Immunofluorescence microscope assay of neutrophils and somatic cells in bovine milk

Zlatina Becheva, Katya Gabrovska and Tzonka Godjevargova

Department of Biotechnology, University “Prof. Dr Asen Zlatarov”, Bourgas, Bulgaria

ABSTRACT
Polyclonal antibody specific for bovine neutrophils was prepared. Serum titer was observed at 75th and 105th day of injection period. The first titer was 1250-fold serum dilution and the second – 31,250-fold serum dilution. Antibody was purified with affinity chromatography. Antibody reactivity to neutrophil fragments, Ficoll-separated bovine blood cells – granulocytes (neutrophils, basophils and eosinophils) and mononuclear cells, and bovine milk somatic cells (SC) were studied. The antibody has detection limit 5 µg/mL neutrophil fragments and reacts only with neutrophils. The antibody was conjugated to fluorescein isothiocyanate (FITC). The specificity of the fluorescent conjugate was analyzed with Ficoll-separated blood cells and milk SC by fluorescence microscopic analysis. The microscope images prove the conjugate reactivity with blood and milk neutrophils. It was found that when SC count (SCC) was 100,000 cells/mL neutrophils were 30%, but when SCC exceeded 800,000 cells/mL neutrophils were 88%. The proposed antibody–FITC method was compared with commercial Giemsa method.

Introduction

The somatic cell count (SCC) in bovine bulk tank milk is presently used as an indicator of raw milk quality, reflecting the udder health status of the herd. Untreated mastitis results in economic losses due to costs arising from reduced milk production, discarded milk, treatment and premature culling or death. The disease is found in four main forms: acute, chronic, clinical and subclinical. Subclinical mastitis, which shows no visible signs of disease, causes the greatest financial loss to dairy farmers.

Screening and evaluation of mastitis has been routinely performed utilizing the following procedures: California Mastitis Test, Wisconsin Mastitis Test, strip test and milk cultures. But the last of these tests are non-specific indicators of inflammation and do not detect specific infectious agents.

There is a variety of direct and indirect methods, such as direct microscope counting, automated particle size analysis, automated nuclei fluorescent staining, for evaluation of the somatic cells (SC) (lymphocytes, neutrophils, macrophages and epithelial cells) in milk. Nowadays, cooperatives and dairies most commonly used electronic counters for total SCC, like Coulter and Fossomatic cell counters. These apparatuses are expensive,
require skilled personnel and offer not enough information for the health of the animals. Some authors recognized that the total SCC increased in mastitic milk, and the predominantly cell type were neutrophil cells. Therefore, neutrophil cell counting in infected milk provide more information for the health of the udder than the total SCC (Cullen, 1966; Kitchen, 1981; Meek, Barnum, & Newbould, 1980; Ruffo, 1968; Schalm, Carrol, & Jain, 1971).

Several methods are developed for neutrophil cell determination by differential counting of the milk SC. Cytological methods are mainly used for visual indentification of the main cell types, by optical microscopy (Baumert, Bruckmaier, & Wellnitz, 2009; Lindmark-Mansson, Bränning, Aldén, & Paulsson, 2006; Sarikaya, Prøgymet, Pfaffl, & Bruckmaier, 2004). Various flow cytometric methods for differentiating of the leukocytes are available for high SCC milk analysis as well as for low SCC milk, which is a technological breakthrough (Dosogne, Vangroenweghe, Mehrzad, Massart-Leên, & Burvenich, 2003; Koess & Hamann, 2008; Miller, Paape, Filep, & Link, 1993), but that methods require expensive equipment and qualified personnel. Enzyme-linked immunosorbent assay (ELISA) was also applied for detecting and quantifying of the SC, for example, by using specific antibody to neutrophils. O’Sullivan, Joyce, Sloan, and Shattock (1992) suggested to use a direct capture ELISA to diagnose bovine mastitis, but the method is time consuming. In order to separate each type of SC, immunomagnetic separation was used for labeling the cell subpopulations. The method was successfully applied to isolated epithelial cells from bovine milk (Boutinaud, Ben Chedly, Delamaire, & Guinard-Flament, 2008), macrophages and neutrophils from sheep milk (Albenzio et al., 2009). There is not enough information about determination of milk neutrophils by fluorescent labeled polyclonal antibody and application of a fluorescent microscopy.

