IDENTIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN B IN DAIRY PRODUCTS BY IMMUNOCHROMATOGRAPHY WITH VISUAL AND DIGITAL VIDEO DETECTION

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Detection of staphylococcal enterotoxins in food products is an important task of food poisoning prevention. The study was aimed to develop immunochromatography tests (ICTs) for detection of staphylococcal enterotoxins A (SEA) and be B (SEB), as well as to improve sensitivity of immunochromatography detection of staphylococcal enterotoxins (by the example of SEB) in dairy products relative to visual assessment by recording the analysis results with digital video recorders (DVR) using the principle of processing digital immunochromatogram images acquired using illumination in various spectral ranges. ICTs for detection of enterotoxins were designed as sandwich tests based on highly specific monoclonal antibodies (MABs) against staphylococcal enterotoxins. Milk, cream, sour cream, cheese artificially contaminated with SEB were analyzed. The analysis results were recorded visually or by DVR. DVR of immunochromatograms of the enterotoxin-containing dairy products acquired using illumination with white light in the wavelength range of 400–800 nm ensures a 4-fold increase in the SEB detection sensitivity, while that involving illumination with green light in the wavelength range having its maximum at 525 nm ensures a 4–8-fold increase relative to visual recording. The use of the "Reflecom" and "Zondazh" digital video immunochromatogram analyzers multiplies sensitivity of SEB detection by immunochromatography when assessing dairy products relative to visual recording.

Keywords: staphylococcal enterotoxin types A and B, immunochromatography, video digital registration of results, dairy products

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ВЫЯВЛЕНИЕ СТАФИЛОКОККОВОГО ЭНТЕРОТОКСИНА ТИПА В В МОЛОЧНОЙ ПРОДУКЦИИ ИММУНОХРОМАТОГРАФИЕЙ С ВИЗУАЛЬНОЙ И ВИДЕОЦИФРОВОЙ ДЕТЕКЦИЕЙ

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Выявление стафилококковых энтеротоксинов в продуктах питания является важной задачей профилактики пищевых отравлений. Целью исследования были разработка иммунохроматографических тестов (ИХТ) для обнаружения стафилококковых энтеротоксинов типов А (SEA) и В (SEB), а также повышение чувствительности иммунохроматографического выявления стафилококковых энтеротоксинов (на примере SEB) в молочных продуктах по сравнению с визуальным наблюдением за счет регистрации результатов анализа приборами видеоцифровой регистрации (ВЦР), использующими принцип обработки цифровых изображений иммунохроматограмм при освещении в различных спектральных диапазонах. ИХТ для выявления энтеротоксинов были сконструированы в «сэндвич»-формате на основе высокоспецифичных моноклональных антител (МКА) к стафилококковым энтеротоксинам. Анализу подвергались молоко, сливки, сметана, сыр, искусственно контаминированные SEB. Результаты анализа фиксировали визуально и с помощью ВЦР. Осуществление ВЦР иммунохроматограмм молочных продуктов, содержащих энтеротоксин, при освещении белым светом в диапазоне длин волн 400–800 нм повышает чувствительность выявления SEB в 4 раза, а при освещении зеленым в диапазоне спектра при максимуме длины волны 525 нм — в 4–8 раз по сравнению с визуальной регистрацией. Использование видеоцифровых анализаторов иммунохроматограмм «Рефлеком» и «Зондаж» кратно повышает чувствительность выявления SEB иммунохроматографическим методом при анализе молочных продуктов по сравнению с визуальным методом регистрации.

Ключевые слова: стафилококковые энтеротоксины типов А и В, иммунохроматография, видеоцифровая регистрация результатов, молочные продукты

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Staphylococcal enterotoxins produced by the Staphylococcus aureus Gram-positive bacterial strains cause food poisoning of varying severity in humans [1–3]. Ingestion of infected food is the main root of staphylococcal enterotoxin entry. According to the guidelines of Rospotrebnadzor MUK 4.2.2429-08 "Method to Determine Staphylococcal Enterotoxins in Food Products" and amendment (MUK 4.2.2879-11) [4, 5], food products with the toxin levels ≥ 100 μg/kg of product are considered to be toxicogenic in the Russian Federation. The same norms are set forth by the U.S. Food and Drug Administration (FDA) [6]. Immunochromatography tests (ICTs) are widely used for detection of staphylococcal enterotoxins in raw materials and processed foods, along with enzyme-linked immunosorbent assay. The ICT efficiency for detection of SEA [7], SEA and SEB

Fig. 1. Zondazh reflectometer-fluorimeter

in food products has been shown [8]. Undoubted advantages of ICT are as follows: compact test system design, quick and simple procedure, possible visual assessment of the results. At the same time, the issue of increasing ICT sensitivity is extremely relevant. Such studies focused on the staphylococcal enterotoxin detection by using silver ions and bifunctional gold nanoparticles have been conducted [9, 10].

