPF300 (κ = 0.30, P < 0.01), and C100 compared with C500 and EST (κ = 0.35, P < 0.01). Figure 3 shows the correlation and agreement between the determined proportions of PMN of the different methods as scatterplots.
Correlation of the results of the proportion of PMN determined by 6 different counting methods: C100 = counting 100 cells, C300 = counting 300 cells, C500 = counting 500 cells, HPF100 = counting 10 high power fields on 100 cells, HPF300 = counting 10 high power fields on 300 cells, EST = estimation of the PMN percentage

3.3 Prevalence of SE determined by different evaluation methods

The greatest prevalence was determined by C100 (33.0%), and the least by HPF300 (10.0%). The Kappa test and correlation coefficients indicated a good agreement between the methods for the diagnosis of SE (Table 3). The greatest correlation coefficient was detected between C500 and EST ($r = 0.91$, $P < 0.01$), whereas the least correlation was found between C100 and HPF300 ($r = 0.44$, $P < 0.01$). The highest agreement was detected between C500 and EST ($\kappa = 0.91$), whereas the comparison of C100 and HPF300 found the least Kappa value ($\kappa = 0.33$).

3.4 Comparison of two different examiners

The comparison of results determined by two examiners according to method C300 showed that median proportion of determined PMN was similar (1.3% vs. 2.0%), whereas maximum values differed (66.7% for Ex1, 91.3% for Ex2). The correlation coefficient was $r = 0.86$ ($P < 0.01$). The inter-observer comparison showed a Kappa coefficient of $\kappa = 0.79$. Figure 4 shows a good agreement in the lower range of proportion of PMN, whereas in the upper range Ex2 tended to find greater proportions of PMN than Ex1.
Figure 4
Correlation of the cytological counting of 100 samples from cows 20-30 dpp examined by 2 different examiners (examiner 1 and 2).

With regard to the detection of SE, Ex1 and Ex2 found a prevalence of SE of 22.0% and 20.0%, respectively. The calculated correlation coefficient ($r = 0.72$, $P < 0.01$) and Kappa value ($\kappa = 0.87$, $P < 0.01$) of SE revealed a strong compliance between both examiners.
4. Discussion

The objective of this study was to evaluate the deviations of different counting methods for the detection of PMN in cytological endometrial samples. Previous studies focused on the definition of thresholds of PMN for the diagnosis of SE, but often used different counting methods for PMN (BARLUND et al., 2008; BARAŃSKI et al., 2012; KASIMANICKAM et al., 2004). There is no generally accepted gold standard for the evaluation of cytological smears for the diagnosis of endometritis. To our knowledge, this is the first study that evaluated different counting techniques for the cytological diagnosis of endometritis in dairy cows.

In this study, we used the correlation and a Kappa statistic for different counting methods to show the agreement between them. The correlation indicated a good compliance between the counting methods in general and between the inter-observer examinations, too. The comparison of the methods, however, showed some differences that are worth to be noticed. The methods revealed in the lower range of PMN a better accordance than in the upper range, where the deviation from the mean increased.

Counting 100 cells under the microscope has been used as diagnostic technique by several authors (BARLUND et al., 2008; BARAŃSKI et al., 2012; KASIMANICKAM et al., 2004). This method C100 showed a good correlation with other methods with correlation coefficients between $r = 0.77$ and $r = 0.90$. The Kappa values of C100 and other methods, however, were generally lower compared with other constellations. A reason for this could be that the deviation of C100 from all other methods was particular high in the low range of PMN, and that the maximum percentage of PMN was not greater than 46.0%. Kasimanickam et al (KASIMANICKAM et al., 2004) showed a good repeatability ($r = 0.84$) for counting 100 cells. Barlund et al (BARLUND et al., 2008) detected values between $r = 0.65$ to $r = 0.85$ as correlation coefficients with this counting method, whereas Dubuc et al (DUBUC et al., 2010) reported a value of 0.82 for the Kappa statistic.
With regard to the diagnosis of SE, this method showed lower agreement with other methods than other constellations and the highest prevalence of SE. This might indicate that method C100 overestimates the prevalence of SE. This is of importance when prevalence of SE, and a threshold for PMN is discussed but when diagnostic methods differing between studies.

