A Novel ELISA Format for the Rapid and Sensitive Detection of Staphylococcal Enterotoxin A

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Staphylococcal food poisoning is one of the leading causes of bacterial food poisoning each year. Detection kits for staphylococcal enterotoxins are commercially available and the assays can require from one and a half to twenty-four hours to complete with detection limits ranging from 0.5 to 2 ng enterotoxin per gram of food. We have successfully demonstrated a microsphere-packed capillary (MPC) ELISA for the detection of staphylococcal enterotoxin A (SEA) and have compared it to two commercially available kits. The MPC assay detected a lower amount of SEA in ham, chicken, cheese, and bean sprouts than either of the two commercially available kits. In addition, the novel MPC assay was completed in less than ten minutes, as compared to three and twenty-four hours for the two commercially available kits. This research also demonstrated that the MPC ELISA can contain integrated positive and negative controls and has the potential to simultaneously detect and identify multiple enterotoxins.

Key words: Staphylococcus aureus; enterotoxins; immunoassay; microspheres; capillary

Staphylococcal food poisoning is the second leading cause of bacterial food poisoning in the United States. It affects 1.2 million people annually, resulting in a total economic burden of 1.5 billion dollars. Staphylococcal bacteria secrete proteinaceous enterotoxins A through E, and H (SEA through SEE, SEG, and SEH) and as little as 0.1 μg of enterotoxin can produce the clinical symptoms of staphylococcal food poisoning which include nausea, retching, vomiting, and diarrhea.

The symptoms of staphylococcal food poisonings are almost entirely a result of enterotoxin formation before ingestion because under normal conditions, almost no S. aureus growth occurs in the intestines. Staphylococcus aureus enterotoxins (SEs) are a heterogeneous group of heat-stable, water soluble, single-chain, globular proteins with molecular weights between 28 and 35 kDa. Seven distinct enterotoxins have been immunologically identified including: SEA, SEB, SEC1, SEC2, SEC3, SED, and SEE. However, other enterotoxins exist. A gene [seg +] encoding an uncharacterized SE (termed SEG) has been identified from S. aureus FRI-572, although this protein has not been isolated and purified for full enterotoxin characterization. Recently, a new enterotoxin, SEH, was identified and characterized. It is antigenically different from all seven previously mentioned toxins.

Staphylococcal enterotoxins are extremely potent and SEA is one of the most commonly involved in staphylococcal food poisoning outbreaks. The most common foods associated with staphylococcal food poisoning are cold cooked meats such as ham, turkey, and roast beef, fermented meats such as salamis, and bakery products such as cream confections.

The presence of these toxins on such a wide variety of foods has driven the development of analytical tools to identify the type and extent of contamination. Several commercially available diagnostic kits exist for the detection of SEs, the most prominent are listed in Table 1. Most of the kits are based on ELISA technology except for RPLA (agglutination) and can detect enterotoxins A through E except SET-EIA and RPLA. Only three of the six kits can differentiate toxin type and most kits require special equipment, especially VIDAS which requires a considerable initial investment. All kits can detect at least 1 ng/ml SEs but they all have assays that require at least 1.5 hours and some as long as 24 hours or more to complete. It should be noted that TECRA is the only kit that has been adopted as an AOAC International Official Method.

The purpose of this study was to develop a rapid, sensitive, and disposable ELISA format that would ultimately permit the simultaneous detection and identification of multiple enterotoxins.

Materials and Methods

Coating Microspheres with Capture Antibody. Five hundred milligrams of polystyrene (0.5% divinylbenzene) microspheres (Polysciences, Inc., Warrington, PA) ranging in diameter from 30 to 60 μm were placed in a 50-ml conical polypropylene centrifuge capillary. Twenty ml of 0.1 m sodium borate, pH 8.5, was added and the mixture was sonicated for 20 minutes to disperse the microspheres. The suspension was centrifuged for 5 minutes at 4000 rpm in a Hermle Z 360 K centrifuge to pellet the microspheres. The supernatant was decanted and discarded. The microspheres were washed twice more with 20 ml of 0.1 m sodium borate, pH 8.5, with centrifugation between each wash to exchange the buffer. The washed and pelleted microspheres were reconstituted in 20 ml of 0.1 m sodium borate, pH 8.5,
Table 1. Commercially Available Diagnostic Kits/Systems for the Detection of Staphylococcal Enterotoxins