Given the above, the study was aimed to develop domestic ICTs for detection of staphylococcal enterotoxins A and B, as well as to demonstrate the possibility of improving sensitivity of the above enterotoxin detection relative to visual recording of the results by using digital video immunochromatography analyzers based on processing of immunochromatogram digital images acquired using illumination in various spectral ranges.

METHODS

The procedure of producing ICTs for detection of SEB and the materials used have been previous reported [10]. ICTs for detection of SEA were constructed by the same method using various combinations of MABs produced by the 329D9B3, 329D9B3 and 329A11F6 clones (Table.1). MABs produced by the 357E10E9 and 357A8C1 clones (48th CSRI of the Defence Ministry; Russia) were used to manufacture ICTs for detection of SEB. MABs S222, S643 were produced by RCMDT (Russia). Colloidal gold nanoparticles (CGNs) with an average diameter of 30 nm conjugated to MABs were used in ICTs, and the SEB preparation was used as a concomitant (SRCAMB of Rospotrebnadzor; Russia). The following domestic dairy products were also used: cow's milk, 3.2% fat (GOST 31450-2013), cream, 10% fat (GOST 31450-2013), thermostatic sour cream, 10% fat (GOST 31452-2012), "Rossiysky" cheese, 50% fat (GOST 314521-2012).

Sample preparation for analysis was performed as follows. A total of 1 mL of the toxin-containing dairy product was placed in the 2 ml microcentrifuge tube, added 50 μL of the 0.5 M citrate buffer (pH = 3.0) and mixed by quick shaking. Samples were centrifuged at 4000 g for 15 min. The sample separated, and sedimentation of milk fat on the test tube bottom occurred. A total of 200 μL were collected from the upper transparent liquid layer and mixed with 200 μL of the concentrated buffer solution for immunochromatography analysis (GosNIIBP of FMBA of Russia; Russia). A total of 140 μL were collected from the resulting sample and applied to ICT. The following procedure was used for cheese samples. A total of 1 g of finely grated cheese was placed in the 10 mL test tube, added 1.0 mL of sterile buffer saline and shaken in vibration shaker for 1 min at maximum speed. Holding the test tube at an angle, wet cheese was squeezed by pressing against the test tube wall with a spatula, so that the liquid could drain into the 2 mL centrifuge tube. Samples were centrifuged at 4000 g for 15 min. A total of 200 μL of transparent supernatant fluid were collected and mixed with the concentrated buffer solution in a volume ratio of 1 : 1 for immunochromatography analysis. Then 140 μL of the resulting mixture were applied to ICT. After 25 min the analysis results were recorded visually or using DVR.

The Reflecom digital video immunochromatography analyzer (Sinteco-Complex; Russia) was used for DVR of chromatograms. Measurements were also conducted with the Zondazh reflectometer-fluorimeter (GosNIIBP of FMBA of Russia; Russia) (Fig. 1). The Zondazh experimental DVR reflectometer-fluorimeter model makes it possible to record intensity of light reflected from the immunochromatography test analytical zone or control zone in four spectral ranges: white — 400–800 nm, red — 650 nm, green — 525 nm, blue — 470 nm. The instrument spectral range enables recording of not only CGN conjugates, but also submicron latex particles of different color often used as a dispersed phase in ICTs. When operated in the luminescence intensity measurement mode, the Zondazh unit enables recording of luminescence

Table 1. ICT analytical properties when detecting SEA and SEB using visual recording of the results

Analyte	Sensitivity, ng/mL	Immunochromatography time, min	Combinations of MABs
SEA (variant I)	50	22	329D9B3 / 329D9B3
SEA (variant II)	25	7	329A11F6 / 329D9B3
SEA (variant II)	10	25	329A11F6 / 329D9B3
SEB (variant I)	10	25	357E10E9 / 357A8C1
SEB (variant II)	16	25	S222 / S643

Note: there were no cross-reactions between SEA and SEB concentrations 100 times higher than the corresponding ICT sensitivity; the toxin solutions were prepared using a buffer solution for immunochromatography analysis.

