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Preliminary report

Cytological and microbiological examination of bovine milk in *Prototheca*-infected dairy herd

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ABSTRACT – In healthy milk the somatic cell count (SCC) is less than 100.000 cells per mL, although in the presence of infectious agents, especially pathogenic bacteria, SCC can be extremely high. Cytological appearance of inflammation in mammary gland depends on the origin and the time-course of the inflammatory response. According to predominant cell type there can be suppurative, pyogranulomatous, granulomatous and lymphocytic inflammation. Prototheca spp. as facultative pathogen creates chronic inflammation in mammary gland with high SCC and reduced milk production. The aim of our study was to elaborate a practical and accurate laboratory procedure to enhance the precision of milk somatic cell differentiation, and to provide quick and easy-to-perform detection for the presence of infectious algae in mastitis cases. In 95 quarter samples SCC was determined by California Mastitis Test, cellular density estimation and somatic cell differentiation were carried out by microscopically evaluation, while presence of Prothoteca spp. was investigated with the help of specific culture medium (PIM agar). According to CMT results, SCC values showed 67.3% prevalence of mastitis, from which 6.3% was clinical and 93.7% subclinical. Comparing CMT results and estimated cellularity values indicated tight correlation. In CMT negative quarters 60% of somatic cells were neutrophil granulocytes, while in CMT positive but Prototheca spp. negative cases these cells were present in 74%. Interestingly in Prototheca spp. positive samples only 60.25% of cells were neutrophil granulocytes, while macrophages and lymphocytes were present in 17% and 18.3%, respectively. Moreover, remarkable cytological pattern was notified in some samples that were positive for Prototheca spp., and statistical analysis showed tight correlation between infectious algae and the ratio of macrophages (p=0.005). In conclusion laboratory techniques applied in our study seems to be helpful supplementary methods to reveal pathological background of mastitis as well as to monitor the progression of inflammatory reactions.

Keywords: mastitis, somatic cell count, infectious algae, cytology, cattle

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INTRODUCTION

Besides reproductive problems, metabolic diseases and foot disorders, mastitis with different origin and severity has the most remarkable economic impact in dairy herds. In mastitis cases economic losses are connected to significant decrease of milk production, as well as the increased number of cullings (*Olechnowicz et al*., 2011). Results of a Hungarian study support that consequences of mastitis in large sized dairy herds can be remarkably reduced milk production together with severe quality degradation, at the same time numbers of premature culling and veterinarian charges can be notifiable elevated (*Ózsvári et al*., 2003).

In both healthy and pathological cases different cells can be found in raw milk, these are the so called somatic cells. Somatic cells are originated from either the mammary gland parenchyma or the blood circulation. In physiological conditions most of the somatic cells are derived from the secretory alveoli and the duct system of mammary gland, while approximately one fourth of somatic cells are white blood cells (*Sharma et al.*, 2011). Generally, in healthy milk the number of somatic cells (i.e. somatic cell count) is less than 100.000 cells per mL, although in the presence of infectious agents, especially pathogenic bacteria, somatic cell count (SCC) can be extremely high as a result of intensive inflammatory reaction in mammary gland tissue (*Kehrli et al*., 1994). Parallel to increasing SCC, milk production shows reduction, and this kind of negative correlation can already be detected at low SCC values, e.g. 50.000 cells per mL (*Pfützner et al.*, 2017).

Milk somatic cells

Epithelial cells are part of somatic cell population; even in physiological conditions they detach basement membrane, and appear in the lumen of glandular alveoli or the duct system. Together with secreted milk, epithelial cells enter the lactiferous sinus, and they are excreted during milk ejection. Basically, epithelial cells can be divided in two groups that are glandular alveolar and ductal cells (*Monteiro-Riviere*, 2006).

Alveolar cells construct secretory units of mammary gland; they can appear as single cuboidal or columnar epithelial cells depending on the specific stage of lactation as well as the quantity of milk accumulated within the alveoli. Glandular alveolar cells perform galactopoiesis, whereby they release milk components via apocrine or merocrine secretion depending on the nature of components (*Baumrucker et al.*, 2016). Ductal cells can be found as single or two-layered cuboidal cells that serve as internal lining of mammary gland ducts from

the smallest intralobular channels until the ultimate papillary ducts (*Boutinaud et al.*, 2002).

The other group of somatic cells in milk is the so called round cells that are derived from the hematopoietic cell lines of the bone marrow. Virtually all types of leukocytes belongs to this group that can be found in the circulation system e.g. neutrophil and eosinophil granulocytes, monocytes and lymphocytes as well as their counterparts existing in mammary gland tissue, like macrophages (*Schwarz et al.*, 2011).

