Becheva and Godjevargova, J Nanomater Mol Nanotechnol 2017, 6:3
DOI: 10.4172/2324-8777.1000217

Journal of Nanomaterials & Molecular Nanotechnology

Research Article

A SCITECHNOL JOURNAL

Preparation of Anti-Elastase Antibody Conjugated With Quantum Dots 710 Nm and Fluorescein Isothiocyanate for Immunoassay of Milk Somatic Cells

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Abstract

A novel immunofluorescence microscopic method for a neutrophil and somatic cell count was developed by using anti-elastase antibody. Two different conjugates - anti-bovine neutrophil elastase antibody-fluorescein isothiocyanate conjugate and anti-bovine neutrophil elastase antibody-Quantum Dots 710nm conjugate were prepared. The coupling of Quantum Dots (QDs) and fluorescein isothiocyanate (FITC) to antibody has been demonstrated by comparison of ultraviolet absorption spectra and fluorescence spectra of conjugate and initial components. The activities of conjugates were measured by indirect ELISA at the same conditions. It was found that the sensitivity of the immunoassay with QDs-conjugate was higher than the sensitivity of the immunoassay with FITC-conjugate. The storage stability of anti-bovine neutrophil elastase antibody-QDs710nm conjugate and anti-bovine neutrophil elastase antibody-FITC conjugate was studied. The residual fluorescence intensity of anti-elastase antibody-QDs was 95% at 30th day, while the intensity of anti-elastase antibody-FITC conjugate was reduced to 60% at same day. The obtained conjugates were applied in real test with bovine milk somatic cells. The fluorescence imaging proved that QDs-conjugate has better fluorescence intensity than the FITC-conjugate, and is more appropriate for determination of somatic cells in milk.

Keywords

Quantum Dots; FITC; Antibody; Conjugate; Somatic cells

Introduction

Quantum Dots (QDs) have already been applied to the analysis of membrane proteins and as a research tool for the molecular imaging of blood cells [1,2]. However, there are no reports on the application of QDs in the study of proteins and somatic cells (SCC) in milk. The SCC of milk is generally considered to be an important parameter for mastitis detection [3]. Milk contains three main somatic cell types: lymphocytes, neutrophils and macrophages. In recent years, somatic cells can be measured by a variety of direct or indirect procedures including direct microscope counting, automated particle size analysis, and automated fluorescent staining of nuclei [4]. Many of these tests are not specific enough. The increased number of somatic cells in mastitic milk samples has been shown to contain predominantly neutrophil cells and some authors have recognized that a measurement of the number of neutrophil cells in milk might provide a more specific inflammation indicator than measuring total somatic cells in milk [5-9]. Neutrophil massive recruitment explains the increase of milk SCC and therefore a part of the increase in proteolytic activity, especially elastase activity. The elastase is a neutral serine-type proteinase mainly associated with neutrophils [10] and it can be used as an indicator for determination of neutrophils and somatic cells in milk. The development of immunofluorescence analysis for determination of elastase concentration is a new specific way for analyzing and control of neutrophils and somatic cells in milk. The sensitivity of immunoanalysis depends on quality of conjugate antibody-fluorescent marker and on intensity and stability of the marker. Recently, QDs get attention due to their good fluorescent properties and they were used for developing of immunofluorescence methods [11]. The role of organic dyes as a marker in the study of biological molecules and cell microscopic analysis is limited due to certain shortcomings, such as narrow excitation spectra, poor photostability and a short fluorescence lifespan [12]. In this paper, we use same antigen–antibody system but with different markers to the antibody (QDs and organic dye) and the obtained results are compared with respect to sensitivity of analysis and to stability of fluorescent conjugates. For that purpose, anti-elastase antibody conjugated with QDs710nm and fluorescein isothiocyanate (FITC) were prepared. The immunofluorescent microscopic assay for determination of elastase and respectively neutrophils and somatic cells in cow milk was developed. The sensitivity and stability of the obtained immunoassay with both conjugates was compared.

