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ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2010

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Simultaneous Detection of Seven Staphylococcal Enterotoxins: Development of Hydrogel Biochips for Analytical and Practical Application

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A method of simultaneous analysis of staphylococcal enterotoxins using hydrogel-based microarrays (biochips) has been developed. The method allows simultaneous quantitative detection of seven enterotoxins: A, B, C1, D, E, G, and I in a single sample. The development of the method included expression and purification of recombinant toxins, production of panels of monoclonal antibodies (mAbs) against the toxins, and design and manufacturing of an experimental biochip for the screening of mAbs and selection of optimal pairs of primary and secondary antibodies for each toxin. The selected mAbs have high affinity toward their targets and no cross-reactivity with unrelated enterotoxins. Finally, a diagnostic biochip was designed for quantitative analysis of the toxins, and the analytical protocols were optimized. The sensitivity of the detection reached 0.1−0.5 ng/mL, depending on the type of enterotoxin. The evaluation of the resulting biochip using spiked food samples demonstrated that the sensitivity, specificity, and reproducibility of the proposed test system fully satisfy the requirements for traditional immunoanalytical systems. The diagnostic biochips manufactured on reflecting metal-coated surfaces shortened the time of analysis from 17 to 2 h without loss of sensitivity. The method was successfully tested on samples of food and biological media.

Actively growing staphylococci can produce toxins, of which the most harmful are enterotoxins.[1] Intoxication with food products contaminated with staphylococcal enterotoxins is very common, and its incidence is second only to intoxications caused by salmonella infections.[2−4] Food intoxications may lead to complications, including various autoimmune conditions, such as rheumatoid arthritis,[5] atopic dermatitis,[6,7] and allergies. In more complex clinical situations, staphylococcal enterotoxins (SEs) are sometimes involved in toxic shock syndrome.[8]

SEs are very stable proteins capable of retaining their biological activity after treatments that inactivate the SE-producing bacteria. This makes it especially important to develop sensitive and specific methods for their direct detection.

SEs are relatively small proteins with molecular mass from 27 to 30 kDa which belong to the family of superantigens.[9] The homology of primary sequences of different SEs is 20−80%, and their spatial structures are very similar.[10] The most frequent causes of food poisoning are SEs A, B, C1, D, and E. Enterotoxins G and I can not only cause food poisoning but also induce toxic shock.[8] SEs must be also considered as potential biological weapons. This possibility adds urgency to the development of sensitive, specific, and robust methods of simultaneous detection of known SEs.

The necessary sensitivity of detection of enterotoxin A is 200 ng in 100 g of food.[11] When children's intoxication is concerned, even higher sensitivity of detection in foods and biological liquids is required.[12]

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10.1021/ac1016634

Currently, a wide variety of methods are being used for the detection and analysis of both SEs and toxin-producing staphylococci. These methods include microbiological tests[13] and detection of staphylococcal DNA using polymerase chain reaction (PCR),[14−16] as well as immunological tests. These include direct and indirect radioimmunological detection,[17] immunofluorescent,[18] and enzyme linked immunosorbent assay (ELISA)[19−21] analyses. The most sensitive and convenient method is solid-phase immunoassay.[19] However, most commercially available detection kits are not quantitative, are too costly for routine screening, and often require special equipment.[22]

One disadvantage of conventional immune detection methods is the inability to test the sample for the presence of several toxins simultaneously. To overcome this limitation, a new analytical tool for parallel immune detection of multiple substances, a biological microchip (biochip), has been proposed. Microarray-based technologies are actively developed throughout the world. One such technology has been introduced by our group at the Engelhardt Institute of Molecular Biology (Moscow, Russia). It employs arrays of hydrogel elements (biochips) which can contain diverse immobilized probes (proteins, DNA, RNA, and oligosaccharides) and allow one to carry out several different reactions simultaneously on a single chip. The possibility of parallel quantitative immunofluorescent analysis of multiple substances on the same biochip has been demonstrated earlier.[23−26] During further development of the method, we have demonstrated the possibility of parallel analysis of several toxins of animal, plant, and bacterial origin on a single biochip.[27]

The purpose of this work was the development of a diagnostic test system for simultaneous quantitative detection of staphylococcal enterotoxins A, B, C1, D, E, G, and I. For practical applications, a test system for the detection of pathogens and enterotoxins must satisfy rather strict requirements: simple procedures of preparation of samples and analysis, high sensitivity and specificity, and reproducibility, as well as reasonable cost.[28] Here, we describe the development of a system for quantitative detection of seven staphylococcal enterotoxins using hydrogel biochips that satisfies all these demands.

MATERIALS AND METHODS

Materials. Inbred specific pathogen-free (SPF) mice BALB/c were obtained from the animal facility of the Pushchino Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry of Russian Academy of Sciences. All work conformed with the regulations of humane treatment of animals.

Equipment and reagents were obtained from the following suppliers: 96-well medium binding plates for ELISA (plates GR-655001) from Greiner Bio-One GmbH (Frickenhausen, Germany); cell culture plates and flasks from NUNC (Thermo Fisher Scientific, Rochester, NY); streptavidin−peroxidase and streptavidin−Cy5 conjugates from GE Health Care (Little Chalfont, Buckinghamshire, UK); Dulbecco's modified eagle medium (DMEM; cell media), SFM4Mab (serum-free media), and fetal bovine serum (FBS) from Hyclone (Logan, UT); hypoxanthine aminopterin thymidine medium (HAT), hypoxanthine thymidine medium (HT), antibiotic−antimycotic, L-glutamine, and sodium pyruvate from Invitrogen (Carlsbad, CA); molecular mass markers for polyacrylamide gel (PAAG) electrophoresis from Helicon (Moscow, Russia); *N*-hydroxysuccinimidobiotin, acrylamide, methacrylamide, *N*,*N*′-methylene bisacrylamide, glycerol, phosphate-buffered saline (PBS) tablets, tris-(hydroxymethyl)-aminomethane, Tween 20, Sephadex G-25 coarse, *o*-phenylenediamine (OPD), polyethylene glycol (PEG) 1300-1600, "Hybri-Max," Pristane, and *Staphylococcus aureus* enterotoxins A and B from Sigma-Aldrich (St. Louis, MO); Micro Bio-Spin chromatography columns from Bio-Rad (Hercules, CA).

