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Development of Staphylococcal Enterotoxin B Detection Strips and Application of SEB Detection Strips in Food

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Background: To present a novel sensitive method, named lateral flow assay (LFA), for detecting staphylococcal enterotoxin B (SEB), and to investigate its application to food samples. Methods: LFA was performed based on an immunochromatographic procedure that utilizes antigen-antibody properties and double-antibody sandwich format on a porous nitrocellulose membrane. **Results:** Results from a series of sensitivity and specificity tests showing that LFA can successfully identify SEB in a wide variety of food samples in 10 min at the level of 10 ng/mL. **Conclusions:** This study has proved that the SEB strip assay plays an excellent role for SEB detection in food specimens and may prove particularly important as an early warning tool for prevention of food poisoning in consumers.

Key words: *Staphylococcus aureus*, staphylococcal enterotoxin B, colloidal gold, lateral flow assay

INTRODUCTION

Staphylococci are gram-positive bacteria that cause a wide range of diseases. They can cause illness directly by infection or indirectly through their products, such as the toxins that are responsible for food poisoning and toxic shock syndrome (TSS).[1] Various types of staphylococcal enterotoxins have been identified. Among these, five major antigenic types (SEA, SEB, SEC, SED, and SEE) are categorized as classical types,[2] whereas the others (SEG-SEU) are grouped as newly developed types.[3-5] The low-molecular-weight staphylococcal enterotoxins (27 to 30 kDa) are the main cause of gastroenteritis resulting from ingestion of contaminated foods. Ingestion of any one of these enterotoxins may induce emesis, abdominal cramps, and diarrhea within a few hours.[6] Although the illness is typically mild, fatalities may occasionally occur in weakened, elderly patients (50% of the lethal dose was calculated to be 0.02 μg/kg by both inhalation and intravenous routes).[7-8] Moreover, hospital-acquired infection caused thousands of deaths in one year.[9] Previous studies revealed that as little as 100 ng of SEB may make a person ill with symptoms of classic food poisoning (nausea, vomiting and/or diarrhea).[10]

It is also well known that SEB can not only function as a super-antigen that has the potential to be a biological warfare agent for contaminating food or water supplies,[11] but also be a prevalent cause of food poisoning in the United States and other countries.[12] In 1974, Reiser *et al*. claimed that in food industries, detection limit of SEs should be under 125 ng per 100 g of food.[13] Thus, the need to develop a highly sensitive and specific detection system for monitoring the toxin is warranted.

Numerous investigators have reported highly sensitive SEB immunoassay methods, such as enzyme-linked immunosorbent assay (ELISA),[14,15] enzymatic bionanotransduction,[16] radioimmunoassay (RIA),[17,18] polymerase chain reaction (PCR),[19,20] and even the chromogenic macroarray system.[21] These techniques are all sensitive and specific. However, they are either time-consuming (e.g., overnight incubation), requirement of special equipment (ELISA reader) and highly trained personnel, or involve complex assay procedures, thus limiting their use in the field. To overcome these drawbacks, an ideal method is urgently warranted for rapid and sensitive detection of the toxin in foods, and the lateral flow assay (LFA) seems to be a good candidate that meets these requirements.

The LFA, also called the immunochromatographic assay or strip assay, has been used as a diagnostic tool for several years.[22-25] This technique is based on an immunochromatographic procedure that utilizes antigen-antibody properties and enables rapid detection of the analyte.

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Although the sensitivity of the LFA (10-20 ng/mL)[26] was found to be much lower than that of ELISA assays (100-1,000 pg/mL),[15] it offers several benefits, such as a user-friendly format, rapid results, long-term stability over a wide range of weather conditions, and relatively low manufacturing costs. These characteristics render it ideally suited for on-site testing by untrained personnel.

Based on this assay, recent studies had been accomplished in detecting ricin[25] and sulfonamides[27] with monoclonal antibodies, and in botulinum neurotoxin with a polyclonal antibody (Pab).[28,29] In this research, a rapid and sensitive SEB test strip for detecting SEB was developed. By investigating the presence of SEB in various types of food samples, SEB test strip as a tool for detecting SEB was evaluated. The findings thus obtained provide evidence that the LFA is an excellent tool for SEB detection in food samples.

