Application of Flow Cytometry and Fluorescence Techniques in Somatic Cell Analysis of Raw Milk

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Abstract: Monitoring the quality and safety of milk requires careful analysis of somatic cell count (SCC). High level of SCC is a signal of diseases such as mastitis. Mastitis can be detected not only by measuring the inflammatory components and pathogens but also evaluating the SCC in milk. In this study, somatic cells are counted with direct microscopy and flow cytometry. For SCC analyses, a centrifugal lipid clearing process is applied. Cleared samples are stained with two fluorescent dyes acridine orange (AO) and ethidium bromide (EtBr), which have different binding mechanism to cell. These two dyes and their different applications are investigated to reveal the effect on somatic cell count. The results are also compared with direct microscopy method. The results of flow cytometry counting method are well correlated with the ones obtained by direct microscopy technique. In conclusion, AO can be an alternative chemical dye for EtBr to be used in enumeration of SCC by flow cytometry technique.

Keywords: Acridine orange, Ethidium Bromide, Flow cytometry, Raw milk, Somatic cell.

1. INTRODUCTION

Monitoring microbial and somatic cells (white blood cells) is essential for assurance of the safety of milk and dairy products. The number of somatic cells is an important indicator for detection of mastitis [1, 2]. In addition to this, it is a useful method when it is run together with rapid screening methods to reduce false-positive outcomes of antibiotic residue tests [3].

All milk contains white blood cells known as leukocytes which constitute the majority of somatic cells. These cells consist of neutrophils, lymphocytes and macrophages. Macrophages comprise the major cell type in milk obtained from uninfected cow [4]. The cell count for "normal" milk is less than 200,000 cells/mL (even it is lower for first lactation cows). Higher counts are considered abnormal and indicate probable infection *e.g.* mastitis caused by a pathogenic bacteria like *Staphylococcus aureus*, tissue damage or other inflammation processes affecting the mammary tissue [5, 6]. The increase in somatic cell count causes to transfer white blood cells from blood to mammary gland [4]. As a result, the level of neutrophils present in milk increases significantly to fight with disease and to repair the damaged tissue. Their main function is to protect the udder from bacterial infections. During the mastitis, neutrophils are transported from the peripheral blood system into milk via the mammary epithelium in response to chemotactic stimuli produced locally as a reaction against microorganisms.

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Currently, the enumeration of somatic cells relies on two methods including direct microscopic counting technique and automatic counting based on flow cytometry technology in raw milk [7]. Direct microscopic counting is known as a conventional culture method used to detect and enumerate the somatic cells or microbial contamination. This well-known method is based on staining milk with trypan blue, spreading uniformly as a thin film onto a foursquare area marked on the surface of a piece of microscope slide and examining under a light microscope [8]. But this is a time consuming method and does not provide complete quality and safety assurance. Additionally the instrumentation is limited in its range of application [9]. Variations in the results related with the skill level of an operator can be another drawback of this method. Discrepancy between the results is also caused by lack of specificity between cells and cytoplasmic particles [10].

There are some automated somatic cell counting (SCC) systems developed based on a staining of the sample and direct microscopic counting. Flow cytometry offers an automatic and objective counting of somatic cells in milk samples. It minimizes the discrepancy of the results via its highly-sensitive property [11]. It can also give information about viability of cells. Automation of this process means that large number of samples can be analyzed per hour in milk-testing laboratories [8].

Flow cytometer can be used for enumeration of somatic cells in raw milk. Flow cytometry uses the principles of light scattering, light excitation, and emission of fluorochrome molecules to generate specific multi-parameter data from cells. It works with a

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combination of fluorescent stains or fluorogenic substrates and the sample pumped through a flow cell of very small diameter which allows only one cell to pass at a time [11, 8].

Somatic cells are counted according to the number of their nucleus DNA in flow cytometer. DNA is stained with fluorescent dyes such as acridine orange (AO), ethidium bromide (EtBr) or propidium iodide (PI) or syto 13 [7, 12]. According to dye property, different pre-processing procedures can be applied. For instance, EtBr and PI can penetrate only DNA of dead cells. In order to evaluate the live and dead somatic cells together, it is necessary to create pore on the cell wall by using a detergent with a salt solution. On the other hand, AO can penetrate into both live and dead cells; the salt solution can be used with AO to penetrate into DNA [13, 12].

Flow cytometry allows the enumeration of large number of somatic cells in a certain time and eliminates variations in the results arising from operator skills. Being expensive, requiring an expertise and lacking of a standard method for staining the cells are the drawbacks of this method [14, 15, 7].