In this study polyclonal antibody specific to bovine neutrophils was prepared. Antibovine neutrophil antibody was conjugated to fluorescein isothiocyanate (FITC) and neutrophil counting in bovine milk was developed.

**Materials and methods**

**Reagents**

Ficoll-Paque Premium (density 1.077 g/mL) and HiTrap Protein G HP column were submitted by GE Healthcare, Sweden. Tris(hydroxymethyl)-aminomethan (TRIS) was provided by Merck, Germany. Sepadex G25 Medium was purchased by Pharmacia Fine Chemicals, Sweden. Eosine yellowish (for microscopy); Giemsa Stain (Modified Solution); Fluorescein 5(6)-isothiocyanate (FITC); 3,3′,5,5′-Tetramethylbenzidine (TMB); Propidium iodide (PI); Bovine Serum Albumin; Anti-Sheep IgG whole molecule – Peroxydase antibody; Tween 20; Triton X-100; Freund’s Adjuvant; Methanol; Glycerin; Ethylenediaminetetraacetic acid disodium salt dehydrate (Na₂-EDTA); dimethylformamide (DMF); Hydrogen peroxide solution; Sulfuric acid and Glycine were delivered by Sigma-Aldrich, Germany.

**Separation of bovine blood and milk neutrophils**

Ficoll-separation of the neutrophil cells was performed by using a slightly modified version of the procedure of Maqbool, Vidyadaran, George, and Ramasamy (2011) and
following the manufactures instructions. Briefly, bovine blood (400 mL) was collected in a flask containing anticoagulant (blood: 3.8% sodium citrate – 9:1). Then blood was diluted with equal volume balanced salt solution. They were mixed by inverting the tubes three times. 15 mL Ficoll-Paque Premium was added to a centrifuge tube and carefully was laid the diluted blood sample (30 mL) on it. These tubes were centrifuged at 400×g for 40 min at 20°C. There were four layers obtained. Plasma and platelets were in the upper layer, second layer contained mononuclear cells (layer 2), third layer was Ficoll-Paque Premium and the bottom layer had granulocytes (neutrophils, eosinophils and basophils) and erythrocytes. They were collected in different flasks. Mononuclear layer was washed twice with balanced salt solution and suspended in 15 mL of the same buffer. The layer containing granulocytes and erythrocytes was collected. The erythrocytes were removed by the addition of 0.87% NH₄Cl. Then cells were washed in balanced salt solution and suspended in 15 mL of the same buffer.

The isolated cell suspensions were counterstained with Giemsa and Eosin-Methylene blue for microscopic examination followed by modified version of the procedure of Marshall, Bentley, and Lewis (1975). First working solutions of Eosin-Methylene blue and Giemsa were prepared. Eosin Y (13 mg) and Methylene blue (9 mg) were dissolved in equal volumes glycerin and methanol (total volume 20 mL). Then 1 mL of the solution was diluted in 4 mL Sörensen buffer. Giemsa working solution contains stock Giemsa and Wies buffer in 1:40 (v/v) ratio.

Ten μl of cell suspension was placed on a glass slide and was air dried, after that it was fixed with methanol (8 min). The fixed cell sample was colored with working solutions of Eosin-Methylene blue (10 min) and Giemsa (20 min). The smears was washed with tap water, air dried and finally counted using light microscopy with magnification 100.

Ficoll-separated bovine milk cells from healthy cow were prepared in the same conditions as described above.

Production of anti-bovine neutrophil polyclonal antibody

All animal experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Immunization was performed precluding unnecessary pain and distress and to minimize infection. Anti-bovine neutrophil antibody was prepared by immunization of a sheep with purified bovine blood neutrophils and neutrophil fragments in saline. Antigen-adjuvant emulsion was used. The first dose contained 16 × 10⁶ neutrophil cells. The other seven doses contained 0.17 mg/mL lyophilized neutrophil fragments. The fragments were prepared by usage of lyses buffer (155 mM NH₄Cl, 1 mM NaHCO₃, 0.13 mM Na₂-EDTA, pH 7.3) as described by Joyce, O’Sullivan, Shattock, and Sloan (1992). The titers of antisera were tested by indirect ELISA (described below). When the titer of antisera was stable, last injection was then given. An apparatus ÄKTAprime plus equipped with HiTrap Protein G HP column was used for purification of the Anti-bovine neutrophil antibody, immunoglobulin G (IgG) type, from the sheep blood serum (according to the manufacturer’s instructions). The column size was 7.5 cm length and 1 cm inner diameter. Binding buffer was 20 mM sodium phosphate (pH 7.0) and elution buffer was 0.1 M glycine-HCl (pH 2.7). The buffers were filtered by passing them through a 0.45 μm filter before use. The serum sample was diluted with binding buffer (1:1, v/v) before loading on the column. IgG-containing fractions were
collected and the pH value was correct with 1M Tris-HCl (pH 9.0) to approximately neutral. Buffer exchange was made to the IgG fractions. Finally, the antibody was in 20 mM sodium phosphate (pH 7.0) and they were lyophilized.

