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The relationship of bovine milk somatic cell count to neutrophil level in samples of cow's milk assessed by an automatic cell counter

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Research Article

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Abstract

This research communication describes the application of a fluorescent automatic cell counter Lactoscan SCC for simultaneous determination of somatic cell count and neutrophils in bovine milk. The obtained results were compared with results obtained by a flow cytometer and a light microscope. The Pearson correlations between the methods were calculated. A comparison between the main characteristics of the three kinds of analysis was made – the assay duration and the intra-assay precision. A relation between the SCC and neutrophil cells was observed in 55 milk samples. The obtained results confirm that the simultaneous determination of SCC and neutrophil analysis are necessary and support the early diagnosis of mastitis, the timely treatment of the animal and the avoidance of major economic losses.

Somatic cell count (SCC) in milk is one of the main parameters for product quality accepted by countries all over the world. European Union has regulation about milk hygiene – Commission regulation EC No 1662/2006. The legal limit is 400 000 cells/ml for SCC in cow milk. There is a standard for somatic cell determination in milk (ISO, 13366-1, IDF 148-1: 2008) that is used for a reference method. Somatic cells in milk are of several types. It is noticed that there is a significant increase in milk neutrophils in sub-clinical and clinical mastitic cows (Damm *et al*., 2017). That change of the milk cell composition is due to the major role of the neutrophils in immunological defence.

A number of methods have been developed for differential cell counting in milk, in particular for the enumeration of neutrophils in bovine milk. The objectives are diagnosis of mastitis and study of the immune status of the udder. These methods have primarily been flow cytofluorimetric or differential cell staining techniques (Kelly *et al*., 2000; Mehne *et al*., 2010). However, these methods have some limitations such as expensive instrumentation, high reagent costs and discrepancies between technicians and between laboratories. It was of interest to investigate the ability of the Lactoscan SCC (Milkotronic Ltd, Bulgaria) a newly-developed image-based cell counter, to enumerate total somatic cells and neutrophil count in milk.

The predominant somatic cell response during mastitis is an increase in neutrophils (Schwarz *et al*., 2011, 2019). However, Wald *et al*. (2019) noticed that some udder infections are not associated with increased SCC, especially cases associated with *Staphylococcus aureus*. These samples have SCC below 200 000 cells/ml. Similar observations were reported from other authors too (Carrillo-Casas and Miranda-Morales, 2012; Oliveira *et al*., 2012). Consequently, neutrophil cells may be a more specific indicator of inflammation than total SCC. In this study, the application of a fluorescent automatic cell counter for simultaneous determination of somatic cell count and neutrophils in bovine milk was studied. The obtained results were compared with results obtained by flow cytometry and light microscopy.

Material and methods

The experimental procedures followed the Bulgarian Law on Animal Protection and were in accordance with EU Directive 2010/63/EU for animal experiments. Detailed materials and methods are given in the online Supplementary File.

Preparation of a standard SC sample

The standard sample was prepared from a milk sample with initial SCC 500 000 cell/ml. Centrifugation of milk sample was performed at 600 ***g*** for 15 min. Fat layer and supernatant were discarded, without interrupting the cell pellet. Then, the cell pellets was washed with cold sterile saline buffer containing 7.5 mM EDTA. Finally, the washed cell pellets were re-suspended with the same buffer to the final concentration of 1 000 000 cells/ml, measured by reference ISO standard method.

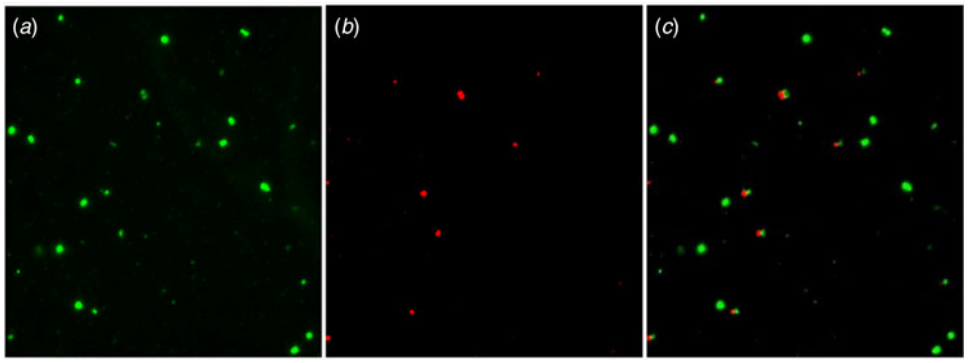

Fig. 1. Image of SC in standard cell sample stained in green with YO, extinction 470 nm (A), Image of neutrophils stained in red with anti-neutrophil antibody-ATTO620, extinction 627 nm (B) and a combination of both colors (C), using automatic cell counter Lactoscan SCC. The initial SCC of the used milk was 500 000 cells/ml.