In addition, the following reagents were used in the manufacturing of biochips: polyvinyl alcohol (PVA), 50 kDa, polyvinylpyrrolidone (PVP), 360 kDa, and Corning 2947 Micro Slides from Corning, NY; microarray slides with gold film coating 25 × 75 mm, coat thickness 120 nm, from NUNC (Roskilde, Denmark, distributed by Thermo Fisher Scientific).

Production of Enterotoxins. Genes for enterotoxins were cloned, and enterotoxins were isolated as recombinant proteins. The original enterotoxin-producing strains of *Staphylococcus aureus* were obtained from M. Bergdoll (University of Wisconsin, Madison, WI) and stored in the collection of L. A. Tarasevich State Institute of Standardization and Control of Biomedical Preparations (Moscow, Russia). The following sequences for the expression of recombinant toxins were PCR-amplified from the corresponding strains: 1-773 from Q6GFA8 (SEA), 2441044 from Q5HHH9 (SEB), 202-914 from P01553 (SEC1), 442-1064 from Q197 × 4 (SED), 82-770 from P12993 (SEE), 228-926 from Q6GFN2 (SEG), and 226-878 from Q7X0E8 (SEI). Appropriate restriction sites were added at the ends of the fragments during amplification; the products were cloned in the vector pET28, and sequences of the resulting plasmids p28-SEA, p28-SEB, p28-SEC1, p28-SED, p28-SEE, p28-SEG, and p28-SEI were confirmed by sequencing and NCBI BLASTA search.

Cultivation of Recombinant Strains, Purification of Enterotoxins, and Purity Control. Recombinant plasmids containing enterotoxin genes were transformed into *E. coli* BL21.

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Enterotoxins were isolated on Ni-NTA agarose columns (Qiagen, Germantown, MD) according to the manufacturer's instructions. If necessary, additional purification was carried out on Mono Q column (FPLC System, Pharmacia Biotech, Uppsala, Sweden).[29] The resulting SEs were analyzed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[30] with subsequent staining with Coomassie Brilliant Blue G-250. The purity of the final preparations of SEs was 95−98%.

Monoclonal Antibodies to Enterotoxins. Monoclonal antibodies (mAbs) to SEs were obtained as described.[31] The reaction product was measured on a Titertec Multiscan plate reader (Flow Laboratories; currently ICN Biomedicals, Aurora, OH) at 492 nm.

Purification of mAbs. Hybridomas were grown as ascites. Antibodies were isolated using ammonium sulfate precipitation and chromatography on Mono Q columns eluted with NaCl gradient.

Conjugation of Antibodies with Biotin. Seventy microliters of antibody solution (5 mg/mL in 10 mM bicarbonate buffer, pH 9.5) was mixed with 10 μL of *N*-hydroxysuccinimidobiotin solution in dimethyl formamide (5 mg/mL). The reaction was carried out for 1 h at 20 °C. The conjugate of antibody with biotin was purified by gel filtration in PBS on a Micro Bio-Spin column with Sephadex G-25.

Sandwich Immunoassays. Sandwich immunoassays were performed as described by Pujol et al.[32] The affinity of antibodies was determined according to Beatty et al.[33] To determine classes and subclasses of mAbs, a commercial Mouse Typer Isotyping Panel was used (Bio-Rad).

Manufacturing of Biochips. Glass Micro Slides (see Materials) were treated with NaOH and H_2SO_4, washed with water, then submerged in 1% solution of Bind Silane (Amersham Pharmacia Biosciences, Piscataway, NJ) in ethanol, washed in ethanol and again in water, and air-dried. Gold-coated slides were used without additional treatment.

Hydrogel biochips were manufactured using a polymerization immobilization reaction as described earlier.[27,34,35] A polymerization mixture containing gel-forming monomers together with the proteins to be immobilized was spotted as 0.1 nL drops onto the activated surface of glass slides using a QArray pin robot (Genetix, UK). The concentration of antibodies in the polymerization mixture was between 0.4 and 1.0 mg/mL. Polymerization of gel elements was carried out under UV light with a maximum wavelength of 350 nm (Sylvania GTE lamp F15T8/350Bl, UK) at a distance of 8 cm from the lamp for 50 min at 20 °C in nitrogen flow. The 150 pm pin produced semispherical drops of 120 pm in diameter. After polymerization, the biochips were washed for 40 min in PBS containing 1% Tween 20 (PBST) and then rinsed with water. To suppress nonspecific binding, the biochips were additionally treated with blocking solution PBSP (PBS containing 1% PVA).

All biochips underwent a computerized quality control procedure as described earlier.[26] Biochips with intrachip variations of radii of individual gel elements exceeding 20% were discarded. Similarly, batches with variations of average radii of individual chips exceeding 20% were considered faulty.

Calibration Curves. For calibration curves, the following standard solutions of enterotoxins were used: SEA, 0−150 ng/mL; SEB, 0−100 ng/mL; SEC1, 0−125 ng/mL; SED, 0−100 ng/mL; SEE, 0−100 ng/mL; SEG, 0−200 ng/mL; and SEI, 0−300 ng/mL.

