The relation of bovine milk somatic cell count and neutrophil level in samples of cow milk

Zlatina Becheva, Yavor Ivanov, and Galina Grigorova

SUPPLEMENTARY FILE

Reagents

Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), methylene blue, oxazole yellow (YO), N,N-dimethylformamide (DMF), ATTO620NHS ester, ethanol (absolute, for HPLC, ≥ 99.8%), tetrachloromethane, glacial acetic acid were ordered from Sigma-Aldrich (82024 Taufkirchen, Germany).

Preparation of milk suspended cell samples

Ten milk samples (10 ml) with initial SCC from 160 000 to 4 230 000 cells/ml were transferred in a ten centrifuge tubes. Then, the milk samples were separately centrifuged at 600 *g* for 15 min. Fat layer and supernatant were discarded, without interrupting the cell pellet. Then, the cell pellets in each tube was washed with cold sterile saline buffer containing 7.5 mM EDTA. Finally, the washed cell pellets was re-suspended with the same buffer to the final concentration of SC 1.000.000 cells/ml, measured by the reference ISO standard method.

Determination of SCC and neutrophil count by a fluorescent automatic cell counter Lactoscan SCC and a flow cytometer

The automatic cell counter Lactoscan SCC had two light sources. The first light source (470 nm) had light power 100%, gain 1, exposition 0.1, and focus 2768. The second light source (627 nm) had light power 100%, gain 47, exposition 0.1, and focus 2888.

The parameters of the flow cytometer Guava easyCyteTM 8HT in a program Guava® ExpressPlus were optimized. The program allowed to acquire and analyzed up to three fluorescence parameters (GRN – green, YLW – yellow, RED – red) in combination with forward scatter (FSC) and side scatter (SSC). Gain and photomultiplier tubes (PMT) voltages were set: FSC Gain x 32, SSC 413 V, GRN 485 V, YLW 250 V, RED 706 V. Also, compensation was made: GRN-%RED 13, RED-%GRN 54.9.

Determination of SCC and neutrophil count in standard somatic cell suspensions and milk by a microscopic method

The methylene blue solution contained for 50 ml: methylene blue 0.5 g, ethanol 27 ml, tetrachloromethane 20 ml, glacial acetic acid 3 ml. The staining procedure was standard. Sample (cell suspension or whole milk) 10 µl was placed over a microscope slide (area 10 x 10 mm) and air dried. Then, methylene blue solution was placed over the dried sample directly while passing the solution through syringe filter with 0.2 µm pore size (GVS life sciences, USA). After 5 min incubation, the slide was rinsed with tap water, air dried, and observed by the light microscope (Olympus BX51 microscope, equipped with QImaging Retiga 2000R camera).

Oxazole Yellow and ATTO620 used with Lactoscan SCC and Guava easyCyteTM 8HT

YO is a dye with green emission and virtually nonfluorescent in solution. It consists of only a single aromatic moiety and binds to DNA through intercalation. That mono-intercalator increases its quantum yield, consequently its fluorescence, when bound to DNA (Murade et al. 2009). The extinction and emission maximums of the dye are at 491 nm and 509 nm wavelength (manufacturer specification data).

The ATTO-fluorescent dyes, like ATTO620, have bright enough signal, desired resolution and photostability (Zheng et al. 2014). Protein conjugates with ATTO620 provides red fluorescence. The extinction and emission maxima are at 620 nm and 642 nm wavelength (manufacturer specification data).

The set parameters of the Lactoscan SCC, flow cytometer Guava easyCyteTM 8HT and light microscope Olympus

The bright signals obtained by Lactoscan SCC were due to the two lasers in the instrument that were suitable for the used markers: 470 nm wavelength for YO, and 627 nm for anti-neutrophil antibody-ATTO620 conjugate. The green emission from YO passes through a filter 538/30 nm wavelength, and the red emission from the conjugate passes through a filter 685/30 nm wavelength. The signal is visualized on the screen like a green and red cells corresponding to total somatic cells and neutrophil cells, respectively.

The flow cytometer Guava easyCyteTM 8HT has two lasers: 488 nm and 642 nm. The filter for the DNA-intercalator YO in the instrument is 525/30 nm. And the filter for the ATTO620-conjugate is 661/19 nm. Both emissions generate signals expressed as events.