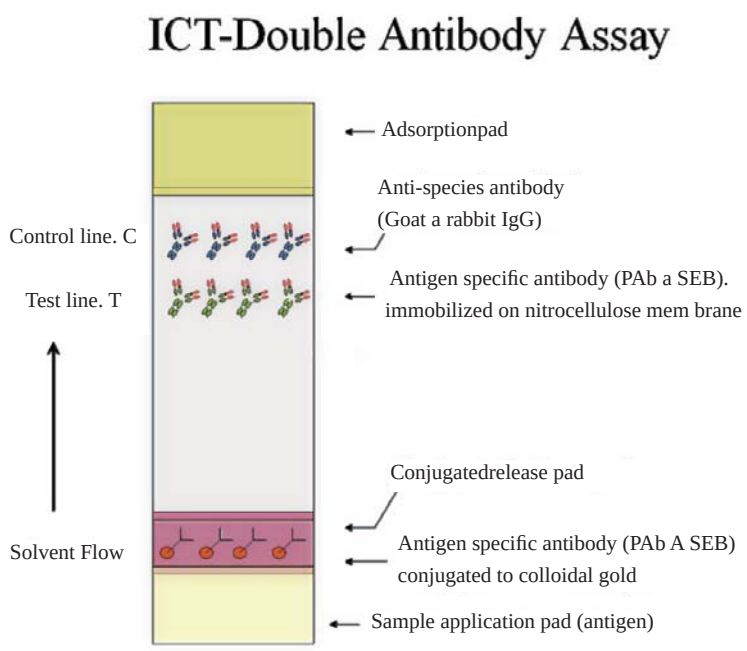

Fig. 1. Schematic description of immunochromatographic test device. The device comprises a nitrocellulose membrane, a sample pad, a conjugate release pad and an adsorption pad. Sample and conjugate pads provide adequate mixture of the liquid sample and colloidal gold particles and serve as a reservoir for the assay reagents. The adsorption pad adsorbs extra liquid and ensures sufficient flow through.

MATERIALS AND METHODS

Materials

Purified SEB toxin was purchased from Sigma-Aldrich, St. Louis, MO, USA. The frozen toxin was thawed, diluted in phosphate-buffered saline (PBS), and stored at 4 °C. Other SE toxins (A~E) were obtained commercially from Denka Seiken Co., Ltd. (from a reversed passive latex agglutination (RPLA) kit; Tokyo, Japan) and stored at 4 °C according to the manufacturer's instructions.

Anti-SEB IgG was purified from anti-SEB sera by thiophilic gel (T-gel, Pierce, Rockford, IL, USA), while the anti-SEB sera were obtained from SEB-immunized rabbits (New Zealand). High-flow nitrocellulose (NC) membranes (HiFlow Plus HFB18004) were commercially obtained from Millipore Co. Ltd., Ireland; glass fiber conjugated pad (AccuFlow™ G), sample application pad (#12-S) and reagent adsorption pad (470 Zuschnitte/Cuts) were purchased from Schleicher & Schuell GmbH (Dassel, Germany). Goat anti-rabbit IgG were obtained commercially from Sigma.

Preparation of colloidal gold probes and conjugation of antibody

A modified citrate reduction method was employed to prepare colloidal gold probes.[30] The size of colloidal gold particles was analyzed by transmission electron microscopy (TEM) (H-600; Hitachi Instrument Co., Tokyo, Japan). The pH value of the colloidal gold solution (1%, w/v) was adjusted to 8.5 with NaOH and the particles can be stored at 4 °C for several weeks in a dark glass bottle.

For antibody conjugation, T-gel purified anti-SEB IgG (0.2 ml; 2 mg/mL; in 5 mM potassium carbonate buffer; pH 8.5) was added to 40 ml pH-adjusted colloid gold solution. The mixture was gently mixed and centrifuged for 30 min (4 °C, 1550 x *g*; 8178 swing-out rotor, Labofuge 400R; Heraeus Instruments). After centrifugation, the colloid gold probes were suspended in 20 mM Tris/HCl, pH 8.2 containing 1% BSA. After the optical density was adjusted to 5.0 at O.D.520, the anti-SEB IgG-coated colloidal gold nanoparticles were ready for use.