**Screening of antibody serum**

ELISA was made for determination of the serum antibody titer. An indirect method for analysis was made to verify antibody in the production steps. Neutrophil fragments (5 μg/mL) were diluted in 50 mM carbonate bicarbonate buffer (pH 9.6). This suspension was added to 12 microplate wells (100 μL in a well) in a shaker for 60 min at 37°C. The plates were washed four times with 200 μL washing buffer (50 mM Phosphate Buffered Saline (PBS), pH 7.4). They were blocked with 200 μL buffer (50 mM PBS, containing 1% BSA) in each well in the shaker for 60 min at 37°C. The plates were washed as aforementioned. Different concentrations bovine serum (1:1; 1:10; 1:50; 1:1,250; 1:6,250; 1:31,250; 1:156,250; 1:781,250 and 1:3,906,250) were prepared with 50 mM PBS, containing 1% BSA and 0.05% Tween 20 (Phosphate Buffered Saline Tween (PBST) 1% BSA) (100 μL in a well) and were set in the shaker for 60 min at 37°C. Then they were washed. Secondary antibody (Anti-Sheep IgG whole molecule – Peroxidase antibody) was added at dilution 1:10 000 in PBST 1% BSA (100 μL in a well) in the shaker (60 min, 37°C) and after that were washed. Then substrate solution was added (100 μL in a well). The solution contains 0.5 mL 3,3′,5,5′-TMB in dimethylformamide 1 mg/mL, 15 μL 3% H₂O₂, 4.5 mL 50 mM citrate buffer, pH 5.0. The plate was incubated in the shaker (30 min, 37°C) and 2 M H₂SO₄ (50 μL in a well) was added to stop the reaction. The samples were analyzed in a microplate reader Rayto RT-2100C (450 nm wavelength).

**Specificity of the anti-bovine neutrophil polyclonal antibody**

Cross-reactivity of the obtained polyclonal antibody was determined with Ficoll-separated bovine blood cells. Blood mononuclear cells, blood granulocyte cells were used as antigens in the first step of the indirect ELISA. Dilutions of 2 × 10³–2 × 10⁶ isolated blood granulocyte and mononuclear cells were prepared by resuspending the cells in 50 mM carbonate bicarbonate buffer (pH 9.6). The used anti-bovine neutrophil antibody concentration was optimal, preliminary determined, 500 μg/mL in PBST 1% BSA.

The same experiments were performed by Ficoll-separated bovine milk cells from healthy cow for analyzing cross-reactivity of the anti-bovine neutrophil antibody (500 μg/mL) with milk granulocyte and mononuclear cells.

The other steps of the indirect ELISA were as described above.

**Indirect ELISA of blood neutrophil fragments**

Indirect ELISA of different concentrations of the neutrophil fragments (0.5–5000 μg/mL in 50 mM carbonate bicarbonate buffer, pH 9.6) was performed with 500 μg/mL antibovine neutrophil antibody in PBST 1% BSA. The indirect ELISA was performed by method described in Screening of antibody serum.
**Indirect ELISA of bovine SC in milk**

Indirect ELISA was performed with different count of SC in milk and in 10 mM PBS buffer (pH 7.4). The initial SC count in mastitic cow milk was $600 \times 10^3$ cells/mL. Solutions containing variety of SCCs ($6.25 \times 10^3$–$800 \times 10^3$ cells/mL) were prepared by dilution with low SCC milk ($6 \times 10^3$ cells/mL). The cells were separated with centrifugation of 200 mL milk at $400 \times g$, 4°C for 20 min. Then obtained cells were suspended in 10 mM PBS pH 7.4 (10 mL). Samples with the same concentrations of SC ($6.25 \times 10^3$–$800 \times 10^3$ cells/mL) in 10 mM PBS buffer pH 7.4 were prepared. The other steps of the indirect ELISA were the same as in Screening of antibody serum. In the experiments, 500 µg/mL anti-bovine neutrophil antibody in PBST 1% BSA were used.