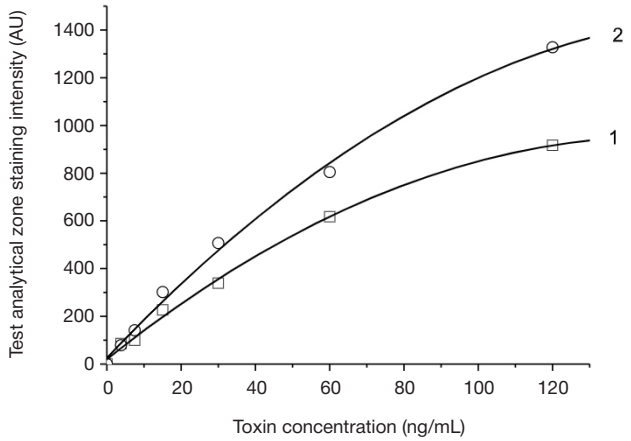

Fig. 2. Graphs of the relationship between the test analytical zone staining intensity and the SEB concentration in buffer solution measured using the Zondazh reflectometer-fluorimeter: 1 — illumination with white light (λ = 400–800 nm); 2 — illumination with green light (λmax = 525 nm). Chromatography time 25 min

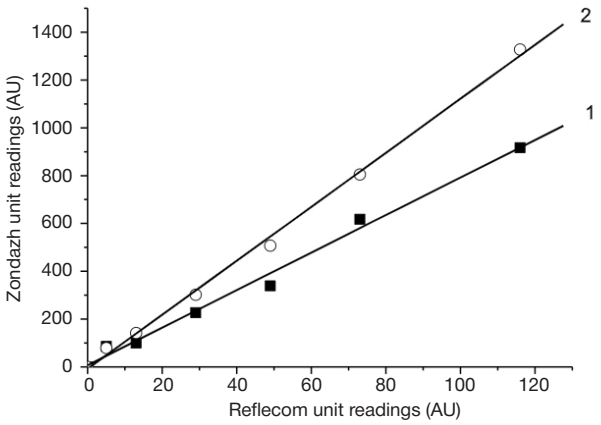

Fig. 3. Graphs of the correlation between the readings of the Reflecom and Zondazh reflectometers used to measure the test analytical zone staining intensity in the SEB concentration range of 0–120 ng/mL. 1 — illumination with white light, 2 — illumination with green light, correlation coefficients of linear relationships R = 0.995 and R = 0.999, respectively

chromatograms, it ensures luminescence excitation wavelength of 380 nm and emission recording wavelength of 490 nm. The instrument operation principle is based on reflectometry of digital chromatogram images or luminescence intensity recording when used in luminescence tests. Light-emitting diodes were used as light sources. A solid state video camera was used as an image receiver.

The unit ensures calculation of the integral intensity of the analyzed and control ICT areas along with the automated baseline correction. When performing DVR with the Zondazh reflectometer-fluorimeter, the increase in the intensity of the test analytical zone staining over the average background value obtained during the blank experiment considering the measurement error at the 95% confidence level was considered to be a criterion of positive result:

$$[X_{av} - t_s \times SE]_{signal} \geq [X_{av} + t_s \times SE]_{background},$$

where $X_{av.}$ was the mean of n measurements, t_s was the Student's t-distribution coefficient for n measurements, SE was the standard error at the 95 % confidence level.

RESULTS

Immunochromatography tests for detection of staphylococcal enterotoxins were designed as sandwich-format tests using MABs. The operation principle of sandwich-format ICTs has been extensively described in the literature [7–10]. A liquid sample potentially containing antigens of toxins is applied to the substrate for sample application. Liquid moves through the multimembrane composites due to influence of capillary forces. First, the CGN conjugate with specific antibodies is solubilized. It is cherry-colored, therefore its movement through the membrane can be visually traced. Moreover, when there is analyte antigen, antigenic immune complex is formed in the sample that starts moving across the test membrane with the fluid flow along with excess conjugate. Then the immune complex is immobilized on the test membrane by specific antibodies in the analytical zone (AZ) to form a "sandwich", and the unbound antibodies of the conjugate are immobilized by antibodies in the control zone (CZ) of the test strip, which results in the emergence of two stained lines. When there is no antigen in the sample, no antigenic immune complex is formed, therefore only one visible line is formed due to binding of the conjugate antibodies and the CZ antibodies (antispecies antibodies against the conjugate antibodies) in the CZ only.

Two ICT options for SEA detection and two for SEB detection were produced based on various combinations of MABs. The ICT sensitivity was dependent on the immunochromatography assay time, it increased as the process progressed throughout 25 min (Table 1).

ICT for detection of SEB variant I was selected for further research due to higher sensitivity shown during the 25 min assay.