Materials and Methods

Reagents and chemicals

SP Sepharose, Sephadex G75, Sephadex G100, N-succinyl-Ala-Ala-Ala-p-nitroanilide, Complete Freund's Adjuvant, Bovine Serum Albumin, Fluorescein-5-Isothiocyanate, Tween 20, Triton X100, Anti-sheep IgG whole molecule - Peroxydase antibody, 3,3',5,5'-tetramethylbenzidine, Protein G Sepharose, HiPrep™ 26/10 Desalting, N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride, hydroxysuccinimide, Sephadex G25 Medium were delivered from Sigma-Aldrich (Germany). Quantum Dots 710nm were delivered from PlasmaChem GmbH (Berlin).

Neutrophil isolation from bovine blood

Isolation of bovine blood neutrophil cells was performed as per method described by Carlson and Kaneko [13]. Isolated bovine blood neutrophils were observed with Olympus BX51 microscope for cell count estimation.

Neutrophil elastase extraction

Neutrophil elastase was isolated from the cells by using a slightly modified version of the procedure of Stoll [14]. The whole process is

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Received: February 23, 2017 **Accepted:** April 12, 2017 **Published:** April 17, 2017

described in detail in our previous paper [15].

Purification of neutrophil elastase

The neutrophil elastase extract was loaded on an ion exchange SP Sepharose column (1 × 8 cm) balanced with 25 mM Na-acetate, 0.2 M NaCl, pH 7.0 on ÄKTAprime plus instrument. A NaCl gradient was applied 0.2-1.0 M in 143 mL [14].The flow rate was 1 mL/min, fractions of 3 mL were collected. The proteins in fractions were monitored by UV absorbance at 280 nm.

Neutrophil elastase selective fractions were concentrated with Vivaspin 20 (10,000 MWCO) and loaded on a Sephadex G75 gel filtration column (1.1 × 55 cm) balanced with 25 mM Na-acetate, 0.2 M NaCl, pH 7.0. The flow rate was 0.5 mL/min and fractions of 2.5 ml were collected [16]. Each fraction was tested for elastase activity by color reaction with specific elastase substrate N-succinyl-Ala-Ala-Ala-p-nitroanilide according to the manufacturer's instructions.

Production and screening of anti-bovine neutrophil elastase polyclonal antibody

A single sheep was vaccinated with 8 doses bovine neutrophil elastase (1 mg/mL) with sterile saline (1 mL each). Doses were mixed with Complete Freund's adjuvant (1:1) for the first injection and with Incomplete Freund's Adjuvant (1:1) for the others. The animal experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments. To estimate the antibody concentration in the sheep blood serum, an indirect enzyme-linked immunosorbent assay (ELISA) was performed and absorption was read with microplate reader Rayto RT-2100C at 450 nm [17].

Purification of anti-bovine neutrophil elastase polyclonal antibody

Anti-bovine neutrophil elastase antibody is IgG type. Affinity chromatography was used for their purification. A column (7 × 1 cm) with Protein G Sepharose was used. Fractions were 2.5 mL, flow rate 5 was mL/min. Binding buffer contained 1.732 g Na_2HPO_4 and 1.217 g NaH_2PO_4 in 1L deionizer water. Elution buffer contained 3.75 g glycine and 1.744 mL hydrogen chloride in 1L deionizer water. Then buffer exchange was made for the obtained IgG-fractions with HiPrep™ 26/10 Desalting on ÄKTAprime pluse. Finally, the anti-bovine neutrophil elastase (Anti-BNE) antibody was lyophilized.