<table>
<thead>
<tr>
<th>Diagnostic Kit/System</th>
<th>Detection Method</th>
<th>Enterotoxins Detected</th>
<th>Differentiate Toxin Types</th>
<th>Sensitivity (ng/ml)</th>
<th>Assay Time (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIDASCREEN</td>
<td>ELISA</td>
<td>A to E</td>
<td>Yes</td>
<td>0.2 to 0.75</td>
<td>3</td>
</tr>
<tr>
<td>SET-EIA</td>
<td>ELISA</td>
<td>A to D</td>
<td>Yes</td>
<td>0.1 to 1.0</td>
<td>20</td>
</tr>
<tr>
<td>TECRA</td>
<td>ELISA</td>
<td>A to E</td>
<td>No</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>TRANSIA</td>
<td>ELISA</td>
<td>A to E</td>
<td>No</td>
<td>0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>RPLA</td>
<td>Agglutination</td>
<td>A to D</td>
<td>Yes</td>
<td>0.5 to 1.0</td>
<td>20 to 24</td>
</tr>
<tr>
<td>VIDAS</td>
<td>ELFA</td>
<td>A to E</td>
<td>No</td>
<td>1</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Kit manufacturers: Ridascreen; R-Biopharm GmbH, Darmstadt, Germany; SET-EIA, Diagnostische Laboratorien, Bern, Switzerland; TECRA, Bioenterprises Pty. Ltd., Roseville, New South Wales, Australia; TRANSIA, TRANSIA-DIFFCHAMPS S.A. Lyon, France; RPLA, Denka Seiken Co; Ltd., Tokyo, Japan; VIDAS, bioMerieux Vetik, Inc., Hazelwood, Missouri.

and 0.4 mg of anti-SE IgG (Toxin Technologies, Sarasota, FL) dissolved in deionized water was added. The antibody/microsphere suspension was incubated overnight at room temperature with gentle end-to-end mixing to adsorb the antibody onto the surface of the microspheres. The following morning, the suspension was centrifuged to pellet the microspheres; the supernatant was discarded. The antibody-coated microspheres were then blocked by resuspending them in 20 ml of 10 mg/ml BSA in 0.1 M sodium borate, pH 8.5, and incubated at room temperature with end-to-end mixing for 30 minutes. Following centrifugation and removal of the supernatant, the pellet was washed twice more with 20 ml of 10 mg/ml BSA in 0.1 M sodium borate, pH 8.5, with centrifugation between each wash to exchange the BSA solution. The resulting coated and blocked microspheres were resuspended in 10 ml of 10 mM PBS, pH 7.4, containing 10 mg/ml BSA, 0.1% NaN3, and 5% glycerol (storage buffer) and stored at 4°C. Using a similar protocol, microspheres were also coated with normal sheep IgG or BSA to be used as negative control microspheres in the MPC assay.

Packing the Glass Capillary with Microspheres. A 1.2 m length of Pyrex glass tubing (ID = 1.8 mm, OD = 3.0 mm) was cut into 6 cm segments with a glass tubing cutter. A small piece of cotton was put into the capillary near one end to form a 2 to 3 mm plug. Two hundred microliters of the 50 mg/ml (10 mg) normal IgG coated polystyrene microspheres was added to the capillary with a 1-ml tuberculin syringe fitted with a small section of Tygon tubing to form a tight junction between the syringe and the glass capillary. A 200-μl volume of 10 mM PBS-Tween, pH 7.4, was then added to pack the microspheres against the cotton plug, thereby forming a 2 to 3-mm section of microspheres. The normal sheep IgG coated microspheres served as a negative control region in the MPC assay. Using the 1-ml tuberculin syringe fitted with Tygon tubing, 200 μl of the 50 mg/ml (10 mg) anti-SEA coated polystyrene microspheres was added to the capillary and packed with the subsequent addition of 200 μl of 10 mM PBS-Tween, pH 7.4. This 2 to 3-mm section will ultimately bind SEA in the sample and will serve as the test region. Then 200 μl of the normal IgG coated polystyrene microspheres were added again followed by another piece of cotton. The resultant regions in the capillary are in the following order: 1) cotton plug, 2) negative control region, 3) test region, 4) negative control region, and 5) cotton plug.

This glass capillary packing method describes the simplest version of the new assay method. More complex packing schemes involving more layers were done and simply involve repeating some of the steps described above using microspheres coated with different capture antibodies.