Preparation of milk suspended cell samples

The milk samples were stored at 4°C before analysis, but all of the analyses were made on the day of the arrival. Ten milk samples (10 ml) with initial SCC from 160 000 to 4 230 000 cells/ml were transferred into ten centrifuge tubes. The ten milk samples was separately treated in the same way as the standard sample, but the washed cell pellets in ten tubes were re-suspended with the same buffer to to the final concentration of 1 000 000 cell/ml, measured by reference ISO standard method.

Milk samples

Raw cow milk samples were collected under sterile conditions and transferred to the laboratory on the same day. Sampling was performed according to the ISO 707 (2008). Selection of the cows ensured milk samples with SCC from 90 000 to 5 640 000 cells/ml. The total number of the samples was 55. The total SCC in initial milk samples was analyzed by the standard method ISO 13366-1:2008, using a light microscope (Olympus BX51 microscope, equipped with QImaging Retiga 2000R camera).

Determination of SCC and neutrophil count by the fluorescent automatic cell counter

For the double staining of the cells, a fluorescent dye Oxazole Yellow (YO) and a fluorescent conjugate anti-neutrophil antibody-ATTO620 were used. The antibody and its fluorescent conjugate were prepared in our laboratory. The procedure for polyclonal anti-neutrophil antibody obtaining was described previously (Becheva *et al*., 2017), and the conjugate was prepared as recommended by the manufacturer of the ATTO620NHS ester. The fluorescent analysis was performed by adding 50 µl of each sample (cell suspension or whole milk) in microcentrifuge tubes and 10 µl anti-neutrophil antibody-ATTO620 conjugate (1 mg/ml). The tubes were incubated at 4°C for 30 min. After that, 2 µl YO (1 mg/ml in DMF) were added and the second incubation was performed for 5 min at RT. The colored cells (8 µl) were analyzed by an automatic cell counter Lactoscan SCC (Milkotronic Ltd, Bulgaria). The set parameters of the instrument are described in the online Supplementary File. The obtained results were compared with results obtained by a flow cytometer and a light microscope (see methodology in Supplementary File).

Statistical analysis

Analysis and visualization of the data were done with Microsoft Office Excel 2010 (Microsoft Corporation, Redmond, WA, USA). For all tests, statistical significance was defined at $P \leq 0.05$.

Results and discussions

The total number of cells and neutrophils in the standard sample were analyzed by Lactoscan SCC and results compared with those from a flow cytometer and a light microscope with the goal of comparing the methodologies in the absence of the milk matrix. The total cell count was determined using the fluorescent dye YO at concentrations varying from 20 to 100 µg/ml. The optimal dye concentration was determined to be a working concentration of 32 µg/ml. Figure 1A presents a fluorescent image of total cell count (green colored cells) of the standard sample analyzed by the automatic cell counter. The neutrophil cells in the same standard sample were determined using a fluorescent conjugate (anti-neutrophil antibody-ATTO620) and the same automatic cell counter. The conjugate binds specifically to the surface of neutrophil cells and a signal is obtained due to the fluorescent dye covalently linked to the antibody. The amount of added fluorescent conjugate was optimized through a series of tests performed with a variety of conjugate concentrations from 50 to 500 µg/ml. The optimal conjugate concentration was determined as 161 µg/ml working concentration. The neutrophil cells can be seen colored in red in Fig. 1B.

Lactoscan SCC permits one to simultaneously determine neutrophils and total number of cells (Fig. 1C), working as an automated fluorescent microscope. It provides an exact view of the stained sample. If the cell has green nucleus and red surface, the cell is counted as a neutrophil. The results for the standard sample 1 were: SCC 1 010 000 cells/ml and 27% neutrophils. These results for standard sample by Lactoscan SCC were compared with the results obtained by flow cytometry (Guava easyCyteTM 8HT), depicted in online Supplementary Figs S1A (total cell count) and S1B (nutrophils). Using the same YO dye and anti-neutrophil antibody-ATTO620 conjugate, the counts we obtained were: SCC 1 000 783 cells/ml and 26.5% neutrophils, demonstrating close agreement.