In healthy udder quantity of these cells are usually low, however in the case of mastitis their absolute and relative numbers can dramatically increase (*Leitner et al*., 2000). For instance, in case of subclinical *Staphylococcus aureus* infection number of somatic cells in milk can be over than 100.000 cells per mL, while in clinical mastitis SCC values can reach 1.000.000 cells per mL or even higher (*Bytyqi et al*., 2010). In mastitis with of bacterial origin neutrophil granulocytes become rapidly predominant, their proportion among somatic cells may increase from the normal 19% to over 75% (*Alhussien et al.*, 2016). In acute bacterial infections relative number of neutrophil granulocytes often exceeds 90% (*Harmon*, 1994).

Cytology of inflammation

Inflammatory processes manifest in the same way in different tissues; their cytological appearance in mammary gland depends mostly on the origin (infectious or non-infectious), as well as on the time-course of the inflammatory response (acute, subacute, chronic).

Inflammatory reactions, in which neutrophil granulocytes account more than 70% of leukocyte population is called neutrophilic inflammation. In those cases where the proportion of cells is higher than 85% so called purulent or suppurative process is present. Since neutrophil granulocytes possess strong phagocytic activity, their presence in most bacterial infections could be remarkable. However, these cells are the important members of innate immune response, and as a result of chemotactic mediator release they are able to leave blood circulation and migrate in tissues to the scene of inflammation. Thus, neutrophil granulocytes predominate in the acute phase of inflammation regardless of its location or cause (*Alhussien et al*., 2016).

Fungal and atypical bacterial infections cause pyogranulomatous inflammation, in which proportions of neutrophils are generally lower than 60%, while mononuclear cells i.e. macrophages are present in 20-50%. The same cytological pattern can be seen in chronic inflammatory reactions, where re-

generative or reparative processes predominate. In protracted cases or in special inflammatory reactions e.g. *Mycobacterium bovis* infection, so called granulomatous inflammation is formed, in which proportions of macrophages can be higher than 50%, and epitheloid macrophages or multinucleated giant cells appear (*MacNeill*, 2017).

In contrast of other tissues e.g. salivary gland or subcutaneous tissue, lymphocytic inflammation is not characteristic in mammary gland. This kind of lesion usually occurs as a consequence of viral infections, late hypersensitivity reactions or immune-mediated disorders, and it means a high mononuclear infiltration where lymphocytes sometimes together with plasma cells predominate. In healthy udder and milk proportion of lymphocytes are usually moderate or low, according to different publishers they are generally present in 15% (*Alhussien et al*., 2015) or between 10-27% (*Riollet et al*., 2002). As SCC increases proportion of lymphocytes tend to decrease, if SCC is higher than 400.000 cells per mL lymphocyte ratios are merely 8.6% (*Koess et al.*, 2008).

Protothecal mastitis

Prototheca spp. algae as ubiquitous organisms can be found widespread in nature such as in soil, water sources or plants. However these single cell organisms are saprophytic occasionally they can act as facultative pathogenic microbes both in humans and animals.

Prototheca spp. can infect dairy cattle and create chronic inflammation in mammary gland as well as in sentinel lymph nodes (*Pal et al.,* 2014), which results in high SCC and reduced milk production. *Prototheca* spp. remain long term in udder tissue, they can persist during the dry period and be excreted via milk in the next lactation. Detection of infectious algae is relatively simple on different culture media e.g. blood agar or Sabouraud dextrose agar, although applying selective and differentiating medium could be more beneficial (*Cremonesi et al.,* 2022).

The aim of our study was to elaborate a practical and accurate laboratory procedure in order to enhance the precision of milk somatic cell differentiation in diverse forms of mastitis, as well as to develop a specific culturing and reliable microscopic evaluation method to provide quick and easy-to-perform detection for the presence of infectious algae in mastitis cases.

MATERIAL AND METHODS

Animals

Milk sample collection was carried out in a conventional free stall dairy farm that applies traditional machine milking technology. The study involved 12 dairy cows being at different stages of lactation, the animals were kept free in a group of sixty within an external paddock and stable wing, they were milked twice a day in a herringbone arranged milking parlor.

Altogether 32 udder quarters of the 12 animals were selected fortuitously, and these were sampled three times. Sample collection was performed during morning milking, between the first and second sampling 48 hours elapsed, while between the second and third sampling 7 days passed.

California Mastitis Test

In order to determine somatic cell counts and to reveal the presence of subclinical mastitis we used California Mastitis Test (CMT) described below.