**ELISA in the MPC.** The innovation, as described in Fig. 1, is a new and unique ELISA format consisting of polystyrene microspheres coated with either anti-SEA IgG or normal sheep IgG packed in an alternating arrangement in a glass capillary between two cotton plugs (cotton plugs not shown). The assay is done by passing the typical sandwich ELISA solutions (sample, wash buffer, conjugate, and wash buffer) through the packed capillary in the classical order. Instead of culminating the assay with a soluble substrate, a precipitating substrate is added. In the microsphere-packed capillary

![Fig. 1. The MPC ELISA Format.](attachment:image)

The MPC consist of antibody coated microspheres sequentially packed into a glass capillary. Classical ELISA solutions are added in the correct order to the MPC culminating with a precipitating substrate to yield a localized color development.
(MPC) described in Fig. 1, SEA present in the sample only binds to the microspheres in the middle section. Upon addition of the antibody/enzyme conjugate followed by the chromogenic precipitating substrate, a signal develops only in the region where the entire sandwich complex is present. The precipitating property of the substrate traps the colored product in the interstitial spaces between the microspheres, generating a localized signal.

A 200 μl volume of sample (buffered at pH 7.4) was put into the MPC with a 1 ml tuberculin syringe fitted with a 1 cm segment of Tygon tubing to interface the capillary and the syringe. The sample was allowed to incubate for 2 minutes at room temperature. The microspheres in the capillary were then washed with 200 μl of 10 mM PBS (0.05% Tween), pH 7.4. Two hundred microliters of a 1:200 dilution of anti-SEA IgG/HRP conjugate (Toxin Technologies, Sarasota, FL) was then added to the capillary. The sample was allowed to incubate for 2 minutes at room temperature. Following another wash with PBS-Tween, 200 μl of either metal enhanced 3,3’-diaminobenzidine tetrahydrochloride (ME/DAB, Pierce Chemical Co., Rockford, IL) or 3,3’5,5’-tetramethyl benzidine (TMB, Pierce Chemical Co., Rockford, IL) was added. The color development occurred within seconds. The capillaries were then prepared for long-term storage by washing them with water followed by an acetone rinse. Air was then flushed through the capillary to evaporate the acetone. Capillaries have been stored at room temperature for at least five months with no noticeable fade in color.

Spiking Food. To 10 g of ham, cheese, chicken, and bean sprouts were added 10 ng of SEA in 100 μl PBS and a second set were left unspiked as a negative control. Both sets were allowed to set at room temperature for one hour. The samples were then liquefied in a blender with an equal volume of PBS. The mixture was centrifuged at 4,000 rpm in a Hermle Z360 centrifuge. The supernatant was tested for the presence of SEA.

Measuring Results. The signal from the MPC assay can be evaluated visually or can be more precisely measured with a desktop scanner or even a more sophisticated densitometer. In this research, a Hewlett Packard ScanJet 3c scanner attached to an IBM compatible PC and Image PC (an image analysis software package downloaded from the NIH Image Home Page, http://rsb.info.nih.gov/nih-image/) was used to measure the results. The capillaries are scanned into the software as a black and white bitmap and the software assigns individual pixels in the image a value from 0 (white) to 255 (black). The user manually selects the region that is to be measured and the software averages all the pixels in the region and returns a gray-scale color value from 0 to 255. It has been estimated that the standard error in the grey scale color value ranging from 5 to 20%.

Results and Discussion

In most ELISA-based detection formats, the assay is done in a well within a 96-well microtiter plate; this format will subsequently be referred to as a well-based format. The RIDASCREEN, TECRA and SET-ELISA kits use a well-based enterotoxin detection format in their kits. These and all other well-based kits suffer from long incubation times and a color development step as long as 30 minutes. This is not surprising since the rate of protein binding in solution to their target protein absorbed onto the surface of the well is primarily diffusion controlled. Diffusion rate limitations of biospecific reactions at solid surfaces have been shown experimentally for a variety of interactions including enzyme-substrate reactions, binding of cholera toxin to ganglioside GM1, protein adsorption, and binding of polyclonal antibodies to protein antigen.

TRANSIA and VIDAS have attempted to overcome this diffusion limitation by doing the ELISA in a cylindrically shaped reaction vessel (known as the tube in the TRANSIA kit and the solid phase receptor or SPR in the VIDAS system). The higher surface area to volume ratio of the tube-based detection formats decreases the distance biomolecules must travel to bind to antibodies adsorbed on the surface. This minor change in the dimensions of the assay vessel reduces the incubation times, thereby, generating results in half the time required by the well-based formats (see Table 1).