**FITC-conjugation of the anti-bovine neutrophil antibody**

Antibody-fluorescent dye conjugate was prepared by the method of The and Feltkamp (1970). Lyophilized anti-bovine neutrophil antibody 2 mg (calculated IgG weight) in 1.18 mL Reaction buffer (500 mM carbonate buffer, pH 9.5) was mixed with 320 µL FITC in DMF (1 mg/mL). The tube was wrapped in foil and was incubated in a shaker for 60 min at room temperature. Then it was left to react at 4°C, overnight. Finally, the conjugate was filtered from uncoupled reagents. Sephadex G25 Medium was used in a column 1 cm diameter, 37 cm length. Flow rate was 0.5 mL/min and fractions were 2 mL each. The applied sample was 1.5 mL. Elution was performed with Storage buffer (10 mM Tris, 150 mM NaCl, pH 8.2). Fractions were analyzed at 280 nm and 495 nm wavelength for protein and FITC, respectively.

Conjugate-containing fractions were analyzed with the following equations: IgG (mg/mL) = $(A_{280} - 0.31A_{495})/1.4$ and $F/P = 3.1A_{495}/(A_{280} - 0.31A_{495})$. The optimal $F/P$ values are 3–10 for any particular IgG.

UV-Vis and fluorescence spectrophotometric analyzes of the fractions containing the conjugate were made to demonstrate the antibody–dye binding.

**Immunofluorescence microscopic analyzes of blood cells with anti-bovine neutrophil antibody–FITC conjugate**

The anti-bovine neutrophil antibody–FITC conjugate specificity was analyzed with Ficoll-separated blood cells (granulocyte and mononuclear cells). Granulocyte cell suspension (100 µL, 500,000 cells/mL in balanced salt solution) was loaded in a vial. Then were added optimal concentrations of the following components: 2 µL Triton X-100, 2 µL propidium iodide (1 mg/mL in DMF), 20 µL anti-bovine neutrophil antibody–FITC conjugate (300 µg/mL in 10 mM PBS pH 7.4). The final solution was gently mixed after each addition. The cell suspension was incubated 60 min at 37°C in a shaker. After that 10 µL of the suspension were placed on a glass slide and were overlaid with a coverslip. The sample was analyzed with a fluorescence microscope Olympus BX51, magnification was ×100. The same procedure was performed with blood mononuclear cells.

PI binds to nucleic acids. The dye has excitation maximum at 535 nm and emission maximum at 617 nm. Therefore a 530 nm-laser was used and the resulting picture has visible red cell nuclei. FITC, in the Anti-bovine neutrophil antibody–FITC conjugate,
has excitation maximum at 495 nm and emission maximum at 519 nm. A 470 nm-laser was used to make visible the green colored neutrophil cell surface.

**Immunofluorescence microscopic analyzes of SCC in bovine milk with anti-bovine neutrophil antibody–FITC conjugate**

The reactivity of the anti-bovine neutrophil antibody–FITC conjugate was observed in real samples (bovine milk SC) with a fluorescence microscope. Cow mastitic milk (530 × 10^3 cells/mL) was used. There are more than 90% neutrophil cells in mastitic milk, established by Giemsa and Eosin-Methylene blue staining (the method was described above). The SC were separated with 400 × g centrifugation for 20 min. Cells were suspended in 10 mM PBS pH 7.4 to the same SCC. Then the sample was analyzed using the immunofluorescence microscopic analysis in the same conditions as Ficoll-separated blood cells.

The six milk samples (n = 6) with different count of SC (from 100 × 10^3 to 800 × 10^3) were analyzed by the developed method.

**Somatic cell counting**

Somatic cells were counted by Lactoscan SCC counter (Milkotronik Ltd). A level of 400×10^3 cells/mL was used as the level to indicate a “mastitic” milk sample, because this is the level currently used in EU directives to indicate milk quality.

**Milk samples collection and storage**

Milk samples were collected from the milking jars at the morning milking and were a mixture of all four quarters from each cow. Samples were transported at room temperature to the laboratory within 4 h of collection. Samples were divided for somatic cell counting (stored at 4°C overnight), and for indirect ELISA were stored at −20°C.