Method C300, that has also been used by some authors (KAUFMANN et al., 2009; WESTERMANN et al., 2010), showed good results of correlations and Kappa statistics \( r = 0.79 \) to \( 0.90 \); \( \kappa = 0.50 \) to \( 0.73 \). Furthermore, C300 found a prevalence of SE close to the mean. Whereas the agreement between C100 and C300 (\( \kappa = 0.59 \)) and between C300 and C500 (\( \kappa = 0.64 \)) was similar, it was poorer between C100 and C500 (\( \kappa = 0.35 \)). Interestingly, the spread of determined proportion of PMN decreased from C100 to C300 and C500. Considering that C500 is a more time consuming technique, C300 can be recommended as a reliable and accurate method.

Other methods for the determination of PMN included high power fields of the microscopic evaluation. Prieto et al (PRIETO et al., 2012) counted 150 cells to assess the percentage of PMN and secondly determined PMN in 10 HPF (average number of PMN). They found a significant correlation between both techniques \( r = 0.84 \) (PRIETO et al., 2012). This study described the second technique as fast, easy and accurate for the diagnosis of SE. Our results showed a good correlation and Kappa statistic for HPF100 and HPF300. For the determination of SE, HPF300 found the lowest prevalence of all tested methods. Surprisingly, estimating the proportion of PMN (method EST) showed very good correlations and agreements with other methods, except the agreement with C100 (\( \kappa = 0.35 \)). The estimation of the proportion of PMN, however, requires a certain degree of experience. With regard to the prevalence of SE, method EST showed a good agreement with other methods, although EST underestimated the prevalence of SE compared with the average. Based on these results, the estimation of the percentage of PMN could be performed in the field by experienced examiners. It can be discussed if thresholds of PMN for the diagnosis of SE have to be corrected for methods C100 and EST.
The inter-observer agreement of method C300 was evaluated by the comparison of Ex1 and Ex2. The results of the correlation and Kappa statistic showed a strong compliance between both examiners. Kasimanickam et al (KASIMANICKAM et al., 2004) and McDougall et al (MCDOUGALL et al., 2011) found similar results for the correlation (r = 0.84 and r = 0.82), respectively. The reproducibility of PMN counting has been described by some authors (MADOZ et al., 2013; SANTOS et al., 2012; MCDOUGALL et al., 2011; GILBERT et al., 2005). Gilbert et al (GILBERT et al., 2005) described an excellent agreement between two examiners (κ = 0.86), similar Dubuc et al (DUBUC et al., 2010) (κ = 0.77). Santos et al (SANTOS et al., 2012) found weighted Kappa coefficient of 0.97 for an agreement between two different examiners. In the present study, the intra-observer reproducibility was not investigated and therefore the results cannot be discussed. Other studies, however described a strong compliance of κ = 0.82 (DUBUC et al., 2010). Also the calculated correlation coefficient showed convincing results (BARLUND et al., 2008; KASIMANICKAM et al., 2004). Whether the findings of this study would be in accordance to data from other working groups or not remains unclear.

Some studies described not only the methods for evaluation cytological samples, but evaluated also different techniques to collect these samples (RODENBUSCH et al., 2007; KASIMANICKAM et al., 2005; SHELDON et al., 2006). Sheldon et al (SHELDON et al., 2006) described the endometrial biopsy which has been established for the diagnosis of endometritis in mares as an invasive, time consuming and expensive technique (BARLUND et al., 2008). It is controversial, however, if biopsy has a negative impact on fertility in cows (RODENBUSCH et al., 2007; HOEDEMAKER et al., 1992; SHELDON et al., 2006). The uterine lavage has been described more often for the diagnosis of bovine SE. Thereby the uterine lumen is flushed by infusing a sodium chloride solution, the fluid is retracted, centrifuged and the sample is evaluated under microscope (KASIMANICKAM et al., 2005). Although this technique captures a large area of the endometrial mucosa, it is time consuming and causes a distortion of collected cells (KASIMANICKAM et al., 2005). With regard to these aspects, the cytobrush technique seems to be the method of choice for evaluation cytological endometrial samples.
In conclusion, the cytological evaluation of samples for the diagnosis of SE is a reliable technique if a sufficient number of cells are counted. This study demonstrated differences in the spread of PMN proportion by different methods and examiners. All methods showed good results in terms of correlation and agreement and were suitable for the cytological evaluation of PMN. For method C100, however, agreement with other methods was the poorest. Nevertheless, the different methods showed varying results with regard to the prevalence of SE. In this study a frequently used threshold of 5% of PMN was applied. It has been hypothesized that this threshold is not suitable for methods C100 and EST. As these methods over- or underestimate the mean prevalence of SE, thresholds should be corrected for these methods. These findings indicate that the threshold of PMN for the diagnosis is not only influenced by time of sampling postpartum but also by the diagnostic method. Results of this study revealed that a threshold of 5% PMN is useful when 300 cells were counted. Considering not only the accuracy and repeatability of the method but also aspects of time needed for the evaluation, method C300 can be suggested as method of choice for the evaluation of PMN.
5. Summary