Before starting test procedure, we cleaned the teats with a specific udder cleaner solution (Sanitizzante Capezzoli, Allegrini SpA, Grassobbio (BG), Italy), then teats were dried with paper towels. The plastic testing scale was flushed with clean water. After discarding the first few squirts of milk from each teat, the testing scale was held underneath the udder and two squirts from every teat were sprayed onto the scale in their corresponding circle. In order to have only the required amount of milk stayed behind, the testing scale was held sideways, and then as much CMT reagent solution (KerbaTEST, Albert Kerbl GmbH, Buchbach, Germany) as milk was put in each circle. By swerving the scale for ten seconds, the CMT solution with the milk was completely mixed. After combining milk with reagent results were read straight away in order to keep its validity and reliability.

As for the test interpretation, according to KerbaTEST description, when liquid remained in unchanged state, somatic cell count was less than 100.000 cells per mL of milk. In the case of mild strand formation, somatic cell count was estimated between 100.000 and 300.000 cells per mL. When strong coagulation occurred somatic cell count was more than 300.000 cells per mL, and as the highest category, in the case of solid gel formation, somatic cell count was above 500.000 cells per mL of milk. According to aforementioned categories, each samples received a numerical 0-4 CMT result, respectively.

Cytological examination

From the selected quarters ca. 80-100 mL milk samples were collected to sterile plastic urine containers and placed immediately at 6-8°C temperature. Samples were submitted to the laboratory as quickly as it was possible, during the storage and transportation period they were kept under the same cooling conditions.

Laboratory procedures were carried out according to *Dosogne et al.* (2003) with some modifications in dilution, centrifugation and staining. The processing started with a gentle, manual homogenization of the samples within the original collecting containers. After that 4 mL aliquot of each samples were transmitted to sterile centrifuge tubes and diluted by 10 mL of Phosphate buffer solution (Sigma-Aldrich Co., St. Louis, USA), pH 7.4. Following multiple manual homogenization, diluted samples were centrifuged at 1500 rpm, 4°C for 5 minutes by VWR Mega Star 600R centrifuge unit (VWR International LLC, Radnor, Pennsylvania, USA). Most of the high fat content supernatant was removed from the samples by manual pipetting, and as a result 1-1.5 mL sedimentation remained in the bottom of the tubes. From the sedimentations 10 µL aliquots were transmitted to specific adhesive glass slides (HistoBond® +M, Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and they were distributed in a circular area with a diameter of 12-15 mm by using the pipette tip in order to create thin smears. Preparations were let dry overnight on room temperature by placing the slides on smooth, horizontal surface.

Smears were fixed and stained by Romanowsky-type fast staining kit (RAL 555®, RAL Diagnostics, Martillac, France). After complete drying, preparations were laid above staining trough and applied by RAL fixative medium sprayed out of a 100mL volume glass atomizer in a way to create a thin covering on the surface. Following 60 minutes of fixation smears were stained by the red component of RAL 555 kit with the same aforementioned spraying technique. After 1 minute staining time, residual dye was discarded and the edges of slides were cleaned by paper towels. The blue component of the staining kit was applied in the same way as the red dye with a quick incubation period as long as 25 seconds. Following the removal of blue colorant, slides were dipped in clean distilled water 5-6 times for 1 second in order to wash off residual dye. Then glass slides were let dry at room temperature in a diagonal standing position for 1 hour. After that smears were treated by Entellan® rapid mounting medium (Merck KGaA, Darmstadt, Germany) and covered by glass coverslips, they were allowed to dry for 1 hour after that.

Smears were evaluated under light microscope (OLYMPUS BX43, OLYMPUS Corporation, Tokyo, Japan) at magnification 100-1000X. Enumeration of somatic cells was carried out by a manual cell counter (ABACUS CC-100, Twinex PRO Kft., Budapest, Hungary) according to *Leitner et al.* (2000). First, in cytological evaluation general cellularity of samples were estimated in low power fields, according to the average numbers of cells in several fields of view, samples were scored 1-4 as low, moderate, high and very high cellularity, respectively. Thereafter at 400-600X magnification somatic cells were analyzed, and depending on their morphological characteristics they were referred into four different groups: neutrophil granulocyte, macrophage, lymphocyte and epithelial cells, respectively. The last category included all the glandular and ductal epithelial cells derived from the mammary gland parenchyma, although other cell types such as red blood cells or squamous epithelial cells were not considered. In order to enhance the accuracy of results 100-400 nucleated cells were counted depending on the cellularity score of the certain sample. In samples with low cellularity 100 cells were counted and classified, samples with moderate cellularity 200 cells, while with high and very high cellularity scores 400 cells were analyzed and referred. The ratio of each cellular type was determined by percentage calculation. Furthermore, when during microscopic evaluation figures of infectious algae appeared among somatic cells, their presence were recorded, as well.