Conjugation of anti-bovine neutrophil elastase antibody with QDs710nm

Sheep anti-elastase polyclonal antibody was conjugated with Quantum Dots 710 nm (QDs710 nm), by using a slightly modified version of the procedure of Yun Xing and collective [18]. First, QDs710 nm were diluted with 10 mM PBS pH 7.4 to concentration 8 μM in a total volume 62.5 μL. Then they were activated with 12.5 μL N-(3-Dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC) (2.2 mM in the same buffer) and 12.5 μL N-Hydroxysuccinimide (NHS) (4 mM in the same buffer). The mixture was leaved for 30 min in a shaker at room temperature. After that, lyophilized Anti-BNE antibody was diluted with 10 mM PBS pH 7.4 to concentration 240 μM in total volume 912.5 μL, and antibody and activated QDs solutions were mixed together. The mixture was leaved in a shaker for 4 hours, then at 4°C overnight. Gel filtration was used for conjugate purification. A column with Sephadex G100 (1 × 35.5 cm) was equilibrated with 10 mM PBS pH 7.4. Flow rate was 0.5 mL/min and fractions were at 2 mL. Fluorescence intensity and optical density measurements were made of each fraction with fluorescence spectrophotometer F96Pro and UV-Vis spectrophotometer JENWAY 6900, respectively. The obtained conjugate peaks were compared with those with pure Anti-BNE antibodies and QDs710 nm and both diluted in the same buffer. Finally, the anti-bovine neutrophil elastase - QDs620 nm conjugate was lyophilized.

Conjugation of anti-bovine neutrophil elastase antibody with FITC

Sheep anti-elastase polyclonal antibody was labeled with fluorescent dye - fluorescein isotiocyanate (FITC) according to the method of The and Feltkamp [19] with some modifications. In brief, 1 mL of antibody at 3 mg/mL in reaction buffer (500 mM carbonate, pH 9.5) was mixed with 480 μL FITC at 1 mg/mL in dimethylformamide (DMFA). The tube was wrapped in foil and was incubated 3.5 hours in a shaker at room temperature. After that, the mixture was leaved overnight at 4°C. The solution was purified by passing over a Sephadex G25 Medium column (1 × 31.5 cm) equilibrated with storage buffer (10 mM Tris, 150 mM NaCl, pH 8.2), flow rate 0.5 mL/min. Fractions of 2 mL were collected. FITC and protein concentrations of each fraction were estimated spectrophotometrically at 495 and 280 nm, respectively.

IgG concentration and fluorescent dye: protein (F/P) ratio was calculated using the equations offered by Aaron:

$$IgG\ (mg/ml) = (A_{280} - 0.31\ A_{495}) / 1.4$$

$$F/P = (3.1\ A_{495}) / (A_{280} - 0.31\ A_{495})$$

The optimal F/P values are 3 - 10 for any particular IgG.

Spectrum analyses of the labeled antibodies were made with UV-Vis spectrophotometer JENWAY 6900 and fluorescence spectrophotometer F96Pro. Absorption and emission peaks of pure anti-elastase polyclonal antibody and FITC (both diluted in storage buffer) were used as references. Finally, the proven conjugate was lyophilized.

ELISA of somatic cells in milk with the Anti-elastase antibody-QDs and Anti-elastase antibody-FITC conjugates

Bovine milk was delivered from local cowherd. Somatic cells were counted with Methylene blue according to ISO 13366-1/2008 [20]. Milk had 100,000 cells/mL. Milk with higher somatic cell count was prepared with addition of separated somatic cells after centrifugation, diluted with cell-free supernatant. Somatic cells were separated after centrifugation (400 × g, 20 min, 4°C) of 100 mL milk. The supernatant was centrifuged again to obtain better yield. Cell-free supernatant was obtained after centrifugation of the second milk supernatant at 800 × g, 4°C, 20 min. The variety of sample concentrations of somatic cells in milk (25,000-1,000,000 cells/mL were prepared by the cell pellet and cell-free supernatant. The samples were loaded in wells (100 μL) and were leaved to adhere at 4°C overnight. After incubation the plate was washed four times with PBS (50 mM, pH 7.4), 200 μL in a well. After that a blocking procedure was performed with 200 μL in each well of 1% bovine serum albumin (BSA) in PBS. The plate was incubated in a shaker at 37°C for 60 min and washed as described above. The conjugates were diluted in PBS, containing 1% BSA and 0.05% Tween 20. The concentration of the Anti-BNE-QDs710 nm and Anti-BNE-FITC was 300 μg/mL. In each well were added 100 μL from the certain conjugate, followed by incubation in shaker at 37°C for 60 min. The plate was washed as described above. A solution of secondary antibody (Anti-sheep IgG whole molecule - Peroxydase antibody) in PBS, con-

taining 1% BSA and 0.05% Tween 20 (1: 10,000 dilution) was added to each well (100 µL). After incubation for 60 min in a shaker at 37°C and washing procedure, substrate solution was added (100 µL). Five mL of the substrate solution contain 0.5 mL 3,3',5,5'-tetramethylbenzidine in dimethylformamide (1 mg/mL), 15 µL 3% hydrogen peroxide and 4.5 mL 50 mM citrate buffer pH 5.0. The reaction was stopped in 40 min by adding 2 M sulfuric acid, 50 µL in each well. The absorption was read with microplate reader Rayto RT-2100C at 450 nm.