Preparation of immunochromatographic test strips

The schematic description and composition of the immunochromatographic test device have been previously described.[25,29,31] Briefly, two antibodies, anti-species antibody and antigen specific antibody, were separately sprayed onto a NC membrane using a Biodot dispensing apparatus (Biodot XYZ 3000 1414) to form a control

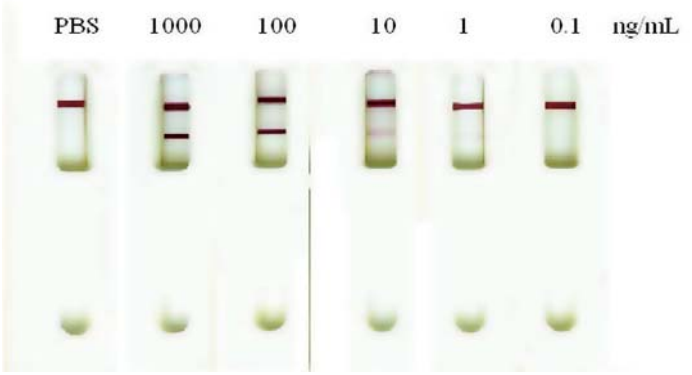

Fig. 2 Immunochromatographic assay of SEB. A series of dilutions (1,000 - 0.1 ng/ml) of SEB were prepared in PBS. The detection limit of SEB toxin was 1 ng/ml. False positive was not detected in the absence of SEB.

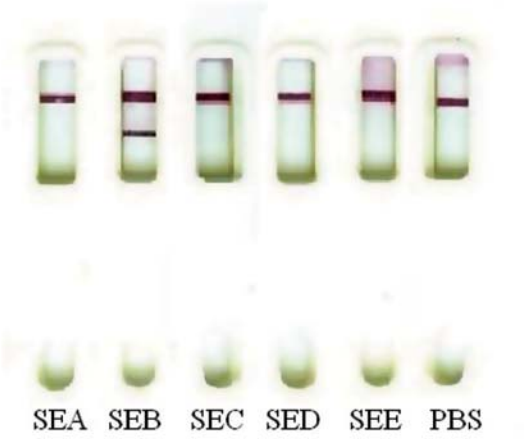

Fig. 3 Cross-reactivity assay of SEB test strip. Samples containing SE toxins (SEA-SEE, 100 ng/ml each) were applied to SEB test strips, and only SEB produced a red band in the test region. Non-specific binding was not visualized in the absence of SEB.

region (C) and a test region (T). The membrane was then mounted onto an adhesive paper plate (2.44×11.81 inches, Adhesives Research Inc., Taiwan) with an additional reagent adsorbent filter, colloidal gold conjugate release pad, and a sample application pad (Fig. 1). The plate was then cut into 5-mm-wide strips (by Biodot CM4000) and then mounted in a plastic cassette to complete the fabrication of the device.

Sensitivity and specificity tests

For the sensitivity assay, 100-micro-liter samples with different amounts of SEB (1,000-0.1 ng/mL) were applied onto the strip. For the specificity test, samples containing various enterotoxins (SEA, SEB, SEC, SED, and SEE) were all assayed by the SEB test strip.

Food sample preparation

A variety of food samples were divided into liquid, solid, and semi-solid categories. Each component was tested separately. For liquid foods, 5 mL of the test sample was fully mixed with pure SEB first (final concentrations, 100 ng/mL); and the mixture was then incubated at room temperature (25 °C) for 30 min. Samples were then centrifuged at 3,000×*g* (8178 swing-out rotor, Labofuge 400R) for 30 min at 4 °C to remove solid particles. Subsequently, 500 μL of the supernatant was carefully mixed with 500 μL of 0.01 M phosphate-buffered saline (PBS; pH 7.4) and stored at 4 °C until use.