Preparation of Prototheca Isolation Medium (PIM agar)

In order to detect *Prototheca* spp. colonies a selective differential medium, *Prototheca* Isolation Medium (PIM) agar was applied according to *Pore's* (1973) description. The components needed for the preparation of the medium are detailed in Table 1.

At first potassium hydrogen phthalate and sodium hydroxide were dissolved in 1 L distilled water, following complete dissolution, the remaining ingredients were added to the mixture.

The medium was boiled to dissolve the components completely by heating magnetic stirrer (HSC Ceramic Hot Plate Stirrer, VELP Scientifica, Via Stazione, Italy). hexachlorocyclohexane (0.01g / liter) is an optional ingredient that was added to eliminate arthropod contaminants. The pH was adjusted to 5.1 ± 0.1 at 25°C. The medium was sterilized by Systec VE 55 autoclave (Systec Ltd., Linden, Germany) at 121°C for 15 minutes. Simultaneously, 0.25 g 5-fluorocytosine (Molekula Limited, Darlington, United Kingdom) was dissolved in 10 mL distilled water and then sterilized by filtering through 0.22 μm PES syringe fil-

ter (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). Immediately before pouring intro Petri dishes, sterile 5-fluorocytosine stock solution was added to *Prototheca* Isolation Medium cooled to between 44 and 47°C.

Until use, poured plates were stored in refrigerator at 4 ± 2°C. At this temperature, the culture medium retained its quality for two weeks.

Table 1
Composition and preparation of *Prototheca* Isolation Medium (PIM agar)

Component	Grams / liter
Potassium hydrogen phthalate	10.0
Sodium hydroxide (NaOH)	0.9
Magnesium sulphate[a] ($MgSO_4$)	0.1
Potassium hydrogen phosphate[a] (KH_2PO_4)	0.2
Ammonium chloride[a] (NH_4Cl)	0.3
Glucose[a]	10.0
Thiamine-HCl[a]	0.001
Hexachlorocyclohexane[b]	0.01
Agar	20.0
Distilled water to	1.0 L
Adjust pH to 5.1 ± 0.1 at 25°C	
Sterilization: 121°C for 15 minutes in autoclave.	
Filter-sterilized component	
5-Fluorocytosine (5-FC) *	0.25

[a] *Essential nutrient*
[b] *Optional ingredient for the elimination of arthropod contaminant*
[*] *Fungal inhibitor especially for Candida and Cryptococcus spp.*

Selective Culturing of Prototheca spp.

The quarter milk samples were aseptically removed from their containers and diluted by mixing 10 mL aliquot with 90 mL 0.1% peptone water. The inoculated plates were incubated in normal atmospheric conditions at 37°C for 72 h.

Colonies suspected of being *Prototheca* spp. were subcultured on PIM plates, and subjected to initial species identification which included macroscopic evaluation of colony morphology as well as microscopic assessment of the colony samples stained by Loffler's methylene blue solution (Merck KGaA, Darmstadt, Germany).

Statistical analysis

Statistical analysis of data was carried out with R software 3.6.4 version (R Core Team [2022]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL1).

In order to determine correlation between CMT, cellularity and different cell types General Lineal Modell (binomial logit function) was utilized under the following formula: $Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4 + \beta_5X_5 + \beta_6X_6 + \varepsilon$, in which CMT and cellularity were dependent variables (Y), while the ratios of lymphocytes, macrophages, neutrophil granulocytes, results of *Prototheca* spp. culturing on PIM agar and their microscopically evaluation (X_{1-5}), the last two as binary variables with either negative (0) or positive (1) dimensions. Sampling as an independent variable (X_6) was involved because sample collection took place at three different times. Although all the circumstances and the personnel were the same, some latent alteration might happen which could have an impact on the final results.

According to CMT and *Protptheca* spp. culturing results three groups were created: 1. CMT and *Prototheca* negative, 2. CMT positive and *Prototheca* negative, and 3. CMT and *Prototheca* positive, respectively. A Shapiro-Wilk normality test was applied to the groups to examine the distribution of the variables (neutrophils, macrophages, lymphocytes and epithelial cells), Frequency distributions of different cell types were also calculated, then a non-parametric; Kurksal-Wallis H test with a Mann-Whitney post-hoc test was applied on the data. In addition, Pearson's product-moment correlation test was used to describe the nature of relation between cellularity and CMT values.

All correlations were considered significant on 95% confidence interval ($p<0.05$).