Demonstration of the MPC ELISA

A photograph of three MPC’s appears in Fig. 2 demonstrating the detection of 1 ng/ml SEA in PBS. The entire ELISA in the MPC was completed in less than 10 minutes. The figure shows three capillaries packed with a small amount of anti-SEA coated polystyrene microspheres. On each end a cotton plug holds the microspheres in place. The top and middle capillaries are negative controls. To the top capillary was added PBS instead of SEA and to the middle capillary was added PBS instead of anti-SEA/HRP conjugate. The bottom capillary is the test capillary to which all components of the assay were include. The dark color present in the bottom capillary is the precipitating ME/DAB substrate trapped in the interstitial spaces of the packed microspheres representing a positive result. Conversely, the lack of dark color in the top and middle capillaries

Fig. 2. Color Development in the MPC ELISA.

An MPC ELISA demonstrating the detection of SEA in PBS. The top and middle capillaries are negative controls while the bottom capillary represents the detection of 1 ng/ml SEA in PBS. The entire ELISA was completed in less than ten minutes.
represents a negative result. (Negative control regions within each capillary as described in Fig. 1 were not included in the capillaries shown in Fig. 2.)

A modest attempt was made to optimize the ELISA. Variables such as blocking agent (BSA, nonspecific sheep IgG, BLOTTO), washing solution (PBS, PBS-Tween, 10 mg/ml BSA in PBS), and washing solution, conjugate, and substrate volumes (200, 400, 800 μl) were manipulated with no significant difference in assay performance (data not shown).

Sensitivity of the MPC ELISA

The extremely high surface area to volume ratio of the MPC ELISA was anticipated to yield a more sensitive ELISA with the possible drawback of a higher background signal from non-specific interactions. An experiment was done to measure the sensitivity of the assay toward SEA in PBS. Sixteen capillaries were packed, with each capillary containing a test region (microwells coated with anti-SEA) and a negative control region (microwells coated with BSA). Eight capillaries were used to measure the detection range with TMB substrate while the other eight were used to determine the detection range with ME/DAB. The data from this experiment is shown in Fig. 3 and shows the grey scale color value as a function of SEA concentration. (It should be mentioned that the detection limit for most kits is the highest level tested in Fig. 3.) The background grey scale color value for the negative control regions in all capillaries was ~30, suggesting that the detection limit is ~0.01 ng/ml for ME/DAB and ~0.03 ng/ml for TMB. (A 200-μl sample containing SEA at 0.01 ng/ml contains 2 picograms or ~73 attomoles of SEA). The dependence of the grey scale color value with respect to SEA concentration was greater for ME/DAB than for TMB. This was expected since ME/DAB yields a brown insoluble product which contrasts more with the white control region than the blue insoluble product produced from TMB. Although a scanner and Image PC software was used to measure the results, all positive results were also visible with the naked eye.

Detection of SEA in Foods

The detection of SEA in PBS is easy and convenient, but it is of limited value. Demonstrating the utility of the MPC ELISA to detect SEA requires the actual use of food extracts. Food was spiked with SEA to yield a final concentration of 1 ng/g SEA in the food. The food was homogenized with an equal weight (volume) of PBS. Following centrifugation, 200 μl of supernatant was assayed for SEA. As a control, unspiked food was processed in the same manner. The foods selected for this experiment were cooked ham, raw chicken, and cheddar cheese, all of which are frequently involved in staphylococcal food poisonings. The results from this experiment are shown in Fig. 4.

It is apparent from Fig. 4 that the MPC ELISA can easily detect SEA at 1 ng per gram of food in all three foods tested. The grey scale color values for all three spiked foods and spiked PBS were all within experimental error, suggesting that the MPC ELISA is independent of matrix effects with these three foods. The reason for the higher color value for unspiked PBS when compared to the three unspiked foods is not clear. A possible explanation may be that the complex food matrix reduces nonspecific interactions between the anti-SEA/HRP conjugate and the microwells. The reduction of these nonspecific interactions indirectly results in a lower color value.

Comparison between the MPC ELISA, TECRA, and SET-RPLA

To evaluate the effectiveness of the MPC ELISA to detect SEA in foods, the assay was compared to TECRA and SET-RPLA, two commercially available SEA detection kits. Cooked ham, raw chicken, cheddar cheese, and bean sprouts were spiked with various amounts of SEA and assayed. The results are shown in Table 2.

The results are represented as positive and negative as judged by an observer, ignorant of the identity of the samples, with the naked eye; this is the manner in which both commercially available kits expect results to be evaluated, although a microtiter plate reader can be used.