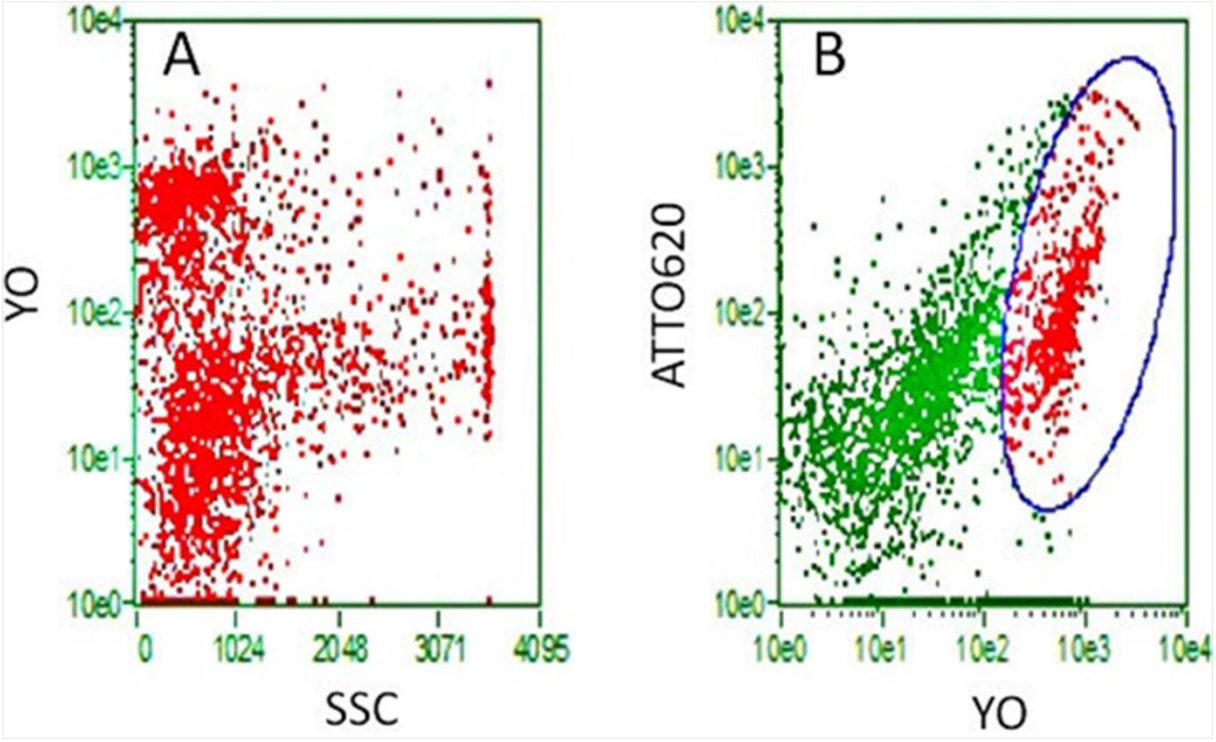


Figure S1. Flow cytometric determination of neutrophil cells and somatic cells, the total somatic cells (**A**) and gated neutrophil cells among the somatic cell variety (**B**) in the standard cell sample.