RESULTS AND DISCUSSION

CMT results

Altogether 96 quarter samples were collected, unfortunately one sample during the second sampling became technically inappropriate, and thus it was excluded from further investigation. 81% of quarter samples had at least one-plus (+) CMT score in the first sampling, while 90% in the second and 31% in the third sampling, respectively. In summary it meant 67.3% prevalence, from which 6.3% was clinical and 93.7% subclinical mastitis.

On individual level prevalence values were much higher, which meant 33% clinical and 100% subclinical mastitis, ergo all the involved animals had minimum one quarter impaired in at least one of the samplings.

Recent CMT based studies show that clinical and subclinical prevalence in dairy herds worldwide are 12% and 43%, respectively. In Europe these values are quite similar, 26% and 41%, respectively (*Krishnamoorthy et al.,* 2021).

These results differ largely from our findings, although in some studies only high CMT scores (+++ and ++++) were considered as positive case.

Cellularity

Comparing CMT results and estimated cellularity values indicated tight correlation. Reliability of microscopically assessment was proved by high correlation coefficient (0.75) in spite of cellularity values were derived from subjective estimation ($p<0.01$) and not from usual microscopic somatic cell count like in other studies (*Takano et al.,* 2018).

Mean values of cellularity show about three times difference between the CMT negative and positive cases (Table 5.). This finding may suggest that however, cellularity results were derived from subjective evaluation instead of automatic somatic cell count, microscopically estimation can possess similar diagnostic values as CMT, although further investigations are necessary to confirm.

Somatic cell differentiation

With microscopically evaluation somatic cells were classified in four different groups: neutrophil granulocytes, macrophages, lymphocytes, and epithelial cells, respectively.

Relative values of cell differentiation were collected separately into three groups based on CMT and *Prototheca* spp. culturing results. Descriptive statistics of the somatic cell types are shown in Table 2., while a summarization of the Kruskal-Wallis H test is illustrated in Table 3. Results show significant differences of somatic cell counts between each group.

Shapiro-Wilk test results of frequency distribution are demonstrated via bar graphs (Chart 1.) which indicate that cell distributions of somatic cells were not normal. Table 4 demonstrates chi-square and probability values of the different cell types in Kruskal-Wallis H test.

The sum of the three sampling showed that in CMT negative quarters 60% of somatic cells were neutrophil granulocytes, which ratio is much higher than results derived from other studies (*Alhussien et al.,* 2016). However in CMT positive but *Prototheca* spp. negative cases these cells were present in 74% that shows good correlation with data from the same publication.

It is interesting that in our study in *Prototheca* spp. positive samples only 60.25% of cells were neutrophil granulocytes, although CMT scores and cellularity were high (Figure 1.).

Table 2
Descriptive statistics of somatic cell types according to three groups of CMT and *Prototheca* spp. culturing results

Group	Variable	Mean	Standard Deviation	N
1	neutrophil gr. %	59,74	19,00	20
	macrophage %	26,34	15,99	20
	lymphocyte %	6,73	3,68	20
	epithelial cell %	7,19	6,41	20
2	neutrophil gr. %	20,80	16,94	70
	macrophage %	5,98	8,44	70
	lymphocyte %	1,49	1,28	70
	epithelial cell %	0,03	0,17	70
3	neutrophil gr. %	64,10	26,60	5
	macrophage %	16,35	7,25	5
	lymphocyte %	18,95	21,66	5
	epithelial cell %	0,60	0,63	5