Endometrial cytology as a reliable diagnostic technique has been established role for the diagnosis of subclinical endometritis (SE) in cows. Several counting techniques have been used to determine polymorphonuclear cells (PMN) in endometrial samples. Information about the agreement between different techniques however, is limited. Therefore the aim of this study was to evaluate the percentage of endometrial and PMN by 6 different counting techniques and in a second step to show reproducibility between two different examiners. All counting techniques were applied to 100 samples each. The applied methods counted a total of 100, 300 and 500 cells (C100, C300, C500), respectively. With method HPF100 and HPF300 10 high power fields per slide were investigated up to a total of 100 and 300 cells. Finally, on method estimated the percentage of PMN by screening the slide under the microscope. Because method C300 showed a good agreement with other methods and has been described in several papers, the inter-observer reproducibility for these methods was analysed with two observers. The comparison between the 6 different methods showed a strong compliance (r = 0.77 to r = 0.90) with the greatest correlation coefficient between C100 and C300. The Kappa statistics showed results between κ = 0.30 to κ = 0.85 with the greatest agreement between HPF300 and EST. Furthermore, the impact of the different methods on the prevalence of SE was calculated, with the greatest prevalence determined by C100 (33.0%) and the least by HPF300 (10.0%). The results of the inter-observer reproducibility showed good results for the correlation r = 0.86 and for the Kappa statistics (κ = 0.79). In conclusion, every method was suitable for the cytological evaluation of PMN, although method C100 showed lowest agreement with other methods. This confirms the hypothesis that a suitable threshold for PMN is not only influenced by e.g. time of sampling postpartum but also by diagnostic method. A threshold of 5% PMN seems useful when 300 cells were counted, whereas counting 100 cells or the estimation of cells seems to overestimate or underestimate the prevalence of SE, respectively. In conclusion, method C300 can be recommended as method of choice for the evaluation of PMN in endometrial samples to detect SE.
References


List of tables

**Table 1:** Different thresholds of PMN for the definition of SE used by several researchers

**Table 2:** Descriptive results of 100 evaluated slides

**Table 3:** Correlation between proportion of PMN (Spearman–Rho–Test, r) and agreement of diagnosis of SE (Kappa statistics, k) for different evaluated methods


**List of figures**

**Figure 1:** The Boxplot – diagram shows the median, minimum, maximum and IR for different methods: C100 = counting 100 cells, C300 = counting 300 cells, C500 = counting 500 cells, HPF100 = counting 10 high power fields on 100 cells, HPF300 = counting 10 high power fields on 300 cells, EST = estimation of the PMN percentage.

**Figure 2:** Deviations for determined proportion of PMN of every sample from the arithmetic mean of all methods (line). The graphic illustrate the counted results up to a value of 25% of PMN. The different methods are indicated by color and symbols.

**Figure 3:** Correlation of the results of the proportion of PMN determined by 6 different counting methods: C100 = counting 100 cells, C300 = counting 300 cells, C500 = counting 500 cells, HPF100 = counting 10 high power fields on 100 cells, HPF300 = counting 10 high power fields on 300 cells, EST = estimation of the PMN percentage.

**Figure 4:** Correlation of the cytological counting of 100 samples from cows 20-30 dpp examined by 2 different examiners (examiner 1 and 2).