**Results and discussions**

**Separation of bovine blood neutrophils and bovine milk neutrophils**

Early diagnosis of subclinical mastitis with a reliable test is a very important task. While the total SCC increases in a mastitic milk sample, neutrophil cells has been shown to be predominantly. Measurement of these cells using a specific polyclonal antibody might provide a more specific indicator of inflammations, which is mastitis, than measuring total SCC in milk. A neutrophil cell isolate from bovine blood was made for anti-bovine neutrophil polyclonal antibody obtaining. For this purpose Ficoll-separated blood cells were sorted in different vessels. The blood was separated into a top layer of plasma, followed by a layer of mononuclear cells and a bottom fraction of granulocyte cells (such as neutrophils, eosinophils and basophils) and erythrocytes. The granulocyte cells can be further isolated by lysing the red blood cells. The isolated blood granulocytes and mononuclear cells were counterstained with Giemsa and Eosin-Methylene blue for microscopic examination (Figure 1). When granulocytes were examined by light microscopy, the cytoplasm was clear and contain numerous light pink cytoplasmic granules (Figure 1 – left). The group
of neutrophils was characterized as cells of 10–14 μm in size and segmented nuclei. They were intensely colored and contained granula in the cytoplasm. Mature neutrophils contained a polymorphic segmented nucleus. The nuclear lobes are connected by short filaments. The isolated blood mononuclear cells had a smaller size than neutrophil size (Figure 1 – right). Mononuclear cells were identified based on their circular form (5–10 μm) and the typical shape of the nucleus that almost filled the cell leaving a very thin rim of cytoplasm. The obtained microscopic images prove that the separation of cells is successfully.

Production of anti-bovine neutrophil polyclonal antibody

The obtained neutrophils and neutrophil fragments were used for immunization of a sheep. Serum titer was observed at 75th and 105th day of injection period (Figure 2). The first titer was 1250 fold serum dilution and the titer at the end of the injection period was 31,250 fold serum dilution. The polyclonal antibody was purified with affinity chromatography.

Indirect ELISA of neutrophil fragments

Indirect ELISA of different concentrations of the neutrophil fragments (0.5–50,000 μg/mL) was performed and the results are shown on Figure 3. The working concentration of antibody (500 μg/mL) for indirect ELISA was previously determined by checkerboard titration. The number of samples that are present in error bars is six. The experimental error in all experiments was from 4.0 to 7.0%. There are two linear ranges under these conditions. The first is from 10 to 1000 μg/mL and the second from 5000 to 15,000 μg/mL neutrophil fragments. The linear equations and correlation coefficients were: \( y = 0.0006x + 0.4080, R^2 = 0.9356 \) and \( y = 5E-05x + 1.4821, R^2 = 0.9389 \), respectively.

The detection limit for determination of neutrophil fragments are 5 μg/mL. In compliance with the International Union of Pure and Applied Chemistry (IUPAC) rules (Inczedy, Lengyel, & Ure, 1998), the limit of detection is calculated as three times the standard deviation of the blank measurements.

Figure 1. Eosin-Methylene blue-Giemsa-stained blood granulocytes (left) and mononuclear cells (right).
Specificity of the anti-bovine neutrophil antibody

The polyclonal antibody produced in this study reacted with bovine neutrophil cells. The reactivity of polyclonal antibody to Ficoll-separated bovine blood cells (neutrophils and mononuclear cells) was studied (Table 1). Since the antibody was intended for mastitis detection by showing elevated neutrophil cell count in bovine milk, it was essential to be tested for reactivity with other cells in normal milk. Ficoll-separated bovine milk cells were used to prove the reactivity of the obtained anti-bovine neutrophil antibody in bovine milk. The polyclonal antibody was tested with whole milk samples, containing added neutrophils and mononuclear cells (Table 1). It is found that polyclonal antibody has no reaction with mononuclear cells and reacts with neutrophil cells.

Figure 2. Serum anti-bovine neutrophil antibody titer before (♦) and after (■) the end of the injection period.