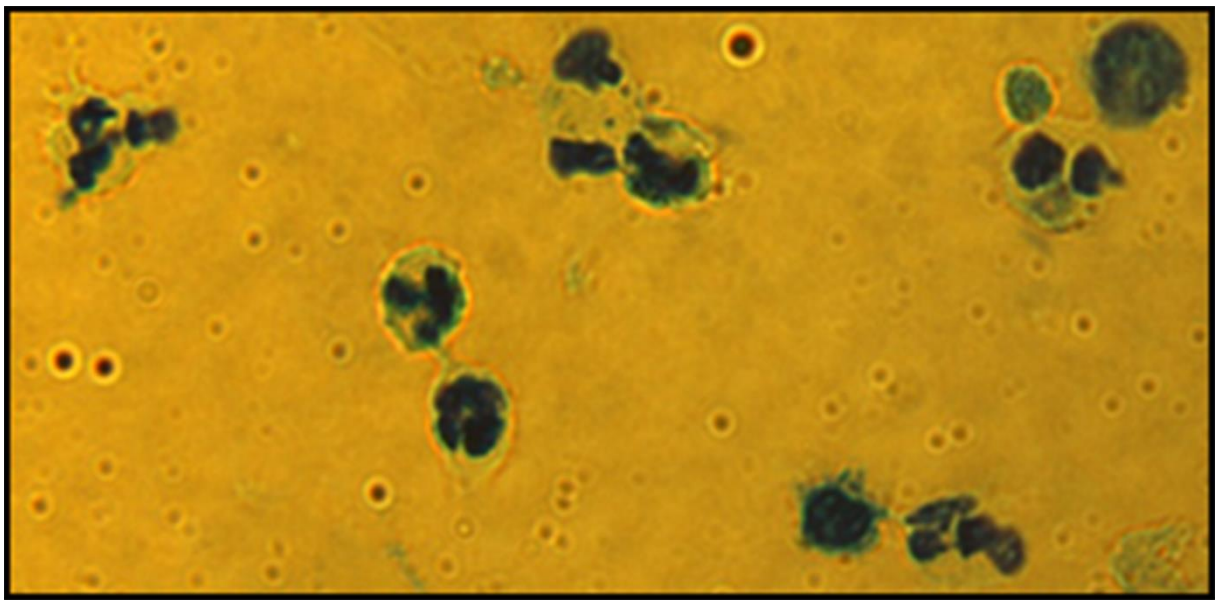


Figure S2. Light microscopic image of somatic cells in standard cell sample, analyzed by a light microscope Olympus (magnification x 100).

The neutrophil cells clearly were differentiated from the other type of somatic cells. The neutrophil nucleus has typical multi-lobulated segmented morphology and it is possible to be observed by a suitable staining technique. The proposed methylene-blue-staining method provided clear differentiation between neutrophils and other somatic cells (Fig. S2).

Relation between SCC and neutrophil count in milk suspended cell samples

Comparison of the results of the ten milk suspended cell samples by used three methods was presented on Fig. S3 (Supplementary).

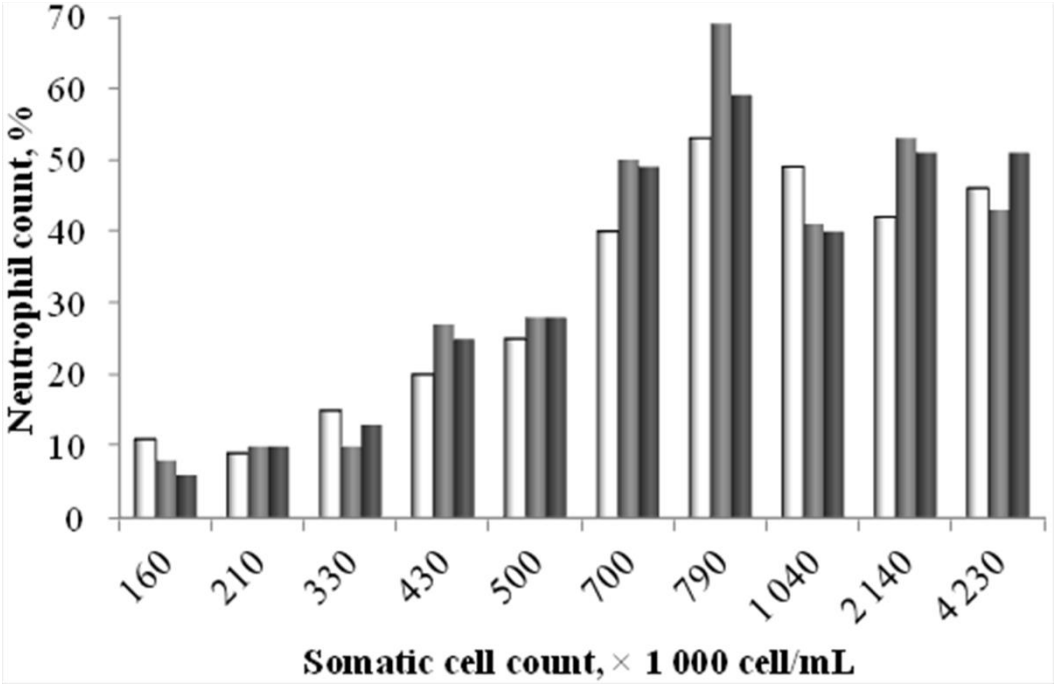


Figure S3. Relation between SCC and neutrophil count in milk suspended cell samples. Comparison of neutrophil count, measured by three different instruments for analysis (light microscope – white, automatic cell counter – grey, flow cytometer – black). The presented SCC in initial ten milk samples was obtained by standard microscopic method with methylene blue (ISO 13366-1:2008 IDF 148-1:2008).