In all versions of the simultaneous sandwich immunoassay of seven SEs on biochips, the following mixture of developing antibodies was used: SEA-6, 14 μg/mL; SEB-6, 9.4 μg/mL; SEC1-4, 10 μg/mL; SED-5, 11 μg/mL; SEE-7, 7 μg/mL; SEI-2, 9.5 μg/mL; and SEG-11, 8.5 μg/mL.

Sandwich Immunoassay of SEs. For the two-step sandwich assay, 60 μL of the analyzed sample or calibration solution were applied on the biochip and incubated for 17 h at 37 °C. The chip was washed with PBST for 10 min; 40 μL of biotinylated antibodies were applied on the chip and incubated for 1 h at 37 °C. The biochips were washed for 20 min with PBST and developed with fluorescently labeled streptavidin for 10 min at 37 °C. Then, the biochips were washed again for 30 min with PBST, and fluorescent signals were registered.

For a one-step sandwich assay (express analysis on gold-coated biochips), 30 μL of biotinylated antibodies were added to 30 μL of the analyzed sample or calibration probe, and the mixture was incubated on the biochip for 2 h at 37 °C. The biochips were developed with fluorescently labeled streptavidin for 10 min at 37 °C. Then, the biochips were washed for 30 min with PBST, and fluorescent signals were registered.

Detection of SEs in Food Samples. Milk (3.2% fat) and cream (10% fat) were spiked with various amounts of the seven SEs. Then, the samples were diluted 20-fold with PBS and centrifuged for 10 min at 3500*g* and 10 °C. The floating layer of fat was removed, and the supernatant was sterilized by filtration and used for further analysis.

To analyze farmer cheese (5.5% fat) and baby food (instant cereal containing dry milk, fat content of 10%), samples were spiked with various amounts of the mixture of seven SEs. Then, 1 g samples were homogenized in 15 mL of PBS (pH 7.4) and put on a shaker for 15 min. The homogenate was centrifuged for 10 min at 3500*g* and 15 °C. The floating layer of fat was removed, and the supernatant was sterilized by filtration and used for further analysis. All food samples were analyzed by a two-step immunoassay on biochips using biotinylated antibodies for development.

Measurements of fluorescence were carried out using a portable biochip analyzer with a laser light source (Biochip-EIMB, Ltd., Moscow, Russia). The analyzer has filters with maximum wavelength 650 nm for excitation and 670 nm for emission.[36] Fluorescent signals from each element of the biochips were processed using ImaGel Research software (Biochip-EIMB, Ltd.,

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Moscow, Russia). Briefly, the software generates a fixed circle around each gel element encompassing on average 300 pixels. The program draws a set of radii from the center of the circle and determines the median intensity for each radius, and then, the median of all radii is considered the actual fluorescence intensity of the element. The same software package was used to calculate the concentrations of the toxins. The intensity of fluorescence of individual data points was always calculated as the median value from four identical elements.

RESULTS AND DISCUSSION

Fast, sensitive, and quantitative detection of biological toxins in food is an important factor in controlling and preventing food intoxications by sanitary services. In particular, a wide range of methods is used for detection of SEs. Although the most sensitive and specific methods are based on the analysis of staphylococcal DNA or RNA, it is preferable to detect the toxins as proteins because of their high thermal and pH stability and resistance to proteolysis. This stability may result in their accumulation and preservation in food during its storage and processing.

Most methods of direct detection are based on an immunochemical approach, which may employ both polyclonal and monoclonal antibodies. Since SEs belong to the same family of proteins, their differential analysis with mAbs is more efficient, providing better specificity while reaching high sensitivity, comparable with polyclonal antibodies.

In this work, mAbs were obtained using recombinant SEs cloned from well-characterized toxin-producing strains of *S. aureus*. The purity of the recombinant SEs was evaluated by electrophoresis in PAAG-SDS.[30]

To obtain a representative panel of mAbs against SEA, two hybridizations were performed. A total of 14 hybridomas were isolated which produced specific antibodies. The other six SEs investigated in this work required one hybridization each, and the following numbers of hybridomas were obtained: 19 against SEB, 15 against SEC1, 17 against SED, 19 against SEE, 14 against SEG, and 15 against SEI. Thus, a total of 113 hybridomas were isolated, which were then used for the selection of optimal pairs of antibodies for immunochemical detection.

All possible pairwise combinations of mAbs against individual serotypes of SEs were tested in sandwich immunoassays on hydrogel biochips, and the most sensitive and specific pairs were chosen as described below. The list of the resulting pairs of mAbs and their individual characteristics are listed in Table 1.

Selection of Optimal Pairs of Antibodies Using the Screening Biochip. The biochip technology allows simultaneous multiparametric analysis of samples. This feature is advantageous in selecting optimal pairs of mAbs for sandwich immunoassay. The specificity, sensitivity, shape of calibration curves, dynamic range of concentrations, and discriminant signal/noise ratio are determined primarily by the characteristics of immobilized and developing antibodies. Choosing pairs of antibodies for simultaneous analysis of several closely related antigens is especially challenging, because it is necessary to avoid cross-reactivity.

For screening of optimal pairs of antibodies, biochips were manufactured for each toxin antigen carrying a complete panel of corresponding antibodies each. Then, each antibody was conjugated with biotin. The resulting conjugates were tested for the ratio between the maximal and background signals.