The aim of this study is to demonstrate the application of flow cytometry coupled with fluorescence techniques for enumeration of somatic cells in raw milk, to use different surfactants to determine different bound mechanisms to cells, to compare the results with the direct microscopic counting method. For this purpose, the effect of fluorescent dyes *i.e*. acridine orange (AO) and Ethidium Bromide (EtBr), a surfactant *i.e*. Triton X-100 (trit-X) and control solution *e.g*. Phosphate-Buffered Saline (PBS) on the somatic cell count of raw milk samples is evaluated. fluorescent dyes *i.e*. acridine orange (AO) and Ethidium Bromide (EtBr), a surfactant *i.e*. Triton X-100 (trit-X) and control solution

2. MATERIAL AND METHODS

Milk and Milk Composition

The raw milk samples were collected during the period from March 2010 to October 2011 from a farm located in Gulbahçe, Izmir, Turkey. Cows in early-lactation (40 to 60 days after calfing) were used. The cows were milked twice daily. The samples from morning milking were aseptically collected and brought to the lab in an hour. Prior to measurements, raw milk samples were preserved with a final concentration of 0.02% Bronopol (Sigma-Aldrich, St. Louis, MO, USA) and stored at +4 ^{0}C.

The percent of fat, protein, lactose and solid not fat (SNF) content of milk samples were determined by using Funke Gerber 3510 Lactostar milk analyzer (Funke Gerber, Berlin, Germany) at 20°C.

Clearing of The Milk and Fluorescent Staining of Somatic Cells

Before the measurement, milk samples were centrifuged at 180×g for 10 min to remove lipids and thereby allow distinction of somatic cells by flow cytometry [2]. The lipid layer collected on top of the samples and adhered to the tube wall was drawn off with a micropipette and a soft swab without disturbing the pelleted material, which contained somatic cells. Cleared milk pellets were suspended in 10 mL phosphate-buffered saline (PBS, Sigma-Aldrich, St. Louis, MO, USA) solution. After this process the pellets were centrifuged at 180×g for 10 min and then re-suspended again in PBS and 0.1% Triton X-100 (trit-X) (Amresco LLC, Solon, OH, USA) prior to staining and analysis.

Two different dyes, *i.e*. acridine orange AO (Sigma-Aldrich, St. Louis, MO, USA) and Ethidium bromide (EtBr) (Sigma-Aldrich, St. Louis, MO, USA) were used for staining the pellets. Stock solutions of AO (0.1 mg/ml) and EtBr (5 mg/ml) were prepared in pure water. The cell pellets suspended in both PBS and Triton X-100 surfactant were stained with AO. However, only Triton X-100 surfactant solution was used to re-suspend the pellets in the staining process with EtBr. Polystyrene flow cytometry tubes in the size of 12×75 mm prepared according to BD Cell Viability Kit and filled with 850 µl of pellet, 50 µl of bead (BD Biosciences, San Jose, CA, USA) and 100 µl of stock dye solution. This mixture was stored for 15 min at room temperature to let dyes to penetrate into the cells.

Flow Cytometry, Microscopy and Data Analysis

The flow cytometer (FACSCanto^{TM}, BD Biosciences, San Jose, USA) equipped with a 15-mW argon laser emitting light at 488 nm was used for somatic cell analyses. The instrument was equipped with forward-angle light scatter (<15°), side-angle light scatter (>15°), and three fluorescence detectors: FL1 (515 to 565 nm), FL2 (565 to 605 nm), and FL3 (>605 nm). For the counting of AO stained cells, excitation and emission wavelength of the argon laser was adjusted to 488 nm and 530 ± 20 nm in Fluorescein isothiocyanate (FITS) channel. On the other hand, for the counting of cells stained with EtBr, the excitation

wavelength was kept at 488 nm and the emission wavelength was set to 585± 20 nm in Phycoerythrin (PE) channel. At least 10,000 cells were monitored using a BD FACSCanto™ flow cytometer and DNA histograms of each sample were further analyzed with FACSDiva 5.0.3 (FACSCanto BD Bioscience, San Jose, USA) software. Total number of somatic cells was determined using Equation (1). Somatic cells were also counted by means of a light microscope (OLYMPUS-CX31, JAPAN) and a hemocytometer to confirm and compare data obtained from flow cytometer measurement.

$$\frac{number\ of\ cell\ events}{number\ of\ bead\ events} \times \frac{assigned\ bead\ count\ of\ the\ lot\left(\frac{bead}{50}\mu l\right)}{volume\ of\ sample} = concentration\ of\ sample\ as\left(\frac{cells}{\mu l}\right) \quad (1)$$

For direct microscopic count, 100 µl cleared cells and 100 µl of 0.5% trypan blue solution (Code: 03-102, Biorad Lab.) were mixed and spread uniformly onto a slide and counted using a hemocytometer. Somatic cells possessing dark blue nucleus were counted by means of a light microscope (Olympus-CX31, Japan) fitted with a 40X objective. The number of somatic cells screened in 30 different areas on the slide was recorded and reported as the total number per milliliter. The experiment was repeated two times.