Fluorescence imaging of milk neutrophil cells with anti-bovine elastase antibody–QDs and anti-bovine elastase antibody-FITC conjugates

Bovine milk neutrophil cells were isolated by using of the procedure of Hoeben and collective [21]. The neutrophil cells isolated from milk - 100 µL (400,000 cells/mL) were mixed with 0.1% Triton X100 (for cell permeabilization) and then 50 µL anti-bovine neutrophil elastase antibody - QDs710 nm conjugate (1 mg/mL in 10 mM PBS pH 7.4) or 50 µL anti-bovine neutrophil elastase antibody – FITC conjugate in the same concentration. The mixture was stirred at room temperature for one hour. Then 10 µL of the mixture was put on a microscope glass and the sample was observed with Olympus BX51 fluorescent microscope, 365 nm laser for QDs-conjugate and 470 nm for FITC-conjugate, magnification x100 was used and QImaging Retiga 2000R camera.

Microscopic assay of bovine milk somatic cells with anti-bovine elastase antibody–QDs710nm and anti-bovine elastase antibody–FITC conjugates

Somatic cells - 100 µL (with different somatic cell count in 10 mM PBS pH 7.4) were mixed with 0.1% Triton X100 (for cell permeabilization) and then 100 µL anti-bovine neutrophil elastase - QDs710nm conjugate (1 mg/mL in the same buffer) or 100 µL Anti-bovine neutrophil elastase - FITC conjugate in the same concentration and buffer. The mixture was stirred in shaker at 37°C for 60 min. Then 10 µL of the mixture was put on a microscope glass and coverslip was placed over it. The samples were observed with Olympus BX51 fluorescent microscope, 365 nm laser (for the QDs710 nm-conjugate), 470 nm laser (for the FITC-conjugate), magnification x40 and QImaging Retiga 2000R camera.

Result and Discussion

QDs conjugation of anti-bovine neutrophil elastase antibody

QDs conjugation of anti-bovine neutrophil elastase antibody was performed by conjugation procedure described from Xing and collective [18], with some modifications. Hydrophilic Cadmium-Tellurium (CdTe) Quantum Dots 710nm are coated with -COOH group. These QDs are biocompatible, non-toxic and can be used as suitable label of antibody for the identification of antigen. Coupling of Quantum Dots to anti-bovine neutrophil elastase antibody was achieved between -COOH groups of nanoparticles and -NH_2 groups of antibody through EDC-mediated esterefication (Figure 1). The influence of the type of activation reagent of QDs 710 nm antibody on the assay efficiency was investigated. To study this effect, the carboxyl QDs surface was activated by EDC or EDC/NHS reagent mixture (Figure 1). The advantage of using EDC to other carboxyl-activating reagents is that it is water soluble, easily removed by washing with water and the reaction can be performed in physiological solutions without adding organic solvent. Although carboxyl-containing molecule can react directly with amines using EDC, the reaction is much more effective with the addition of NHS, as it forms a stable intermediate.

This is probably due to different modification of the surface charge of QDs, resulting from activation with various reagents. Activation with EDC replaced negative charges of carboxyl groups with a positive, which, to a less extent, leads to repulsion of the protein molecule. It was also considered that EDC-esters are unstable, and unable to react quickly with amines and cause hydrolysis and regeneration of the carboxyl group. Activation with a mixture of EDC and NHS leads to neutralization of negative charge, which increases efficiency and binding of the protein molecule. It was found that the activity of conjugate obtained only by EDC (0.6 absorption unit) was lower than activity of conjugate obtained by EDC and NHS (0.95 absorption unit). The activity of conjugates was measured by indirect ELISA at the same conditions. That why all other experiments were performed by activated QDs with EDC and NHS.