Table 1. Monoclonal Antibodies against Staphylococcal Enterotoxins Used for the Analysis on Biochips

antigen	antibody[a]	K_{aff}, M^{-1}	antibody subclass
SEA	SEA-11	7.3×10^8	IgG_{2a}, κ
	SEA-6	1.3×10^9	IgG_{2a}, κ
SEB	SEB-2	3.6×10^8	IgG_1, κ
	SEB-6	2.5×10^8	IgG_{2a}, κ
SEC_1	SEC_1-7	7×10^8	IgG_{2a}, κ
	SEC_1-4	6.5×10^8	IgG_{2a}, κ
SED	SED-8	7.6×10^8	IgG_{2a}, κ
	SED-5	1.2×10^9	IgG_{2a}, κ
SEE	SEE-8	2.3×10^9	IgG_{2a}, κ
	SEE-7	1.7×10^9	IgG_{2a}, κ
SEG	SEG-7	6.8×10^8	IgG_{2b}, κ
	SEG-11	9.2×10^8	IgG_{2a}, κ
SEI	SEI-9	5.6×10^8	IgG_1, κ
	SEI-2	8.5×10^8	IgG_{2a}, κ

[a] In the "antibody" column, the upper row corresponding to each enterotoxin indicates the coating mAb in sandwich immunoassay, while the lower row indicates the probing mAb.

To do so, each biotinylated conjugate was loaded on the biochip either without the toxin or mixed with the corresponding toxin at 100 ng/mL. The biochips were developed using Cy5-labeled streptavidin. The ratios between positive signals from biochips treated in the presence of SEs and signals from the biochips without the SEs, the latter representing calibration signals at zero concentration of the antigens, were calculated. In all calculations, median measurements from four identical elements corresponding to each experimental combination of antibodies and probes were used. Finally, antibodies that gave the highest ratios were selected as developing antibodies.

At the next stage, calibration curves were obtained for each selected pair by plotting the intensity of fluorescent signals against the concentration of the corresponding antigen in solution. The final choice of optimal pairs was based on the maximal ratio between the positive signal and the zero calibration signal, as well as the best linearity of the complete calibration curve. The notion of "the best linearity" is used here in an empirical sense defined as the longest segment of the calibration curve which does not display saturation and bending of the curve. When the calibration curve is S-shaped, significantly deviating from linear form, this results in decreased sensitivity at low concentrations of the probe and a narrower dynamic range of detection. Therefore, the plotting of the calibration curves was performed using piecewise linear interpolation similar to commercial ELISA kits. The concentration of each analyte in the probe was determined using the corresponding signal and the linear segment of the calibration curve between the two points where the signal was located. Figure 1 shows an example of data used in the process of selection of an optimal pair of antibodies against SEE.

Testing the Specificity of the Selected Pairs of Antibodies. Each selected pair was tested for specificity, i.e., the absence of cross-reactivity with other selected antibodies as well as interaction with unrelated SEs. Figure 2 illustrates this experiment using antibodies against SEE as an example. The specificity was evaluated in two reactions. First, a biochip was manufactured with antibodies against all seven enterotoxins (SEA, SEB, SEC1, SED, SEE, SEG, and SEI) immobilized in individual gel elements

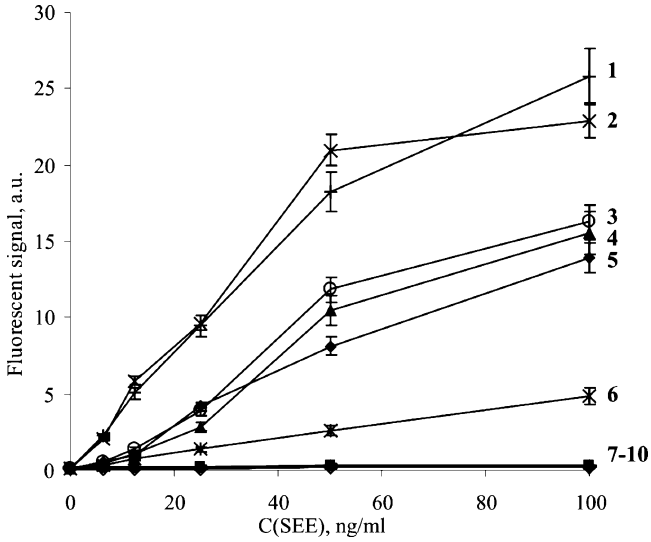

Figure 1. Calibration curves obtained during the selection of an optimal pair of antibodies for the detection of SEE. Biotinylated antibodies SEE-7 were used for development. The curves correspond to the following antibodies immobilized on the biochip: 1, SEE-8; 2, SEE-12; 3, SEE-14; 4, SEE-5; 5, SEE-1; 6, SEE-9; 7, SEE-2; 8, SEE-10; 9, SEE-15; 10, SEE-17. The SEE-8 antibody was selected for further work because of the highest signal and best linearity (curve 1).

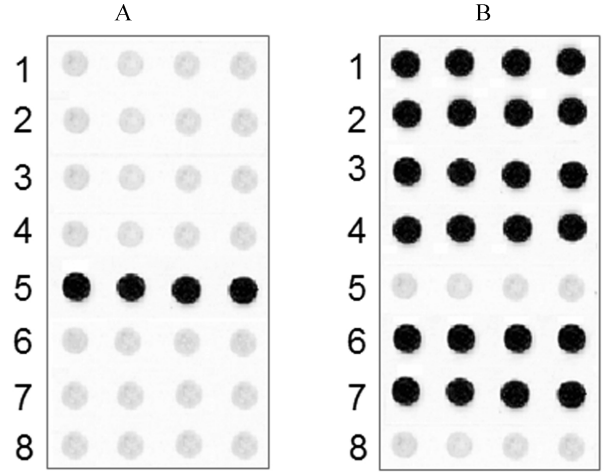

Figure 2. Testing of specificity of the pair of antibodies against SEE. The following antibodies were immobilized on the biochip: 1, SEA; 2, SEB; 3, SEC1; 4, SED; 5, SEE; 6, SEG; 7, SEI; 8, empty gel elements. Panel A shows a fluorescent image of the biochip after the sandwich immunoassay with a mixture of all seven investigated enterotoxins SEA, SEB, SEC1, SED, SEE, SEI, and SEG and a single developing biotinylated antibody SEE-7. Panel B shows fluorescent image of the biochip after sandwich immunoassay with a mixture of six enterotoxins SEA, SEB, SEC1, SED, SEI, and SEG and developing biotinylated antibodies against these six toxins. No cross-reactivity was observed.