The CVs obtained for ten milk suspended cell samples were presented in Table S1.

Table S1. The precision of analysis of neutrophil counting for ten milk suspended cell samples, using three different devices

	Neutrophil cell count – microscopic method				Neutrophil cell count – automatic cell counter				Neutrophil cell count – flow cytometer			
SCC, $\times 10^3$ cells/mL	Mean, $\times 10^3$ cells/mL	s_r, $\times 10^3$ cells/mL	CV, %	r, $\times 10^3$ cells/mL	Mean, $\times 10^3$ cells/mL	s_r, $\times 10^3$ cells/mL	CV, %	r, $\times 10^3$ cells/mL	Mean, $\times 10^3$ cells/mL	s_r, $\times 10^3$ cells/mL	CV, %	r, $\times 10^3$ cells/mL
160	17.6	1.7	9.9	4.9	12.8	0.6	4.9	1.8	9.6	0.2	2.1	0.6
210	18.9	1.9	9.8	5.2	21	1.0	4.9	2.9	21	0.5	2.3	1.4
330	49.5	4.6	9.2	12.8	33	1.5	4.5	4.2	42.9	0.9	2.1	2.5
430	86	7.7	9.0	21.7	116.1	4.6	4.0	13.0	107.5	2.4	2.2	6.6
500	125	11.3	9.0	31.5	140	6.6	4.7	18.4	140	4.2	3.0	11.8
700	280	22.4	8.0	62.7	350	10.5	3.0	29.4	343	9.6	2.8	26.9
790	418.7	33.5	8.0	93.8	545.1	17.4	3.2	48.8	466.1	16.3	3.5	45.7
1 040	509.6	40.8	8.0	114.2	426.4	12.8	3.0	35.8	416	13.7	3.3	38.4
2 140	898.8	88.1	9.8	246.6	1134.2	34.0	3.0	95.3	1091.4	43.7	4.0	122.2
4 230	1945.8	184.9	9.5	517.6	1818.9	50.9	2.8	142.6	2157.3	88.4	4.1	247.7

SCC – somatic cell count; s_r – repeatability standard deviation; CV – coefficient of variation of repeatability; r – repeatability limit ($r = 2.8 \times s_r$).

Relation between SCC and neutrophil count in the whole milk samples

The row data of the samples added in the analyses is presented in Table S2 and comparison of the results of the whole milk samples by the used three methods was presented on Figure S4. There were three whole milk samples with case conditions. In those milks the number of somatic cells was small, but the number of neutrophils was large. Therefore, those milks were excluded from the other milk results.

Table S2. Row data of the analyzed whole milk samples.