Somatic cell count results of raw milk samples were evaluated with one-way analysis of variance (ANOVA) with Tukey's test, individual error rate were performed by means of statistical software (Minitab V14, Minitab Inc., State College, PA, USA). The Tukey`s test was carried out in order to evaluate the effect of AO and EtBr and trit-X and PBS solutions on somatic cell number of raw milk samples at 95 percent confidence interval.

3. RESULTS AND DISCUSSION

Milk Composition

The composition of raw milk samples as fat%, protein%, lactose% and SNF% were measured in the samples withdrawn during winter, spring, summer and autumn season. The statistical testing showed that seasons had a significant effect on the fat%, protein%, lactose% and SNF% content of raw milk. The clear differences were observed on the fat content of raw milk. In summer season, the fat content of raw milk samples was very low (2.56 ± 0.168 %) whereas the highest fat content (8.058 ± 0.081 %) was measured in the samples obtained in autumn season. The protein, lactose and SNF content of raw milk were 7.25 ± 0.039 %, 7.04 ± 0.045 % and 7.13 ± 0.042 %, respectively. These were the highest values and reported in spring season. The lowest values were measured in winter season.

Somatic Cell Counts

In order to determine the number of somatic cells by means of flow cytometry, we first removed the lipids in raw milk. Presence of lipid particles in milk increases the amount of debris causing limited somatic cell counting. Two different DNA-binding fluorescent dyes acridine orange (AO) and ethidium bromide (EtBr) were used to identify the types of inflammatory cells present in milk. Since AO can penetrate into either live or death cell's DNA, cells were suspended in PBS solution before staining. On the other hand, EtBr can penetrate only death cells. It is also advised to be used by AOAC 978.26 method [16] to determine SCC with optical somatic cell count method. Somatic cells are primarily dead white blood cells that are being flushed from the animal through the milk. In order to stain the live cells with EtBr, cells were suspended in Triton-X-100 (trit-X) solution to create pores on their cell wall. Also in order to evaluate the effect of trit-X with AO, again cells were suspended in trit-X than stained with AO. All the flow cytometry results were confirmed with direct microscopic measurements.

The somatic cell count in milk was evaluated in the range of 50×10^3 and 135×10^3 cells/mL. According to Raynal-Ljutovac (2007 [17] non-pathological factors are responsible for variation of SCC in cow milk in the range of 40×10^3 and 100×10^3 cells/mL. This variety in SCC did not depend on directly seasons. High numbers of somatic cells can fluctuate from one day to another particularly in the late lactation and even within days. It was reported that the sample collection during milking or after milking was also responsible for the change of SCC in milk [17, 18].

There was a good correlation *i.e.* $R^2 = r = 0.96$, between the number of somatic cells stained with AO and EtBr using the same surfactant (trit-X) (Figure **1a**). It was revealed that the means of somatic cell number were not statistically different from each other ($p>0.05$) (Table **1**). Moreover the same analyses were done with cells suspended in trit-X and PBS and stained with AO (Figure **1b**). The results showed that there was a good correlation and statistically no significant differences

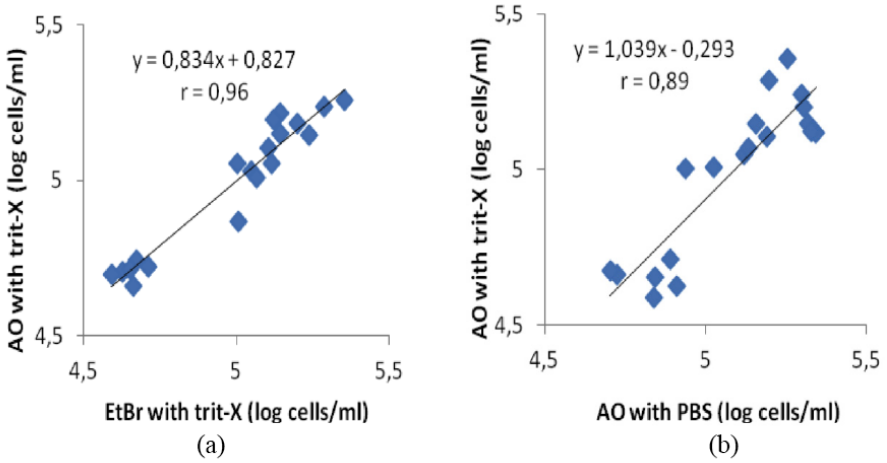

Figure 1: Correlations between the log numbers of stained somatic cells, (**a**) the measurement performed with cells suspended in trit-X surfactant and stained with EtBr and AO, (**b**) the measurement performed with cells suspended in trit-X and PBS and stained with AO.

between data obtained by using the surfactant trit-X and control solution PBS (R^2 = r = 0.89, p>0.05) (Table **1**).