For semi-solid and viscous foods (e.g., honey and ice cream), 10 g of each sample was first diluted with the same volume of 0.01 M PBS (wt/vol); 5 mL of the mixture was then spiked with SEB (final concentration, 100 ng/mL) and homogenized with a blender to generate a homogeneous suspension. Samples were then incubated at room temperature in the same manner as liquid samples. After incubation, the samples were diluted to a ratio of 1:5 in PBS and centrifuged as previously described to remove solid particles and/or the lipid layer. Then, as before, 500 μL of the supernatant was thoroughly mixed with 500 μL of 0.01 M PBS, and stored at 4 °C before performing the assay. For solid food samples, 10 g of each sample was first chopped into tiny pieces, diluted with 0.01 M PBS (1:1, wt/vol), and homogenized with a blender. Afterwards, 5 mL of the slurry was fully mixed with SEB, followed by incubation, dilution, and centrifugation as before. Finally, 500 μL of 0.01 M PBS was added to an equal volume of the supernatant, and the mixtures were stored at 4 °C before use. Before analysis, all samples were left at room temperature for 1 h to allow the toxins to interact with the food matrix.

RESULTS

Sensitivity of SEB test strip

SEB toxins of different concentrations were assayed separately by the SEB test strip. Results were determined by the appearance (positive result) or absence (negative result) of a red line in the test area, under the condition that a red line could be visualized in the control area. Analyses were accomplished in less than 10 min, and the

Table 1 SEB Test Strips for Food Analysis

Food Matrix		No SEB Spiking				Spiked with SEB			
		undiluted		diluted		undiluted		diluted	
		10 min	30 min	10 min	30 min	10min	30 min	10 min	30 min
Liquid									
	orange juice	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	apple juice	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	grape juice	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	plum juice	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	coffee	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	cola	−	−	−	−	+	+	+	+
	black tea	−	−	−	−	+	+	+	+
	soybean milk	−	−	−	−	+	+	+	+
	bottled water	−	−	−	−	+	+	+	+
	fruit-vegetable juice	−	−	−	−	+	+	+	+
Semi-solid									
	honey	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	ice cream	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	yogurt	ND[a]	−	−	−	ND[a]	+	+	+
	ketchup	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	mayonnaise	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
	mustard	ND[a]	ND[a]	−	−	ND[a]	ND[a]	+	+
Solid									
	french fries	ND[a]	−	−	−	ND[a]	+	+	+
	baked macaroni	ND[a]	−	−	−	ND[a]	+	+	+
	burg-meat	ND[a]	−	−	−	ND[a]	+	+	+
	fried chicken	ND[a]	−	−	−	ND[a]	+	+	+
	smoked salmon	ND[a]	−	−	−	ND[a]	+	+	+
	tempura	ND[a]	−	−	−	ND[a]	+	+	+
	sausage	ND[a]	−	−	−	ND[a]	+	+	+
	cured beef	ND[a]	−	−	−	ND[a]	+	+	+
	carp	ND[a]	−	−	−	ND[a]	+	+	+
	prawn ball	ND[a]	−	−	−	ND[a]	+	+	+

ND: not detectable

detection limit of SEB toxin was 10 ng/mL (Fig. 2). All the results were highly reproducible, and no false positive results were obtained throughout the assay.

Cross-reactivity of SEB test strip

The specificity of the SEB strip was examined. Samples contained five different SEs (A through E) were separately diluted in PBS buffer and applied onto the strips simultaneously. When 100 ng/mL of each toxin was tested, only SEB produced a red line in the test region but not the rest four SEs (Fig. 3), which suggests a clear specificity of the SEB strip.

Evaluation of SEB test strip for food analysis

Twenty-six food samples of different categories, all purchased from a local market, were applied to SEB strips for SEB detection (Table 1). Samples that were too viscous or too thick needed to be diluted first to a 1:10

ratio in 0.01 M PBS. Table 1 shows that in 10 min, all diluted samples spiked with SEB toxin present positive results, whereas samples without SEB provide negative results.

Of all 26 food samples tested, only 6 showed positive results within 10 min in their undiluted forms. Some of the liquid samples such as cola, black tea, bottled water, and energy drinks showed positive results within 10 min. Although several liquid samples, such as apple juice, orange juice, and grape juice, were filtered through a Whatman No. 4 filter paper (Whatman Laboratory Division, Springfield Mill, Kent, UK), the strip membrane still turned slightly red, olive yellow, and purple, respectively, making it difficult to read the results after 10 and 30 min of assay time.