**Figure 3.** Indirect ELISA test of different concentrations of the neutrophil fragments (0.5–50,000 μg/mL) and constant concentration of the anti-bovine neutrophil antibody (500 μg/mL).
Indirect ELISA of bovine SC in milk

Indirect ELISA with anti-bovine neutrophil antibody was performed with SC in bovine milk and in buffer to observe cream and milk protein influences. Somatic cell count was varied in the same amount in both cases. The working concentration of antibody (500 µg/mL) for indirect ELISA was previously determined by checkerboard titration. The number of samples that are present in error bars is six. The experimental error in all experiments was from 5.0 to 8.0%. The linear ranges under these conditions are $25 \times 10^3 - 400 \times 10^3$ cells/mL with SC in milk and $12.5 \times 10^3 - 400 \times 10^3$ cells/mL with SC in buffer. Figure 4 shows that the absorption values in milk are lower than absorption values in buffer and this fact proved that milk matrix has influence. The linear equations and correlation coefficients were determined: SC in milk $y = 0.0013x + 0.1940$ and $R^2 = 0.9638$; SC in buffer $y = 0.0015x + 0.2310$ and $R^2 = 0.9707$.

FITC-conjugation of anti-bovine neutrophil antibody

Anti-bovine neutrophil antibody–FITC conjugate was prepared to visualize the antibody-neutrophil capturing. The conjugating mixture has antibody-fluorescent dye conjugate, free antibody and unconjugated dye. The mixture was filtrated with Sephadex G25 Medium for size-exclusion chromatography. The conjugate has the highest molecular weight in the mixture and exits the column first (fraction 5 and fraction 6).

Table 1. Cross-reactivity of the obtained anti-bovine neutrophil antibody to Ficoll-separated bovine blood and milk cells.

| Cell count, cells/mL | Blood neutrophil cells, absorbance intensity | Blood mononuclear cells, absorbance intensity | Milk neutrophil cells, absorbance intensity | Milk mononuclear cells, absorbance intensity |
|---------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|---------------------------------------------|
| 2000                | 0.40                                        | 0                                           | 0                                           | 0                                           |
| 20,000              | 0.70                                        | 0.05                                        | 0.30                                        | 0                                           |
| 200,000             | 0.90                                        | 0.10                                        | 0.45                                        | 0.13                                        |
| 2,000,000           | 1.00                                        | 0.15                                        | 0.55                                        | 0.20                                        |

Figure 4. Indirect ELISA test of SC in bovine milk (♦) and SC in buffer (▪), at constant concentration of the anti-bovine neutrophil antibody (500 µg/mL).
absorptions of the fraction 5 and 6 were calculated with equations to prove the conjugation. Fluorescent dye: protein ratio (F/P) is in optimal values (3–10). Fraction 5 has IgG 1.03 mg/mL and F/P 4.24. Fraction 6 has IgG 0.77 mg/mL and F/P 4.65. Scan of the fraction 5 with 6900 UV/Vis Spectrophotometer JENWAY was made to confirm the anti-bovine neutrophil antibody–FITC coupling (Figure 5). The antibody–FITC conjugate has two absorption maximum: first – at 265 nm and second – at 492 nm. The maximum absorbing wavelength in conjugate spectrum, which are characteristic of protein and free FITC, was slightly shifted from 270 to 265 nm compared with free antibody and from 484 to 492 nm compare with free FITC.

Figure 6 shows fluorescence spectrophotometric analysis of the fraction 5 and free fluorescent dye (FITC) was performed with Fluorescent Spectrophotometer F96Pro. The free FITC has peak at 518 nm and anti-bovine neutrophil antibody–FITC conjugate has peak at 528 nm. This shift proves the antibody-dye coupling.

**Specificity of the obtained anti-bovine neutrophil antibody–FITC conjugate**

The specificity of anti-bovine neutrophil antibody–FITC conjugate was analyzed with Ficoll-separated blood cells (granulocytes and mononuclear cells) by using of fluorescence microscopic analysis. For this purpose anti-bovine neutrophil antibody–FITC conjugate and PI were added to the separated cells. Triton X-100 was used to ensure cell permeability. Fluorescent dye PI binds selectively to the DNA of the nucleus of the neutrophils and stains them in red color (Figure 7). The core of neutrophils turns red. The antibody (anti-neutrophil) labeled with FITC binds to epitopes lying on the surface of the neutrophil cell surface, and the entire cell is colored in green (Figure 7). This staining is successfully only with neutrophil cells.

Consecutive excitation with 470 nm and 530 nm was performed for visualization of neutrophil green staining and red colored cell nuclei, respectively. The microscope software combines both images and formed green colored ring around the red neutrophil nucleus. This double staining was observed only in neutrophils. Mononuclear cells had
only red stained cell nuclei (Figure 7). Obviously, the size of colored in red mononuclear cells was smaller than that of the stained green neutrophil due to single cell staining.