Graphs of the relationships of the Zondazh reflectometer-fluorimeter readings obtained during recording immunochromatograms of SEB diluted in the test buffer illuminated with various spectral ranges were plotted based on the data obtained (Fig. 2). The curves were well approximated by fitting with the following polynomials: $Y = 20.49 + 12.43X - 0.041X^2$ (white light) and $Y = 25.91 + 16.44X - 0.047\ X^2$ (green light); covariation coefficient $R^2 = 0.996$ in both cases. Such relationships are typical for immunochromatograms of SEB [9].

As can be seen in the graphs showing the correlation between the readings of the Reflecom digital video immunochromatography analyzer and the Zondazh reflectometer-fluorimeter yielded when assessing immunochromatograms of SEB, there is a linear correlation between the readings of these instruments (Fig. 3).

Readings of the instruments depending on the SEB concentration in the artificially contaminated dairy products yielded after immunochromatography are provided in Table 2.

DISCUSSION

Enzyme-linked immunosorbent assay (ELISA) with photometry or luminescence detection is recommended by the regulatory documents on detection of enterotoxins in food products [4, 5] as an express method. Facilitation of immunochromatography analysis relative to ELISA is achieved due to rejection of additional processing, washing, incubation with the signal-enhancing substrate, as well as to visual assessment of the results. When CGNs are used as labels, typical ICT time is 10–25 min, and the method sensitivity for protein toxins is usually within the range of 1–100 ng/mL, depending on the toxin type. Since immunochemical reactions on the membrane are nonequilibrium, ICT is considered to be inferior to ELISA in terms of sensitivity. At the same time, there are practices and methods to increase the ICT sensitivity to 0.1 ng/mL when used for protein antigens, however, this requires using additional reagents or luminescent labels together with instrumental recording, which significantly increases the analysis time.

In our studies DVR of the ICT results was used to obtain quantitative data along with visual recording. DVR of

Table 2. The test analytical zone staining intensity depending on the SEB concentration and the light spectral range measured using DVR instruments

SEB cocentration, ng/mL	Staining intensity, AU		
	Reflecom digital video immunochromatography analyzer White light λ = 400–800 nm	Zondajzh reflectometer-fluorimeter	
		White light λ = 400–800 nm	Green light λ_{max} = 525 nm
Milk, 3.2% fat, according to GOST 31450-2013			
0	0.0 ± 0.0	77 ± 4	118 ± 5
3.8	0.0 ± 0.0	80 ± 9	135 ± 7
7.5	0.0 ± 0.0	88 ± 5	156 ± 7
15	0.8 ± 0.1	124 ± 12	250 ± 6
30	1.9 ± 0.2	156 ± 8	303 ± 9
60	3.0 ± 0.2	544 ± 17	729 ± 8
Cream, 10% fat, according to GOST 31451-2012			
0	0.0 ± 0.0	93 ± 8	119 ± 6
3.8	0.0 ± 0.0	119 ± 10	139 ± 10
7.5	0.1 ± 0.1	117 ± 8	138 ± 15
15	0.8 ± 0.1	254 ± 11	272 ± 15
30	2.7 ± 0.2	293 ± 6	571 ± 7
60	3.1 ± 0.2	410 ± 12	510 ± 8
120	6.2 ± 0.3	597 ± 8	968 ± 10
240	8.2 ± 0.3	824 ± 11	120 ± 9
Sour cream, 10% fat, according to GOST 31452-2012			
0	0.0 ± 0.0	97 ± 13	120 ± 5
3.8	0.0 ± 0.0	123 ± 20	354 ± 34
7.5	0.5 ± 0.1	326 ± 16	610 ± 8
15	1.8 ± 0.1	533 ± 18	954 ± 39
30	2.9 ± 0.2	704 ± 8	1037 ± 2
Cheese, 50% fat, according to GOST 314521-2012			
0	0.0 ± 0.0	77 ± 6	118 ± 4
3.8	0.0 ± 0.0	80 ± 12	104 ± 10
7.5	0.4 ± 0.1	91 ± 8	128 ± 4
15	0.8 ± 0.1	187 ± 2	237 ± 3
30	1.2 ± 0.1	384 ± 10	291 ± 12
60	2.4 ± 0.2	541 ± 15	616 ± 13
120	4.4 ± 0.2	723 ± 14	825 ± 14

Note: the instrument readings provided in the table are mean values of five measurements. The error represents a standard error at the 95 % confidence level 95%, multiplied by the coefficient ts = 2.776 of Student's t-distribution for four degrees of freedom. Calculations were performed using MS Excel.