Most of the viscous or semi-solid samples (e.g., ice cream or honey) did not show detectable results in their undiluted form (with/without SEB) because they were too viscous to reach the detection window. These results remained unchanged even after 30 min (except for yogurt). However, the assays were accomplished within 10 min when the samples were diluted with PBS, and the results show a clear positive (with SEB) or negative (without SEB). On the other hand, undiluted solid food samples also failed to show detectable results in the first 10 min, but specific results were observable in 30 min. In the diluted form, all samples in this category showed clear results within 10 min of assay time.

DISCUSSION

The heat-stable staphylococcal enterotoxins (SEs) pose a serious threat to the food industry and human health. Unfortunately, there is no effective vaccine or specific anti-toxin to treat SEB poisoning. Therefore, a simple and rapid method for screening SEB toxin would provide an important tool to prevent food poisoning.

The results presented in this paper verified that an SEB immunochromatographic assay was developed for SEB detection. The criterion for reactivity was judged by the formation of red lines in both test and control areas within 10 min after sample addition and the detection limit was 10 ng/mL. In contrast, Pab-based SEB test strips had no cross-reaction with other SEs (SEA to SEE) even when 1,000 ng/mL of toxins was tested, suggesting that these SEB strips have high specificity for SEB.

In strip analyses, it was not possible to measure the quantity of specific anti-SEB IgG contained in the T-gel-purified IgG because the purified IgG was total IgG rather than the specific IgG. Nevertheless, good specificity and sensitivity were still obtained in the SEB strip test when using this total IgG as a reagent. On the other hand, previous researches discovered that in LFA, some simulated components would interfere with the antigen-antibody reaction and result in a weak signal,[32-33] while in this study, the strips are still with sufficient sensitivity for detecting SEB in food.

Several steps are involved in the immunochromatographic assay. First, two antibodies are immobilized on the membrane: a specific antibody against the test antigen, and an antibody against the animal species IgG from which the pathogen antibody is derived. The antibodies are strongly adsorbed to the membrane and remain attached to the surface throughout the procedure. Second, any remaining protein-binding sites on the membrane are blocked by chemicals to reduce the nonspecific binding of antibody or antigen to the membrane. Third, when samples are applied to the test device, the liquid mixture migrates along the NC membrane. As a result, the antigen sample would react first with the antibody conjugated on colloidal gold probes and then with the two antibodies bound on the membrane, thus forming a visible line(s). The color intensity is proportional to the concentration of the antigen. To avoid nonspecific binding and to prevent undesired cross-reactivity of the antibodies with the test line, all the procedures require conscientious development and optimization of various capture lines.

In the second part of this study, solid and semi-solid samples were used as slurries prepared by combining and homogenizing equal parts of food sample and PBS buffer. Since the undiluted high-viscous-content foods were unable to reach the detection window in 10 min, an extra 20-min treatment was given to allow the samples to flow forward to the detection window. However, previous experience with strip assay revealed that red lines appearing in the test zone 20 min after sample addition were not consequential and should be ignored. Therefore, the results of solid samples (plus the semi-solid yogurt) in undiluted form are regarded as invalid. Only the diluted samples, in which red lines appeared in both test and control areas in less than 10 min, were considered significant.

As shown in Table 1, all food samples in diluted form gave obvious positive results within 10 min. The assay results were sufficiently robust, indicating that SEB strip can successfully detect SEB in different simulated food samples. The detection limits of the SEB strip in various food matrices ranged from 10 to 100 ng/mL (data not shown). Previous studies have reported that as little as 250 ng of SEB may make a person ill,[10,33] and the SEB

strip seems to have enough sensitivity to meet the requirements for detecting the toxin before food poisoning occurs.

This study has described a general assay method for the detection of enterotoxin SEB. The assay is easy to perform, shows no cross-reactivity and gives results much more quickly than ELISA or radioimmunoassay. Furthermore, the results can be read directly by naked eyes. Hence, the strip assay is not only a highly specific and sensitive method for detecting SEB, but also a powerful early warning tool for on-site surveillance to prevent food poisoning in consumers.

CONCLUSION

In summary, this study developed successfully a polyclonal-based strip test kit that can rapidly detect SEB without cross-reaction with antibodies against other SEs; and demonstrated the ability of the SEB strip to detect SEB in complex food matrices. Experimental results show that the strip assay is sufficiently sensitive to support the detection of SEB contamination in food samples.

DISCLOSURE

All authors declare that this study has no conflict of interest.

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