The coupling of Quantum Dots to antibody has been demonstrated by ultraviolet-spectrophotometric method. The ultraviolet absorption spectra of conjugate and spectra of free antibody and free Quantum Dots are shown in Figure 2. The maximum absorbing wavelength in conjugate spectrum, which are characteristic of protein, was slightly shifted from 268 to 272 nm compared with free anti-bovine neutrophil elastase antibody (Figure 2). The slight shifted spectrum is evidence for linking the components. The UV-Vis spectra of the unbounded anti-bovine neutrophil antibody showed peak at 220 nm, and the conjugate - at 225 nm. The letter peak is larger from the presence of QDs in conjugate (Figure 1). The coupling of Quantum Dots to antibody has been proved also by fluorescence scan of conjugate and free Quantum Dots. The fluorescence measurement showed maintaining the overall shape and ~ 40 nm shift of the anti-bovine neutrophil antibody - QDs710 nm conjugate compared with free Quantum Dots 710 nm (Figure 3). These were sufficient evidence of the successful connection of the reaction components. The obtained conjugate has an excitation/emission peak at 365/710 nm.

FITC conjugation of anti-bovine neutrophil elastase antibody

Conjugation of anti-bovine neutrophil elastase antibody with Fluorescein-5-Isothiocyanate (FITC) was carried directly by reaction presented on Figure 4.

FITC can be coupled to distinct antibodies with the help of its reactive isothiocyanate group, which is binding to amino groups on proteins, forming a stable thiourea bond.

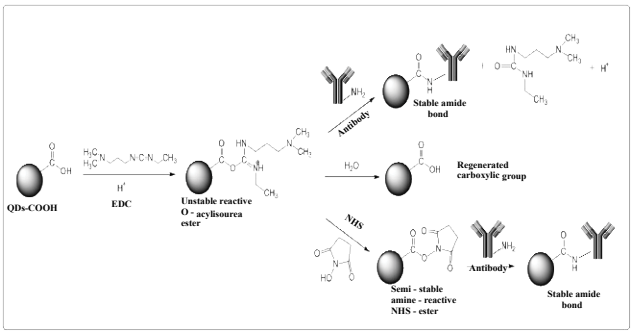

Figure 1: The coupling of Quantum Dots 710 nm to anti-bovine neutrophil elastase antibody by EDC and EDC/NHS method.

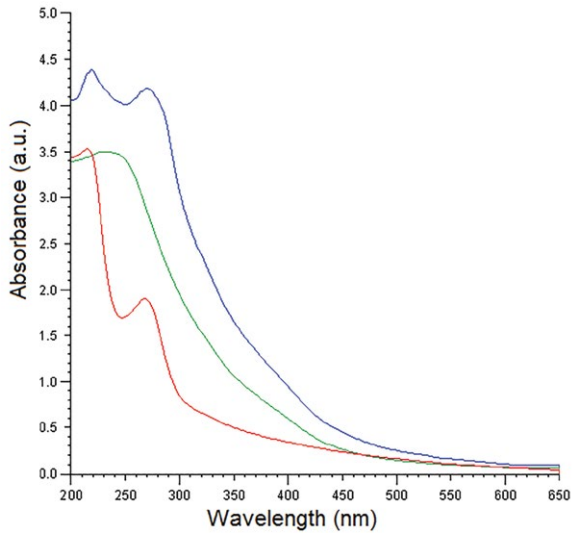

Figure 2: UV-Vis spectrum analysis of free anti-bovine neutrophil elastase antibody (red), free quantum dots 710nm (green) and anti-bovine neutrophil elastase antibody – QDs710nm conjugate (blue).