immunochromatograms is a common method to obtain semi-quantitative and quantitative immunochromatography analysis results when performing laboratory diagnosis of disorders [11]. DVR is based on assessing digital immunochromatogram images using specialized software that enables determination of integrated intensity of light absorbed by the analytical and control zones formed by the stained particles of the CGN conjugate with specific antibodies. A maximum contrast between the membrane background and the stained zone of chromatograms is required to achieve maximum recording sensitivity. Considering the fact that CGNs and their conjugates have broad structureless absorption bands within the range of 500–600 nm, the contrast must depend on the immunochromatogram illumination spectral composition. Based on subtractive color perception theory, a red object illuminated by green light looks almost black. Since there is too little green in red, the red object would absorb the majority of green photons and reflect almost nothing. Red would lose very much in saturation and tone, it would become brown, gray or even black [12]. Illumination of SEB immunochromatograms with green light (λmax = 525 nm) ensures a more intense signal during DVR compared to illumination with white light (Fig. 2). The readings of two different instruments using the same signal processing principle are linearly correlated. Furthermore, DVR response is stronger when using illumination in green spectral range (Fig. 3). The same relationships are observed when performing analysis of artificially contaminated dairy products after sample preparation for immunochromatography analysis (Table 2). The pooled data on the sensitivity of SEB detection using illumination of immunochromatograms in various spectral ranges are shown in the chart (Fig. 4). DVR of immunochromatograms of the enterotoxin-containing dairy products involving the use of illumination with white light four times increases the SEB detection sensitivity, while illumination with green spectral range 4–8 times increases sensitivity compared to visual recording.

It can be expected that the patterns of SEB detection in dairy products would be preserved when performing SEA analysis, considering structural similarity of these proteins.

The analyte-containing matrix has a great impact on the possibility of chromatography analysis and sensitivity [13, 14].

To obtain appropriate results, it is necessary to concentrate a protein enterotoxin in the low-viscosity hydrophilic phase with pH = 5.5–7.0 that is optimal for immunochemical reaction. Such phase would move well through the ICT nitrocellulose membrane and ensure immunochemical binding of reagents. As for dairy products, it is necessary to separate the serum containing proteins (such as staphylococcal enterotoxin) and the milk fat globules by centrifugation. When the product fat content is low, the product can be analyzed directly with no sample preparation. Thus, the results of assessing milk (3.2% fat) with or without sample preparation are almost the same. However, sample preparation involving sample dilution and incomplete toxin extraction to the hydrophilic phase reduces overall sensitivity of the analysis.

Our findings confirm the possibility of using ICT for detection of staphylococcal enterotoxins in dairy products, and the staphylococcal enterotoxin detection sensitivity achieved by using DVR is 3.8–7.5 ng/mL. These values enable the analysis meeting regulatory requirements that the levels of enterotoxins in food products should not exceed 100 ng/g of product.

CONCLUSIONS

We have developed ICTs based on MABs for detection of SEA and SEB showing sensitivity of 10 ng/mL for each toxin when conducting immunochromatography in buffer solutions

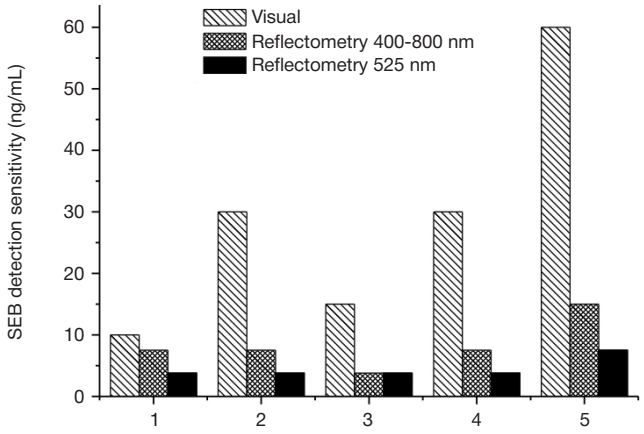

Fig. 4. Chart reflecting sensitivity of SEB detection in dairy products using visual recording of the results or instrumental recording with the Zondazh reflectometer-fluorimeter. 1 — SEB solution in buffer; 2 — milk, 3.2% fat; 3 — cream, 10% fat; 4 — sour cream, 10% fat; 5 — cheese, 50% fat

for 25 min (visual assessment of the results). ICTs show no cross-reactions when used for analysis of enterotoxins A and B with the 100-fold increased concentration of enterotoxin of other type. DVR of enterotoxin-containing dairy product immunochromatograms involving the use of illumination with white light four times increases the SEB detection sensitivity, while illumination with green spectral range results in the 4–8 times increased sensitivity compared to visual recording.

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