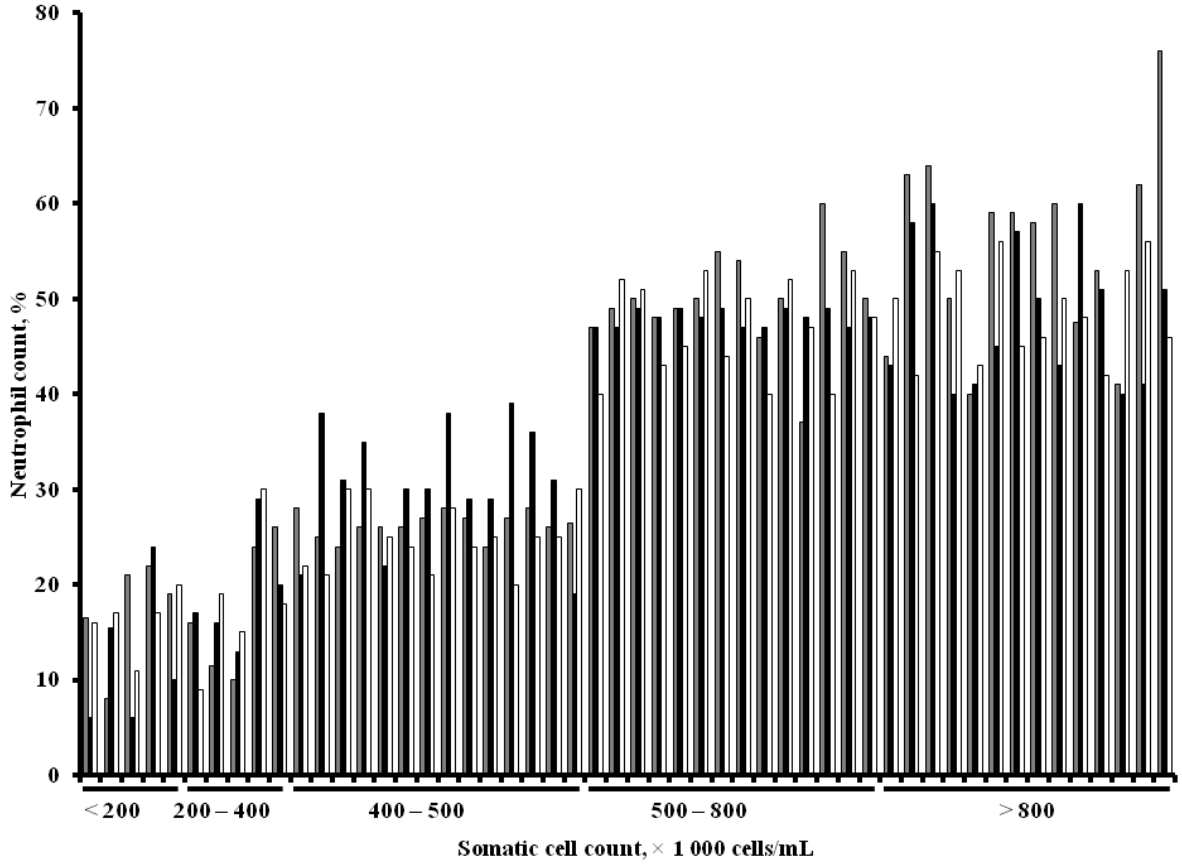
Milk group	Neutrophil count – Lactoscan SCC	Neutrophil count – Flow cytometer	Neutrophil count – microscopic method	Milk group	Neutrophil count – Lactoscan SCC	Neutrophil count – Flow cytometer	Neutrophil count – microscopic method
< 200 000 cells/mL	16.5	6.0	16.0	500 000 – 800 000 cells/mL	47.0	47.0	40.0
	8.0	15.5	17.0		49.0	47.0	52.0
	21.0	6.0	11.0		50.0	49.0	51.0
	22.0	24.0	17.0		48.0	48.0	43.0
	19.0	10.0	20.0		49.0	49.0	45.0
200 000 – 400 000 cells/mL	16.0	17.0	9.0		50.0	48.0	53.0
	11.5	16.0	19.0		55.0	49.0	44.0
	10.0	13.0	15.0		54.0	47.0	50.0
	24.0	29.0	30.0		46.0	47.0	40.0
	26.0	20.0	18.0		50.0	49.0	52.0
400 000 – 500 000 cells/mL	28.0	21.0	22.0		37.0	48.0	47.0
	25.0	38.0	21.0		60.0	49.0	40.0
	24.0	31.0	30.0		55.0	47.0	53.0
	26.0	35.0	30.0		50.0	48.0	48.0
	26.0	22.0	25.0	> 800 000 cells/mL	44.0	43.0	50.0
	26.0	30.0	24.0		63.0	58.0	42.0
	27.0	30.0	21.0		64.0	60.0	55.0
	28.0	38.0	28.0		50.0	40.0	53.0
	27.0	29.0	24.0		40.0	41.0	43.0
	24.0	29.0	25.0		59.0	45.0	56.0
	27.0	39.0	20.0		59.0	57.0	45.0
	28.0	36.0	25.0		58.0	50.0	46.0
	26.0	31.0	25.0		60.0	43.0	50.0
	26.5	19.0	30.0		47.5	60.0	48.0
					53.0	51.0	42.0
					41.0	40.0	53.0
					62.0	41.0	56.0
					76.0	51.0	46.0

102

103

104

Figure S4. Relation between SCC and neutrophil count in the whole milk samples. Comparison of neutrophil count, measured by three different instruments for analysis (light microscope – white, automatic cell counter – grey, flow cytometer – black).



Reference

Murade CU, Subramaniam V, Otto C & Bennink ML 2009 Interaction of oxazole yellow dyes with DNA studied with hybrid optical tweezers and fluorescence microscopy. *Biophysical Journal* **97** 835-43

Zheng Q, Juette MF, Jockusch S, Wasserman MR, Zhou Z, Altman RB & Blanchard SC 2014 Ultra-stable organic fluorophores for single-molecule research. *Chemical Society Reviews* **43** 1044-1056