together with control empty elements. This biochip was incubated with a mixture containing all seven toxins but only one biotinylated antibody against SEE, SEE-7 (Figure 2A). The purpose of this experiment was to prove that the developing antibody SEE-7 did not interact with any enterotoxins except SEE. Second, the same biochip was treated with a mixture of six enterotoxins (SEA, SEB, SEC1, SED, SEI, and SEG) and developing biotinylated antibodies

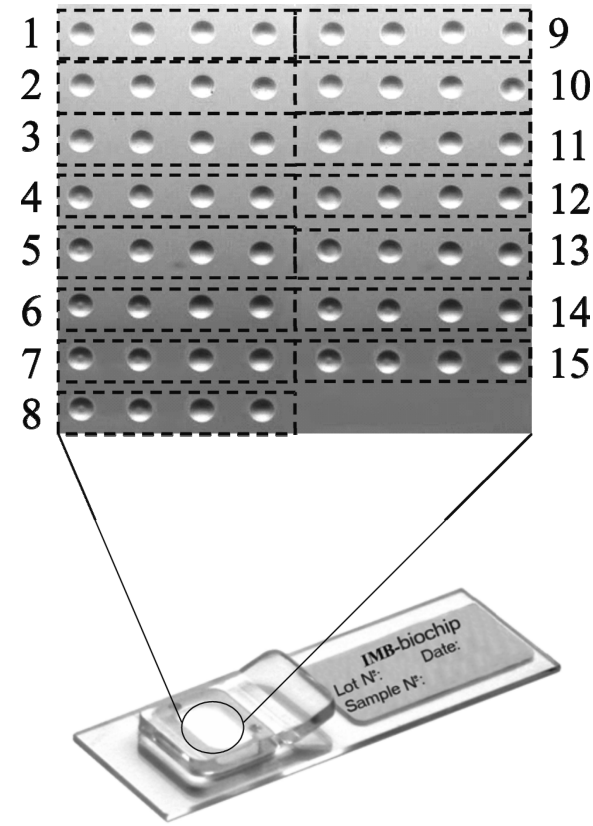

Figure 3. Biochip for quantitative analysis of seven staphylococcal enterotoxins. Top: enlarged image of the biochip in a bright field microscope; bottom: overall view of the biochip. The biochip consisted of hydrogel elements 120 μm in diameter and 0.1 nL in volume. The elements with immobilized mAbs against seven different SEs as well as SEs themselves were placed in groups of four, contoured with dashed rectangles in the following order: (1) Ab SEA; (2) Ab SEB; (3) Ab SEC1; (4) Ab SED; (5) Ab SEE; (6) Ab SEG; (7) Ab SEI; (8) control element without immobilized proteins; (9) SEA; (10) SEB; (11) SEC1; (12) SED; (13) SEE; (14) SEG; (15) SEI.

against all these toxins (without SEE and SEE-7 antibody). In this case, it has been demonstrated that there were no nonspecific interactions of the immobilized antibody against SEE, SEE-8, with unrelated antigens (Figure 2B).

The specificity of six other pairs of antibodies was tested by the same two-stage procedure. In case a pair of antibodies produced nonspecific signals in either of the two tests, it was replaced by another pair, and the specificity tests were repeated. As the result of this screening, four out of 113 mAbs were rejected.

In addition, the specificity of each pair of mAbs was evaluated by comparing the fluorescence of the first (zero concentration) calibration probe with the fluorescence of the empty elements containing no immobilized proteins. The pairs of mAbs were considered acceptable when the signal of the former exceeded the signals from the latter by less than 15%. This figure, 15%, is the maximal variation coefficient for each concentration of the calibration probe observed in our experiments.

Testing the Specificity of Monoclonal Antibodies on Native Enterotoxins. Enterotoxins belong to a group of homologous proteins whose evolution continues.[9] Each recombinant toxin obtained in this work and used for the production of mAbs is but one variant of a group of toxins of the same serotype, while in a natural environment other variants could be encountered. There-