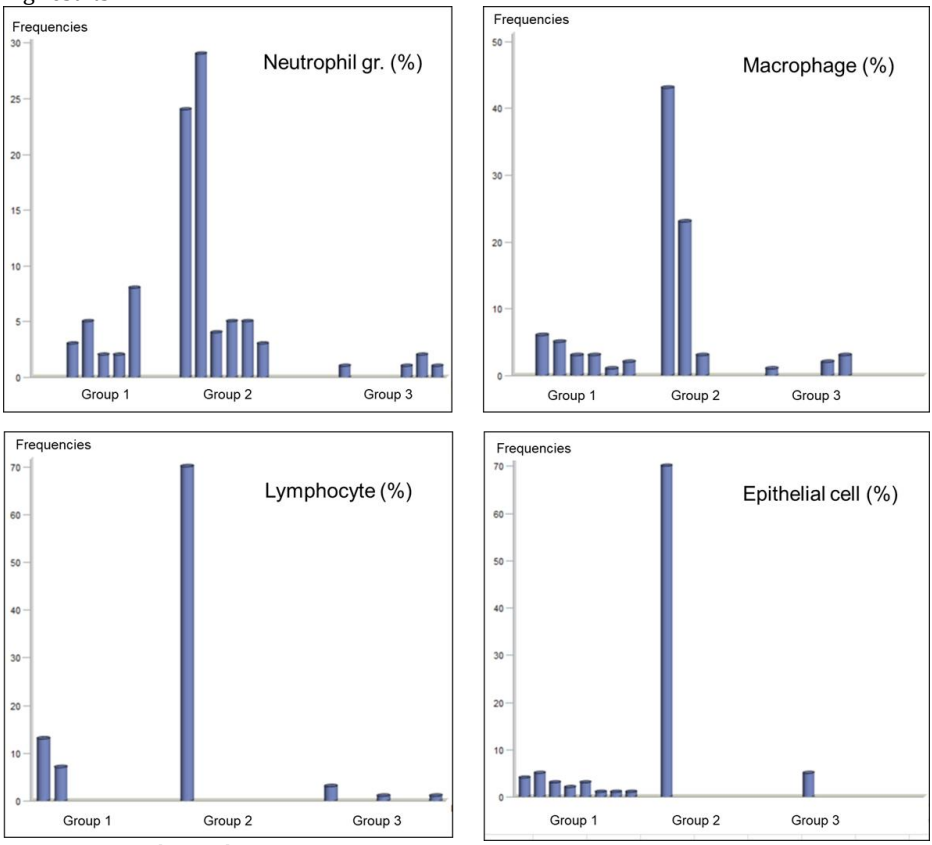
Group 1. CMT and Prototheca negative
Group 2. CMT positive and Prototheca negative
Group 3. CMT and Prototheca positive

Table 3
Kruskal-Wallis H test values on somatic cells according to CMT and *Prototheca* spp. culturing results

Group	N	Mean ranks			
		Neutrophils	Macrophages	Lymphocytes	Epithelial cells
1	20	77.68**b**	79.6**b**	76.97**b**	81.15**b**
2	70	37.36**a**	37.24**a**	37.21**a**	37.63**a**
3	5	78.2**b**	72**b**	83.2**c**	60.6**ab**
Kurksal-Wallis test results	**p-value**	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Group 1. CMT and Prototheca negative
Group 2. CMT positive and Prototheca negative
Group 3. CMT and Prototheca positive
N = number of samples
a, b, c indicate significant differences between groups

Chart 1
Frequencies of cellular distribution in the three groups of CMT and *Prototheca* spp. culturing results



Group 1. CMT and Prototheca negative
Group 2. CMT positive and Prototheca negative
Group 3. CMT and Prototheca positive

Table 4
Chi-square and p-values of different somatic cells in Kruskal-Wallis H test

Somatic cell type	chi-square values	p-values
neutrophil granulocyte	7.563	0.0227
macrophage	10.479	0.0053
lymphocyte	1.529	0.4654
epithelial cell	1.265	0.5311

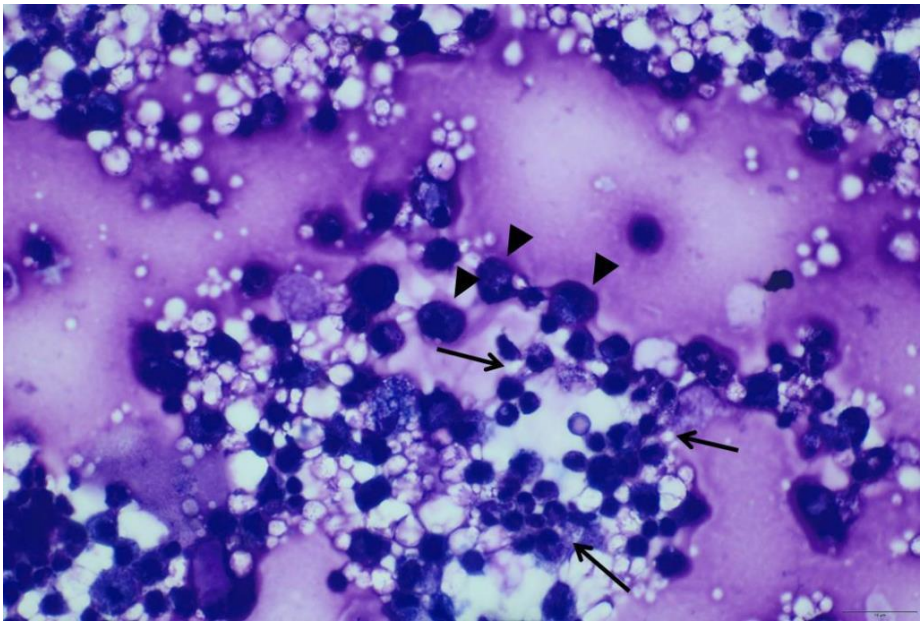


Figure 1. High cellularity milk sample with a mixed population of inflammatory cells: neutrophil granulocytes (arrow) and macrophages (arrowhead). RAL 555 rapid stain, 600X