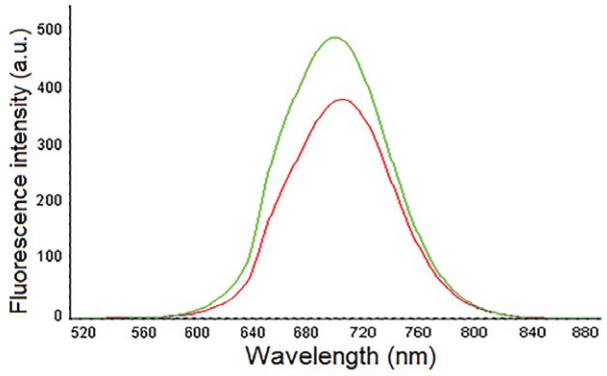

Figure 3: Fluorescence spectra analysis of free quantum dots 710nm (green) and the anti-bovine neutrophil elastase antibody – QDs710nm conjugate (red).

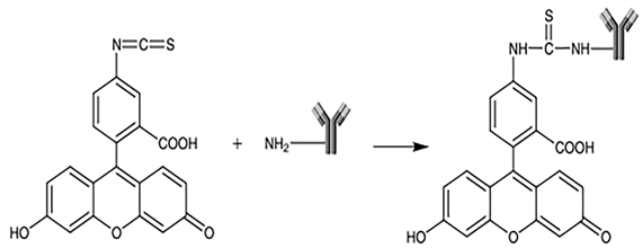

Figure 4: The coupling of FITC to anti-bovine neutrophil elastase antibody by direct method.

Obtained conjugate was proved by ultraviolet absorption spectra of the conjugate, free antibodies and free FITC (Figure 5). It was found that the absorption maximum of the conjugate spectrum are at 226, 266 and 492 nm, which are corresponding to characteristic of FITC (230 nm), antibody (270 nm), FITC (493 nm). The coupling of FITC to antibody has been proved also by fluorescence scan of the conjugate and free FITC (Figure 6). The fluorescence measurement showed maintaining the overall shape and ~ 12 nm shift of the anti-bovine

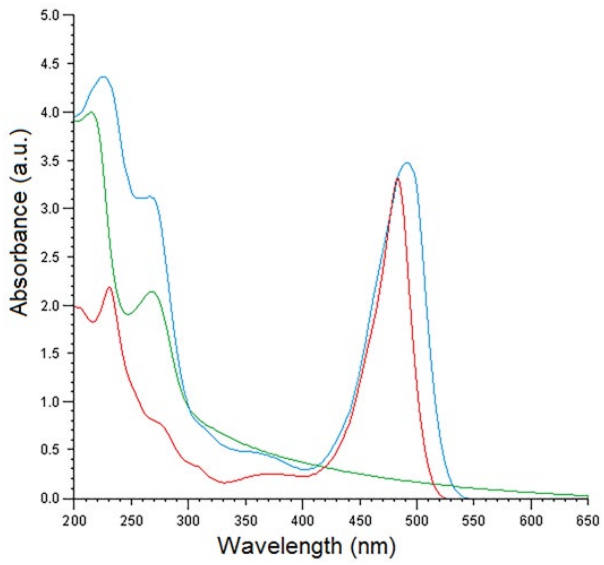

Figure 5: UV-Vis spectrum analysis of free FITC (red), free anti-bovine neutrophil elastase antibody (green) and anti-bovine neutrophil elastase antibody – FITC conjugate (blue).

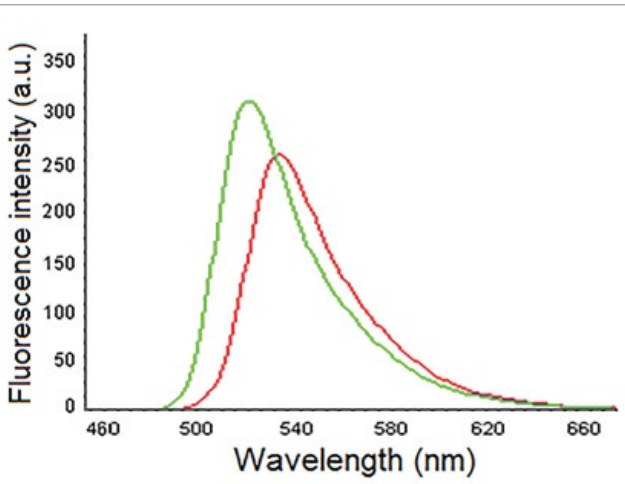

Figure 6: Fluorescence spectra analysis of free FITC (green) and the anti-bovine neutrophil elastase antibody – FITC conjugate (red).

neutrophil antibody - FITC conjugate compared with free FITC (from 518 to 530 nm). These were sufficient evidence of the successful connection of the reaction components. The obtained conjugate has an excitation/emission peak at 493/530 nm.