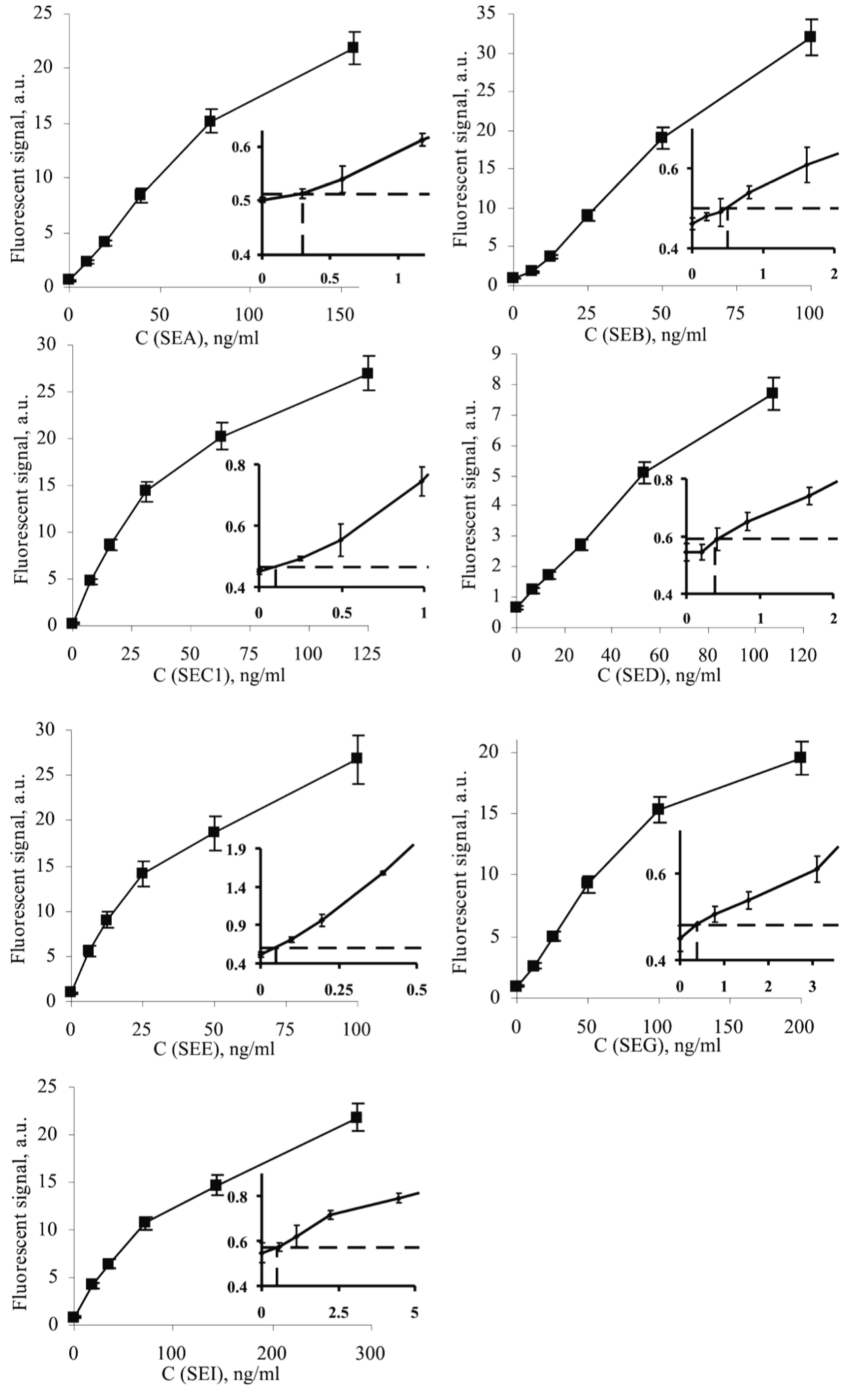

Figure 4. Calibration curves obtained by a simultaneous two-step sandwich immunoassay of seven serotypes of SEs. The biotin−streptavidin system was used for detection. Variation coefficient did not exceed 15%. Calibration curves for low range concentrations are shown in the insets. The dashed lines correspond to fluorescence level exceeding the signal emitted by control elements by two standard deviations. The calculated concentrations of SEs corresponding to this level equals their limits of detection: SEA, 0.3 ng/mL; SEB, 0.5 ng/mL; SEC1, 0.1 ng/mL; SED, 0.4 ng/mL; SEE, 0.05 ng/mL; SEG, 0.4 ng/mL; and SEI, 0.5 ng/mL.

fore, it was important to confirm the validity of the developed detection technique by evaluating the affinity and specificity of the antibodies against recombinant enterotoxins in reactions with toxins isolated from naturally occurring strains.

To assess the immune specificity of the mAbs selected for the analyses on the biochip, a parallel sandwich analysis was performed of recombinant SEA and SEB obtained in this work and natural commercial preparations of the same toxins were isolated from *S. aureus* and purchased from Sigma (see Materials). The sandwich assay was carried out on both standard immunoplates and biochips described in this work. The results fully confirmed the immune specificity of the mAbs against native variants of SEs.

Biochip for Simultaneous Quantitative Analysis of Seven Enterotoxins. Biochips for simultaneous quantitative analysis of staphylococcal enterotoxins A, B, C1, D, E, G, and I consist of two groups of elements (Figure 3). The elements in the left group contain immobilized antibodies against the seven SEs, while the elements in the right group contain immobilized antigens, SEs. Each protein is immobilized in four identical elements for better statistical validity of the data. The biochip also has gel elements

without any immobilized proteins which are used as controls to determine the fluorescence resulting from nonspecific adsorption of reagents.

During the sandwich analysis of the sample, triple complexes "immobilized mAb−antigen−developing mAb" are formed in the elements in the left part of the biochip, while binary complexes of immobilized antigen with developing mAb are formed in the right part. The concentration of the SEs in the samples was measured using signals from the elements in the left part of the biochip. Signals from the elements on the right with immobilized antigens were used as positive control for the developing antibodies and also for the detection of the hook effect during express analysis.

In immunodiagnostic systems based on one-step sandwich assays, a high concentration of the analyte in samples may cause the so-called hook effect, when the relation between the concentration of the analyte and the intensity of the registered signals becomes inverted. Earlier, we have demonstrated that this effect could be detected using an appropriate design of the detection scheme on biochips.[26] In this work, we applied the same approach to the one-step express analysis of the seven enterotoxins.

Analytical Procedure. For quantitative analysis of seven serotypes of SEs on biochips, a two-step sandwich immunoassay with fluorescent signal registration was chosen. A developing system consisting of biotinylated antibodies with subsequent treatment using fluorescently labeled streptavidin has been consistently found to provide the highest sensitivity.