In addition relative numbers of macrophages in our study substantially deviate from data published by *Alhussien et al.* (2016), for instance in our CMT negative samples the mean percentage of macrophages was only 23.8%, which seems much lower than their findings. The cause of difference could have technical background because in our study macrophages were classified separately from epithelial cells, while in other researches the two cell groups had been categorized together as large mononuclear cells (*Alhussien et al.*, 2015).
However in the case of *Prototheca* spp. positive samples ratio of macrophages were very homologous to the aforementioned literature data that was 17%. This is also true for the lymphocyte numbers, because in both CMT negative and positive cases the mean lymphocyte ratio were 22% and 6.2%, respectively.

Cellularity values and somatic cell counts of CMT negative and CMT positive but *Prototheca* spp. negative samples are showed in Table 5. In contrast to cellularity results somatic cell counts do not show remarkable differences between CMT negative and positive groups when samples were free from *Prototheca* spp. High numbers of neutrophil granulocytes in negative samples may

suggest the presence of concurrent bacterial infection which can cause increased relative values, although somatic cell counts remain under the CMT cut off.

Table 5
Mean cellularity scores and somatic cell counts in CMT negative and positive samples from first, second and third sampling

Samples (sampling)	Mean cellularity	Mean neutrophil (%)	Mean macrophage (%)	Mean lymphocyte (%)	Mean epithelial (%)
CMT negative (I.)	1.33	71.00	18.83	5.42	4.58
CMT negative (II.)	1.00	58.00	18.75	10.50	12.75
CMT negative (III.)	1.60	53.58	33.88	6.00	6.53
CMT positive (I.)*	3.83	80.50	12.75	5.20	1.54
CMT positive (II.)*	3.75	71.44	18.38	8.72	1.47
CMT positive (III.)*	3.57	70.10	24.32	4.79	0.75

* *Prototheca-positive samples are not included*

Prototheca spp. positive samples

During microbiological culturing 7 samples were found as *Prototheca* spp. positive, in which 5 cases were confirmed microscopically, as well (Figure 2.).

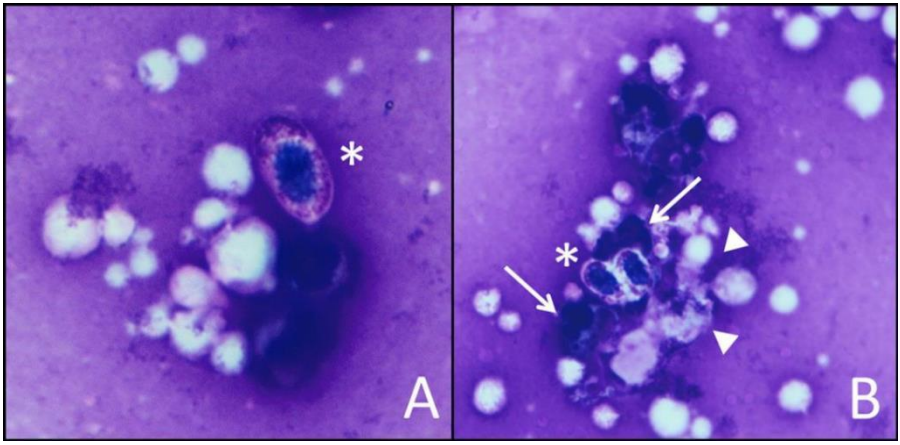


Figure 2. *Prototheca* spp. in mastitis positive milk; A: single *Prototheca* spp. cell (asterix) RAL 555 rapid stain, 1000X ; B: Two *Prototheca* spp. cells (asterix) among neutrophil granulocytes (arrows) and lipid vacuoles (arrowheads) RAL 555 rapid stain, 600X

Table 6
Comparing results of culturing on PIM agar and microscopic examination of *Prothoteca*-positive samples from first, second and third sampling

Sample	Culturing I.	Microscopic evaluation I.	Culturing II.	Microscopic evaluation II.	Culturing III.	Microscopic evaluation III.
14.	+	+	+	+	+	+
15.	-	-	+	-	-	-
28.	+	+	+	-	+	+

One sample was positive for infectious algae in the course of the second sampling, but it was negative during the first and the third occasion. Moreover this sample was derived from the right caudal quarter of a cow, whose right cranial quarter gave constantly positive samples for *Prototheca* spp. during the whole study. Thus positivity of the right caudal quarter is questionable; it is strongly suspicious for cross contamination (Table 6.). Statistical assessment showed significant correlation between results of culturing as well as microscopically affirmation of *Prothoteca* spp. positivity and CMT scores and cellularity values ($p<0.05$), although analysis could not find normal distribution in the case of any cell types.