Our automatic cell counter data were also compared with light microscopic analyses, performed with an Olympus BX51 light microscope using reference methodology and methylene blue (online Supplementary Fig. S2). Counts obtained from the same

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Table 1. Relationships between SCC and neutrophil count in milk samples ($n = 52$) and comparison of three different instruments for analysis

		Milk SCC × 1 000 cells/ml				
		<200	200–400	400–500	500–800	>800
Neutrophils, % (Lactoscan SCC)	Min – Max value	8–22	10–26	24–28	45–55	41–76
	GM*	16.3	16.3	26.3	49.7	54.6
	AM*	17.3	17.5	26.3	50.0	55.5
Neutrophils, % (Flow cytometer)	Min – Max value,	7–24	13–29	19–39	47–49	40–60
	GM*	10.6	18.3	29.9	48.0	48.0
	AM*	12.3	19.0	30.6	48.0	48.6
Neutrophils, % (Microscopic method)	Min – Max value,	11–20	9–30	20–30	40–53	42–56
	GM*	15.9	16.9	24.8	46.7	48.7
	AM*	16.2	18.2	25.0	47.0	48.9

*GM, geometric mean; *AM, arithmetic mean.

standard sample were: SCC 1 000 000 cells/ml and 28% neutrophils, once again demonstrating close agreement.

To further compare the methods, ten samples of suspended cells diluted with saline buffer to a final concentration of 1 000 000 cell/ml were prepared from ten milk samples with initial SCC ranging from 160 000 to 4 230 000 cells/ml. The treatment of milk samples was performed to exclude the matrix effect of milk on the cell counting. Comparison of the results is presented in online Supplementary Fig. S3 (Supplementary). Close agreement between the methods is apparent, as is the relationship between neutrophils and original SCC value. If the starting SCC was higher than 400 000 cells/ml, the neutrophil cell percent was about 30% or higher. It is noteworthy that the highest neutrophil count (65–70%) was obtained by all three methods at an SCC of 790 000 cells/ml. Each of the ten milk samples was analyzed five times on the same three instruments and the intra-assay variation of the results was calculated to be <10%. The Pearson correlations between the methods were calculated. Correlations were high: between the standard microscopic method and the automatic cell counter the correlation was 0.97, between the standard method and the flow cytometry it was 0.96 and comparing the automatic cell counting and flow cytometry, the correlation was 0.98. we compared the time taken for analysis as well as the intra-assay precision. Duration of the analyses performed by standard microscopic technique was about 30 min for one sample and more than 60 min for ten samples. The analysis duration with the automatic cell counter and the flow cytometer was 2 and 15 min for one sample, respectively, and for 10 samples was about 20 min with both instruments. Consequently, the automatic cell counter and the flow cytometer are preferable when there are greater numbers of samples. The intra-assay precision was expressed by coefficient of variations of repeatability (CVs). CVs, repeatability standard deviations, and repeatability limits obtained for ten milk suspended cell samples are presented in online Supplementary Table S1. The microscopic method had CVs lower than 10%, whislt the fluorescent methods performed by the automatic cell counter and flow cytometer both had CVs lower than 5%.

It was of interest to make comparison between three methods for detection of SCC and neutrophil count in whole milk samples. Therefore, 55 milks were analyzed. Ten of them were from clinically healthy cows, and the others were from cows with mastitis (Table 1). All the raw data of the samples are presented in online Supplementary Table S2. Milk from healthy mammary gland had a neutrophil count of less than 20%, whereas milk from cow with mastitis had nuetrophil counts in excess of 30%. Values between 20 and 30% probably depend on the individual and can be discussed if the amount of neutrophil is monitored over a long period for the same mammary gland. Similar observations of the relation between SCC and neutrophil count in milk have been reported in other studies. For example, there are reported 19% neutrophils from a healthy gland, and 75% from mastitis cow (Alhussien *et al.*, 2016). Gargouri *et al.* (2008) observed a trend of rising neutrophil proportion (from 23 to 44%) as a response to inflammatory infection. We also noted that there were three cases from all 55 milk samples, where the number of somatic cells was small (consequently, the milk classified as healthy), but the number of neutrophils was large. That finding suggests that inflammatory processes can appear within an SCC range that is clearly below the legal cut-off value of 400 000 cells/ml, perhaps during the initial phase of an inflammation. Comparison of the results of the whole milk samples using the three methods are presented in online Supplementary Fig. S4. The Pearson correlations between the methods using for measuring of whole milk samples were calculated. The microscopic method compared to automatic cell counting and flow cytometry had correlations of 0.88 and 0.84, respectively and the correlation between the two fluorescence techniques was 0.87. It was observed that the correlation coefficients are lower than those obtained with standard cell suspensions, probably as a consequence of the matrix effect of milk.

In conclusion, this study establishes the potential of the Lactoscan SCC automatic cellcounter for providing consistent and reproducible counts of SCC and neutrophils in bovine milk. Furthermore, the data obtained with this method were highly correlated with those obtained with the standard microscopic method and the flow cytometer. The development of an assay for simultaneous determination of the number of somatic and neutrophil cells in the same sample will allow accurate and definitive diagnosis of mastitis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000534

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