ELISA of Anti-elastase antibody-QDs and Anti-elastase antibody-FITC with milk somatic cells

The activity of Anti-elastase antibody-QDs and Anti-elastase antibody-FITC to the somatic cells was performed by indirect ELISA. The immunoassay was carried out in milk, pH 6.6 with both obtained conjugates. The results were presented in Figure 7. The assay sensitivity is usually expressed in terms of its detection limit and the slope of the curve. It was determined the linear equations and correlation coefficients: for assay with anti-elastase antibody-QDs y=8E-07x + 0.4927 and R^2=0.9368; for assay with anti-elastase antibody-FITC y=4E-07x + 0.4029 and R^2=0.9080. The slope of somatic cells standard curve ob-

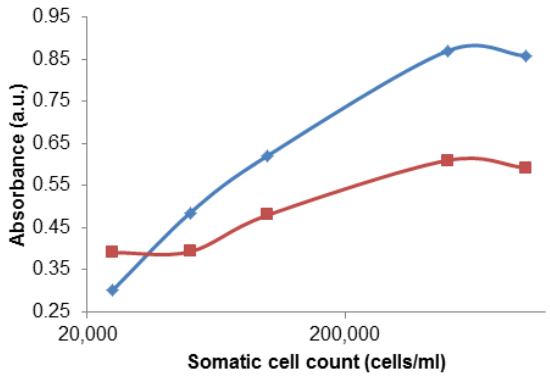

Figure 7: ELISA of milk somatic cells with Anti-elastase antibody-QDs conjugate (blue) and with Anti-elastase antibody-FITC (red).

tained with Anti-elastase antibody-QDs conjugate (8E-07) was higher than the slope with anti-elastase antibody-FITC (4E-07). This means that the sensitivity of immunoassay with the QDs-conjugate was higher than the sensitivity of immunoassay with the FITC-conjugate. The linear interval for determination of somatic cells with antibody-FITC conjugate was shorter than the linear range with antibody-QDs conjugate. The obtained results showed that the Anti-elastase antibody-QDs conjugate activity was higher than that of Anti-elastase antibody-FITC. Probably, the longer spacer between antibody and QDs was ensured the higher activity of the conjugate than activity of Anti-elastase antibody-FITC conjugate.

Milk neutrophil and somatic cells counting with anti-bovine elastase antibody-QDs and anti-bovine elastase antibody-FITC conjugate

The obtained conjugates were applied with isolated bovine milk neutrophil cells. Figure 8 (left) shows fluorescence image of red colored neutrophil cell with the antibody-QDs conjugate (magnification x100). Figure 8 (right) shows green colored neutrophil cell with the antibody-FITC conjugate (the same magnification). Obviously, the fluorescence intensity of neutrophil tagged by antibody-QDs conjugate (red color) is stronger than fluorescence intensity of neutrophil cell tagged by antibody-FITC conjugate (green color). Figure 9 shows that the obtained anti-elastase antibody-QDs is effective reagent for neutrophil cells and respectively for somatic cells, because of the correlation between them. Cuccuru et al. [22] found in individual milk that neutrophils ranged between 30 and 40% when SCC was <100,000 cells/mL but increased to 70% when SCC exceeded 400,000 cells/mL.

The storage stability of anti-bovine neutrophil elastase antibody-Quantum Dots 710nm conjugate and anti-bovine neutrophil elastase antibody-FITC conjugate was studied. The conjugates were remained at 4°C for 30 days. The fluorescence intensity of conjugates was measured at every 2 days. It was found that the fluorescence intensity of anti-elastase antibody-Quantum Dots conjugate was very stable - at 30th day the residual intensity was 95%, while the fluorescence intensity of anti-elastase antibody-FITC conjugate was reduced to 60%.