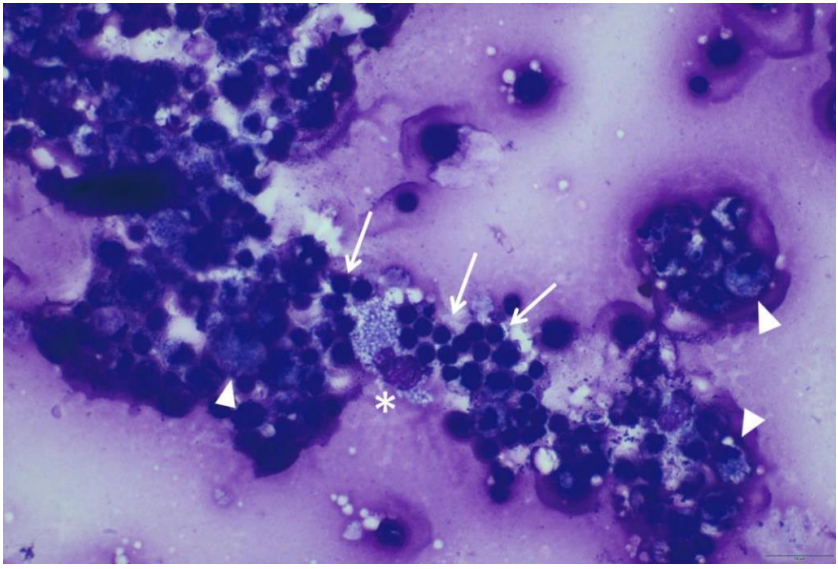


Figure 3. *Prototheca* spp. positive milk sample with high cellularity and large numbers of small lymphocytes (arrows) and macrophages (arrowheads). In the center a mammary foam cell (asterix) can be seen. RAL 555 rapid stain 600X

Besides relative numbers of somatic cell types a remarkable cytological pattern was notified in some samples that were positive for *Prototheca* spp. (Figure 3.). In half of these cases macrophages and lymphocytes displayed strong predominance, moreover medium and large sized lymphocytes were frequently present. In addition statistical analysis of *Prototheca* spp. algae and the ratio of macrophages showed tight correlation (p=0.005).
This finding is supported by the fact that infections caused by fungi, algae or some kind of bacteria (e.g. *Mycobacterium* spp.) produce pyogranulomatous or granulomatous inflammation, in which ratio of macrophages can be more than 50% (*Pal et al.,* 2014).

Table 7
CMT results, cellularity scores and somatic cell counts in *Prototheca*-positive samples from first, second and third sampling

Samples (sampling)	**CMT**	**Cellularity**	**Neutrophil** (%)	**Macrophage** (%)	**Lymphocyte** (%)	**Epithelial cell** (%)
14. (I.)	4	4	73.25	19.75	7.00	0.00
14. (II.)	4	4	76.50	19.00	4.50	0.00
14. (III.)	4	4	89.75	5.75	3.25	1.25
14. mean	4	4	79.84	14.83	4.91	0.41
15. (II.)	4	4	83.25	13.50	3.00	0.25
28. (I.)	4	4	20.25	24.50	54.00	1.25
28. (II.)	4	4	18.00	19.00	62.50	0.50
28. (III.)	4	4	60.75	12.75	26.00	0.50
28. mean	4	4	33.00	18.75	47.50	0.75
Grand mean	**4**	**4**	**66.00**	**15.08**	**18.30**	**0.62**

Although in our *Prothoteca* spp. negative samples high numbers of macrophages seldom occurred, so this presumed unique cytological pattern requires further investigation.

CMT scores, cellularity values and somatic cell counts of *Prototheca* spp. positive samples are showed in Table 7.

CONCLUSIONS

In summary, it has been concluded, that laboratory techniques applied in our study could be helpful supplementary methods besides routine laboratory tests in order to reveal pathological background of mastitis more accurately,

as well as to monitor the progression of inflammatory reactions in the mammary gland tissue.

PIM agar as a selective and differentiating culture medium has been proved to be appropriate diagnostic tool in the detection of *Prototheca* spp. in cow's milk. Although molecular diagnostic tests e.g. Polymerase Chain Reaction (PCR) could be more accurate, because microbial culturing can only detect live agents with adequate dividing ability. It is true that specificity and sensitivity values of microscopically assessment is much lower than culturing, but it could help with the identification of inoperative microbes.

According to the low number of animals and samples being involved, current research should be considered as a preliminary study, which requires more investigations involving much higher number of samples in order to draw further conclusions.

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