Finally, all the flow cytometry counts were compared with direct microscopy results. The data given in Figure **2** showed that there were no significant differences (p>0.05) between these methods (Table **1**).

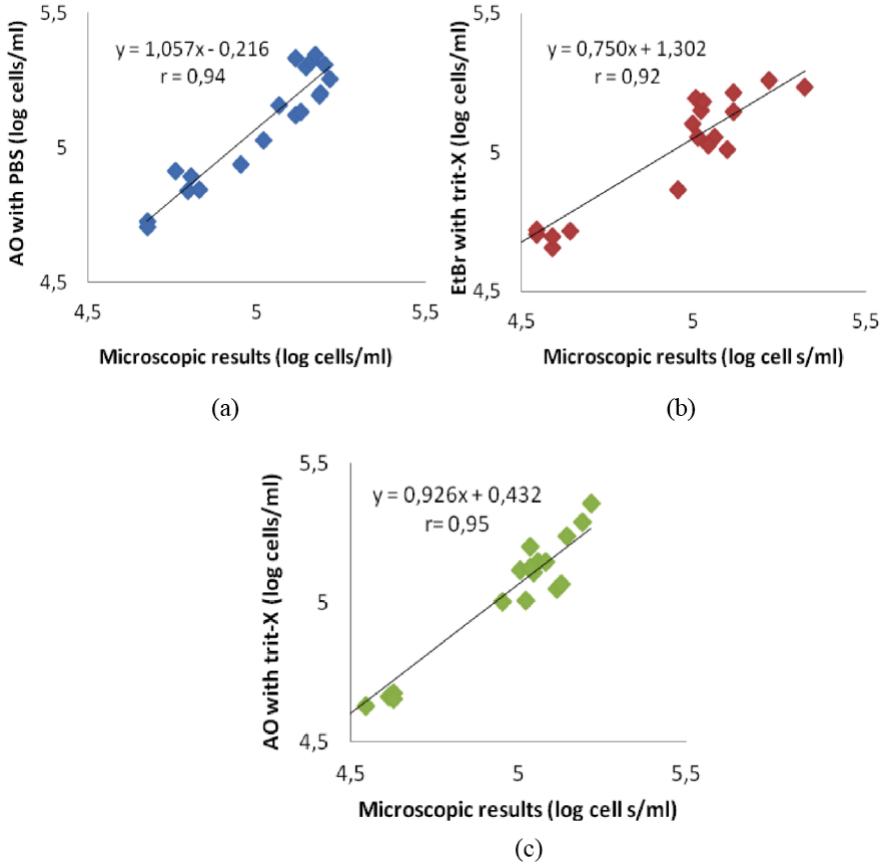

Figure 2: Correlations between somatic cell counts enumerated by the direct microscopy and the flow cytometry, (**a**) cells suspended in PBS and stained with AO, (**b**) cells suspended in trit-X and stained with EtBr, (**c**) cells suspended in trit-X and stained with AO.

Table 1: Comparison of the Flow Cytometric and Direct Microscopic Somatic Cell Count Data Obtained by Using Different Dyes (AO and EB), a Surfactant (trit-X) and Control Solution (PBS)

Somatic Cell Counts with Flow Cytometry	Microscopic Counts	Dye	Surfactant
AO with PBS	130 748[a] ± 7835	-	135 388 [a]± 6937
AO with trit-X	136 523[a] ± 9623	139 637[a]± 10250	131 986[a]± 8425
EtBr with trit-X	133 214[a] ± 4867	131 986[a]± 8425	-

Means within a column with different letters differ statistically ($p<0.05$).

Furthermore, there was a better correlation between the data obtained by the direct microscopy and the method where the cells were suspended in the surfactant trit-X and stained with AO dye ($R^2 = r = 0.95$) compared to the other methods employing different dye combinations for somatic cell counting in raw milk samples.

4. CONCLUSIONS

For SCC analyses, centrifugal lipid clearing was applied. Cleared samples were stained with two fluorescent dyes acridine orange (AO) and ethidium bromide (EtBr) which have different bound mechanism to cell. EtBr stock dye is advised to determine the SCC by AOAC method. AO is an alternative chemical dye for EtBr. These two dyes and their different applications were compared how to affect the count of somatic cells and the obtained results were also confirmed with direct microscopy method. Based on the data, a good correlation was observed between the counts obtained by using these two fluorescent dyes. The results of flow cytometry counting method are also well correlated with the ones obtained by direct microscopy technique. It is concluded that AO can be an alternative chemical dye for EtBr to be used in enumeration of SCC by flow cytometry technique.

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