The obtained conjugates were applied in real test with bovine milk somatic cells. Figure 10 shows the fluorescence images of bovine milk somatic cells with two conjugates (magnification x40). The fluorescence images proved that the QDs-conjugate has better fluorescence intensity than FITC-conjugate, and is more appropriate for determination of somatic cells in milk.

Figure 8: Bovine milk neutrophils colored green with the anti-bovine neutrophil elastase antibody-FITC conjugate and red with the anti-bovine neutrophil elastase antibody- quantum dots 710nm conjugate, magnification x100.

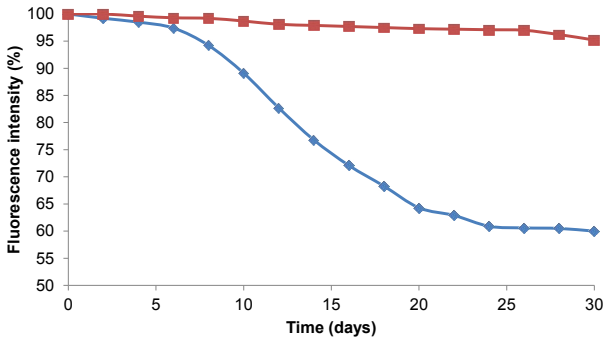

Figure 9: Storage stability of anti-bovine neutrophil elastase antibody-quantum dots 710nm conjugate (red) and anti-bovine neutrophil elastase antibody-FITC conjugate (blue).

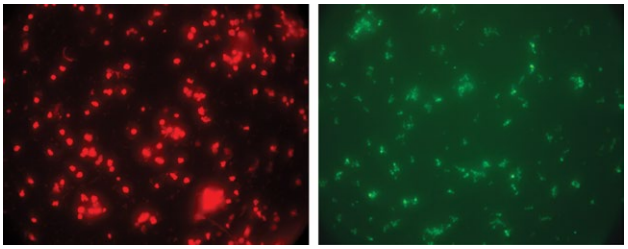

Figure 10: Fluorescence images of bovine milk somatic cells colored red with the anti-bovine neutrophil elastase antibody- quantum dots 710nm conjugate and green with the anti-bovine neutrophil elastase antibody-FITC conjugate, magnification x40.

Forth real milk samples containing increasing somatic cell count from150,000 cells/mL to 800,000 cells/mL were analyzed. Each sample was analyzed 6 times. The count of somatic cells was performed by commercial count Lactoscan SCC of Milkotronik Ltd. The count of neutrophils was carried by proposed method (from fluorescence images). Coefficients of variation were calculated (Table 1). The coefficients of variation have values from 4.2 to 6.3%, which indicate that the reproducibility of the obtained method is high.

Conclusion

In this paper, a novel immunofluorescence microscopic method of neutrophils and somatic cells counting was explored by using anti-elastase antibody. The two different conjugates - anti-bovine neutrophil elastase antibody-FITC conjugate and anti-bovine neutrophil

Table 1: Determination of neutrophils and somatic cells count in real milk by fluorescence microscopic method using the anti-bovine neutrophil elastase antibody-QDs710nm conjugate (n=6).

Milk sample no.	Actual somatic cell count, SCC/mL	neutrophils cells count, neutrophils/mL	Neutrophils cell count, %	Standard deviation,neutrophil cells count, neutrophils/mL	Coefficient of variation, %
1	420,000	285,000	65	10,737	4.2
2	800,000	686,000	84	42,363	6.2
3	150,000	33,000	30	2,074	6.3
4	500,000	390,000	71	20,100	5.2

elastase antibody-Quantum Dots 710nm conjugate, were prepared. The anti-elastase antibody-QDs conjugate activity was higher than that of anti-elastase antibody-FITC. Probably, the longer spacer between antibody and QDs may ensure higher activity of the conjugate than activity of anti-elastase antibody-FITC conjugate. In conclusion, the QDs-conjugate has better fluorescence intensity and stability than the FITC-conjugate, and is more appropriate for SSC in milk.

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