To determine the concentration of an SE in a sample using immunoassay on biochip, it is necessary to have a calibration curve showing the dependence of the fluorescent signal of the gel elements with immobilized antibodies on the concentration of the corresponding antigen in calibration solution. For simultaneous analysis of seven serotypes of SEs, calibration samples were prepared containing all seven antigens at different concentrations. The control probes (zero concentration samples) did not contain any SEs. The developing mixture contained secondary antibodies against all seven SEs, so that each experiment provided data for all seven calibration curves.

Typical calibration curves resulting from these experiments are shown in Figure 4. The reproducibility of the analysis was determined using 10 independent calibration curves. Each calibration sample, including each concentration of all seven antigens separately and each sample containing all seven studied antigens together, was analyzed 10 times on 10 different biochips from the same batch. The graph shown in Figure 4 represents average fluorescence intensities. The variation coefficient for different concentrations of the antigens, i.e., for each data point, did not exceed 15%.

As indicated in the Materials and Methods, we used linear interpolation for all calibration curves. Although in some cases second order polynomial interpolation seemed to work better, it would skew the data at low concentrations and affect the limit of detection (LOD).

For better reproducibility, each biochip has four gel elements corresponding to each immobilized protein, and the median of the four registered signals is calculated as the final value for further analysis. In addition, strict requirement must be satisfied in the process of quality control after the manufacturing of the biochips as described in the Materials and Methods.

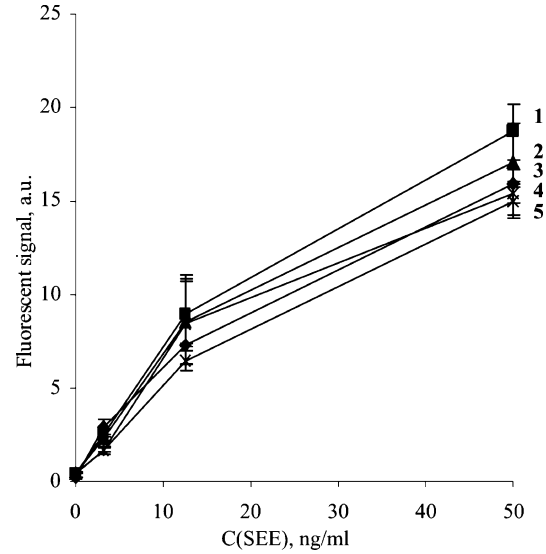

Figure 5. Calibration curves obtained in immunoassays of SEs in food samples: 1, buffer solution; 2, milk; 3, cream; 4, baby food (instant cereal); 5, farmer cheese.

One of the main characteristics of quantitative analysis is its sensitivity (LOD). The LOD was defined as the concentration which yielded fluorescence signal exceeding the average fluorescence of the control probe by at least two standard deviations.[37] This definition of sensitivity corresponds to 95% probability of correct detection and is commonly accepted as sufficiently reliable. The control samples were measured ten times. These calculations resulted in the following LOD values: SEA, 0.3 ng/mL; SEB, 0.5 ng/mL; SEC1; 0.1 ng/mL; SED, 0.4 ng/mL; SEE, 0.05 ng/mL; SEG, 0.4 ng/mL; and SEI, 0.5 ng/mL.

Analysis of Staphylococcal Enterotoxins in Biological Samples. The detection technique described above was tested on food samples spiked with a mixture of seven SEs investigated in this work. The mixture was added to control buffer solution, milk, cream, farmer cheese, and baby instant cereal containing dry milk. Dairy products were chosen because they are often considered a probable cause of food contamination with enterotoxins resulting from the use of milk from cows who have mastitis.[38] Such a contamination in baby food is especially dangerous because infants' poisoning with enterotoxins could result in sudden infant death syndrome (SIDS).[12]

Calibration curves obtained for all SEs in all tested food samples and corresponding controls were virtually identical (Figure 5). Such a reproducibility of the calibration curves in all studied environments suggest that the protocol developed in this work can be used for simultaneous detection of all seven serotypes of enterotoxins in food samples. The LOD for all tested food samples did not exceed the following values: SEA, 0.5 ng/mL; SEB, 0.6 ng/mL; SEC1, 0.4 ng/mL; SED, 0.7 ng/mL; SEE, 0.05 ng/mL; SEG, 1.1 ng/mL; and SEI, 0.8 ng/mL.

In the next experiment, each SE was added to each of the four tested foods in four different defined concentrations. Control food samples underwent identical treatment but were not spiked with

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(38) Asao, T.; Kumeda, Y.; Kawai, T.; Shibata, T.; Oda, H.; Haruki, K.; Nakazawa, H.; Kozaki, S. *Epidemiol. Infect.* **2003**, *130*, 33–40.

Table 2. Concentrations of Staphylococcal Enterotoxins Measured in Food Samples[a]