Figure 6. Fluorescence spectrophotometric analysis of anti-bovine neutrophil antibody–FITC conjugate (A) and free FITC (B).

Figure 7. Differential staining of bovine blood cells – granulocytes and mononuclear cells, with PI ("Red emission" column) and Anti-bovine neutrophil antibody–FITC conjugate ("Green emission" column).
The neutrophil concentrations (7–80%) in milk were determined by the proposed immunofluorescence microscopic method and a commercial Giemsa method. Figure 8 shows the values obtained by both methods and they are in excellent agreement. The correlation coefficient for neutrophil per cent from 7 to 80 in milk was 0.974. The advantages of the developed immunofluorescence microscopic method is clear cell staining and short time (70 min) of analysis compared to Giemsa method (2 h).

**Immunofluorescence microscopic analysis of milk SC and milk neutrophils**

Figure 9 shows milk neutrophils and milk SC stained with anti-bovine neutrophil antibody–FITC conjugate and PI. Excitation with 470 nm and 530 nm was performed for green stained neutrophils and red stained nuclei of all milk SC. It was noticed that the number of stained milk SC is much larger than the number of milk neutrophils. It was noticed that leukocytes (neutrophils, eosinophils, basophils, macrophages and lymphocytes) had red emission (570 nm), and neutrophils had both red (570 nm) and green (470 nm). Combination of the red and green emission images is shown on Figure 9. The microscope images proved anti-bovine neutrophil antibody–FITC conjugate reactivity with milk neutrophil cells.

Six milk samples ($n = 6$) with different count of SC (from $100 \times 10^3$ to $800 \times 10^3$) were analyzed by the developed method (Table 2). Analytical recovery was determined. The actual SC count was measured by commercial Milkotronik counter. The count of neutrophils was measured by the proposed method. It was found that when SCC was $100 \times 10^3$ cells/mL the neutrophil cells were 30%, but increased to 70% when SCC exceeded $400 \times 10^3$ cells/mL and 88% when SCC exceeded $800 \times 10^3$ cells/mL. The coefficient of variation (%) was calculated. While the neutrophil count was higher, the coefficient of variation was smaller. Cuccuru et al. (1997) found in individual milk that neutrophils...
ranged between 30 and 40% when SCC was <100 × 10³ cells/mL but increased to 70% when SCC exceeded 400 × 10³ cells/mL; macrophage concentration had the opposite trend, displaying an increase when neutrophil cells decreased. Other authors described that neutrophils increased constantly from 0% at SCC 1 × 10³ cells/mL to a maximum of 88% at 139 × 10³ cells/mL. At SCC 1824 × 10³ cells/mL the proportion of neutrophil cells was 86%.

The results presented in this study indicate that the specific detection of neutrophil cells using the proposed polyclonal antibody capture assay has significant potential as a new diagnostic test for mastitis on the farm, in veterinary practice and in cooperatives and laboratories.

**Conclusions**

Differential somatic cell counting is helpful for determining the cattle health status. The large number of polymorphonuclear neutrophil cells in milk is reliable evidence for inflammation in the udder. Polyclonal antibody specific for bovine neutrophils was prepared. Anti-bovine neutrophil antibody–FITC conjugate was obtained to visualize the antibody-neutrophil capturing. The specificity of anti-bovine neutrophil antibody–FITC conjugate was proved by using of fluorescence microscopic analysis. The results indicate that the polyclonal antibody marked with FITC has excellent potential in the immunofluorescence microscope determination of bovine mastitis.

**Disclosure statement**

No potential conflict of interest was reported by the authors.
Notes on contributors

Zlatina Becheva is PhD Student in department of Biotechnology at “Prof. Dr Asen Zlatarov” University, Bourgas, Bulgaria. The dissertation title is “Immunofluorescence microscopic analysis of somatic cells and neutrophils in milk”. The immunoanalysis are based on differential determination of cells in milk and blood by antigen-antibody binding.

Katya Gabrovska is associate professor in department of Biotechnology at “Prof. Dr Asen Zlatarov” University, Bourgas, Bulgaria. She works in the field of immunoassays, genetically modified food and molecular biology.

Tzonka Godjevargova is Professor in department of Biotechnology at “Prof. Dr Asen Zlatarov” University, Bourgas, Bulgaria. She is Head of the Biotechnology Department. She works in the field of the immunoassays, enzyme biosensors, nanoparticle producing and food safety.

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