enterotoxin	calculated concentration	milk, 3.2% fat	cream, 10% fat	farmer cheese, 5.5% fat	baby food (instant cereal)
SEA	0	0	0	0	0
	0.6	0.8 (±0.2)	0.7 (±0.1)	0.5 (±0.1)	0.5 (±0.1)
	9	8 (±1)	6 (±1)	5 (±1)	5 (±1)
	37	43 (±8)	59 (±11)	23 (±4)	18 (±4)
	150	159 (±32)	139 (±21)	101 (±20)	132 (±20)
SEB	0	0	0	0	0
	0.8	0.9 (±0.2)	0.7 (±0.1)	0.8 (±0.2)	0.6 (±0.1)
	7	7 (±1)	4 (±1)	6 (±1)	5 (±1)
	26	26 (±5)	31 (±6)	20 (±4)	18 (±4)
	104	101 (±10)	93 (±18)	82 (±16)	101 (±15)
SEC1	0	0	0	0	0
	0.5	0.7 (±0.1)	0.4 (±0.1)	0.6 (±0.1)	0.5 (±0.1)
	8	7 (±1)	2 (±1)	6 (±1)	3 (±1)
	31	30 (±4)	48 (±10)	15 (±2)	30 (±6)
	125	126 (±19)	141 (±21)	126 (±19)	96 (±19)
SED	0	0	0	0	0
	0.8	0.9 (±0.2)	0.6 (±0.1)	0.7 (±0.1)	0.5 (±0.1)
	6	5 (±1)	3 (±1)	6 (±1)	2 (±1)
	27	35 (±7)	45 (±9)	17 (±3)	18 (±4)
	107	95 (±19)	70 (±14)	72 (±14)	69 (±11)
SEE	0	0	0	0	0
	0.1	0.17 (±0.04)	0.21 (±0.04)	0.11 (±0.02)	0.18 (±0.04)
	3	3 (±1)	3 (±1)	2 (±1)	5 (±1)
	13	16 (±3)	18 (±4)	11 (±2)	16 (±3)
	50	62 (±12)	55 (±8)	46 (±7)	47 (±7)
SEG	0	0	0	0	0
	1.6	1.7 (±0.3)	1.5 (±0.3)	1.5 (±0.3)	1.7 (±0.3)
	12	11 (±2)	7 (±1)	8 (±1)	10 (±2)
	50	45 (±5)	48 (±10)	25 (±4)	24 (±4)
	200	192 (±29)	374 (±75)	243 (±37)	264 (±40)
SEI	0	0	0	0	0
	2	1.3 (±0.3)	1.5 (±0.3)	1.7 (±0.3)	1.5 (±0.3)
	18	15 (±3)	9 (±1)	17 (±3)	12 (±3)
	72	70 (±14)	55 (±11)	57 (±11)	
	286	320 (±60)	261 (±27)	251 (±35)	

[a] All concentrations are shown in ng/mL.

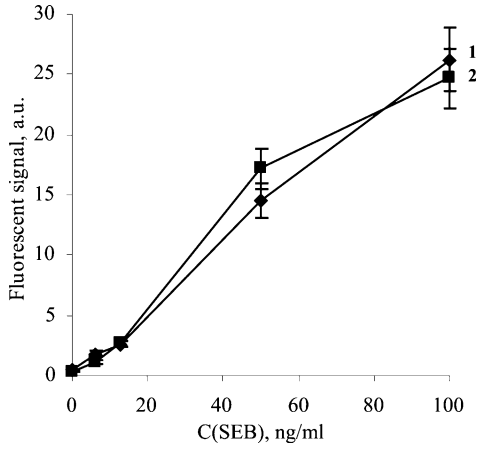

Figure 6. Comparison of calibration curves obtained by sandwich immunoassay of SEB on gold-coated biochips (1, time of analysis: 2 h) and on standard glass slides (2, time of analysis: 17 h).

SEs. The results of this experiment are shown in Table 2. For calculations, calibration curves were obtained using standard solutions of SEs in buffer as described in the Materials and Methods. To avoid false positive results, food samples without added SEs were exactly like spiked samples, and the results of the measurements of SEs levels were considered to correspond to zero concentrations.

The data shown in Table 2 represent the averages of three measurements for each sample; variation coefficient for these measurements did not exceed 20%. In addition, neither positive nor false negative data were obtained. These results demonstrate that the method of simultaneous quantitative analysis of seven SEs using biochips developed in this work allows accurate and reliable assessment of their content in food samples.

Express Analysis of Staphylococcal Enterotoxins Using Metal-Coated Biochips. The time required for the analysis of potential contamination with toxins is an important factor in the evaluation of any new method. Earlier, we have shown that it is possible to significantly shorten the time of immunoassay on hydrogel biochips without any loss of sensitivity using reflecting substrates for biochip manufacture. Fluorescent signals from hydrogel elements with immobilized proteins or oligonucleotides are amplified on biochips with a mirror surface as compared with transparent biochips.[39,40] This effect could be explained by phenomena of geometric optics. The use of hydrogel biochips manufactured on reflecting surfaces enhances the ratio of positive fluorescent signals to background fluorescence. As a result, it

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becomes possible either to increase the sensitivity of the immunoassays or to shorten the analysis.

For express analysis of SEs, the biochips were prepared on gold-plated glass slides (see Materials) or on regular glass slides, and a standard one-step sandwich immunoassay of seven SEs was performed. The procedures differed by the time of incubation: it was 17 h on transparent biochips and 2 h on gold-plated biochips. A typical example of such an experiment, obtaining data for the calibration curve for SEB, is shown in Figure 6. These data illustrate that the use of biochips on reflecting metal-coated substrates shortened the time of the simultaneous quantitative immunoassay of seven enterotoxins from 17 to 2 h without loss of sensitivity.

In summary, we have demonstrated the feasibility of the new approach to the development of test systems for simultaneous quantitative analysis of several toxins. Quantitative detection of the toxins using the developed biochips has been successfully tested on several dairy products which are the most common source of contamination with SEs.

ACKNOWLEDGMENT

The authors are grateful to E. Ya. Kreindlin and O. V. Moiseeva for the help in microchip manufacturing and R. A. Urasov for the elaboration of the programs for processing the experimental data. The authors especially thank Alexander Kolchinsky (Champaign, IL) for his critical comments and assistance during the preparation of the manuscript. This work has been supported in part by Biopatogen State Program/07 538/Natl. Technol. Foundation/K 11 09 2007/Fed.Target Program National Technology (Rus.).

Received for review June 24, 2010. Accepted September 1, 2010.

AC1016634