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**Fig. 3.** Sensitivity of the MPC ELISA.

The dependence of the grey scale color value on SEA concentration using two different precipitating substrates, ME/DAB (circles) and TMB (squares).

**Fig. 4.** Detection of SEA in Food Using the MPC ELISA.

Ham, chicken, cheese and PBS were spiked with SEA at a level of 1 ng SEA per gram of food (or buffer). The figure is a histogram of the grey scale color value for spiked food and the corresponding unspiked controls.
Table 2. Comparison of the Packed Tube Assay with Two Commercially Available Test Kits

<table>
<thead>
<tr>
<th>Food Sample</th>
<th>MPC</th>
<th>TECRA</th>
<th>SET-RPLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA spiked pre-cooked ham at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 ng/g of food</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.0 ng/g of food</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.5 ng/g of food</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>unspiked pre-cooked ham (negative control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SEA spiked raw chicken at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ng/g of food</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>unspiked raw chicken (negative control)</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>SEA spiked cheddar cheese at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 ng/g of food</td>
<td>+</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>unspiked cheddar cheese (negative control)</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>SEA spiked bean sprouts at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 ng/g of food</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2.0 ng/g of food</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>0.5 ng/g of food</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>unspiked bean sprouts (negative control)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

with the TECRA kit.

When the results from Table 2 are compared, the MPC ELISA appears to be more sensitive than either TECRA or SET-RPLA. The sensitivity of TECRA to detect SEA in food extracts reported in Table 2 is as good or slightly better than previously reported data for the kit.22,23 The TECRA kit yielded a false positive with bean sprouts which could be the result of the inherent peroxidase activity in bean sprouts. (The addition of sodium azide to the food extract to eliminate endogenous peroxidase activity was intentionally omitted.) The lack of a false positive result in the MPC assay could be the result of a more thorough manner in which the coated microspheres are washed following sample addition. The values in Table 2 obtained for SET-RPLA do not appear as sensitive as previously reported.22,24-26 How far off the values in Table 2 are from those previous reported is difficult to establish since food spiked with SEA at concentrations between 2 and 10 ng per gram of food were not measured.

Demonstration of Integrated Controls

In commercially available well- or tube-based SEA detection kits, integrated controls are nonexistent. Positive and negative controls must be done separately from samples, occupying valuable wells or capillaries in the kit. If only a few samples are assayed at a time, a considerable number of wells in a 96 well test kit can be consumed running the appropriate number of controls for each assay.

Figure 5 demonstrates the ability of the MPC ELISA to contain integrated positive and negative controls. The three capillaries in Fig. 5 were all packed in the same manner and all contained the following regions (from left to right): BSA coated microspheres (negative control); anti-SEA coated microspheres (test region), BSA coated microspheres (negative control); SEA coated microspheres (positive control); BSA coated microspheres (negative control); cotton plug. The flow was from left to right. The assay was done in the same way for all capillaries, except that to the top capillary was added 1 ng/ml SEB in PBS, to the middle capillary was added 10 ng/ml SEB in PBS and to the bottom capillary was added 1 ng/ml SEA in PBS. In all three capillaries, the positive control region (the colored region in all three capillaries to the farthest right) demonstrated that the anti-SEA/HRP conjugate successfully associated with the SEA coated microspheres generating color upon addition of substrate. In only the bottom capillary did color development occur in the test region; this was expected since SEA was only added to the bottom capillary. The top two capillaries demonstrate that SEB at 1 and 10 ng/ml failed to produce a false positive in the SEA test region. That 10 ng/ml SEB did not produce a false positive in the MPC ELISA is particularly significant since the high surface area to volume ratio of the MPC format had the potential to increase nonspecific binding, thereby exacerbating false positives associated with antigen/antibody cross reactivity.

It has been demonstrated that the MPC ELISA format can successfully detect SEA in spiked foods samples at concentrations equal to or lower than two of the most sensitive commercially available kits. It was also demonstrated that the MPC ELISA can detect SEA at concentrations near 10 pg/ml in PBS, which is at least 10 times lower than the most sensitive kit available. (An attempt to detect picogram per milliliter levels of SEA in foods was not made.) Also, the MPC ELISA can be completed in less than ten minutes and can also include integrated positive and negative controls.

A more complex version of the MPC can be envisioned. It would include a detection region for each of the seven antigenically distinct enterotoxins sequentially packed into a single capillary. This “multiplexed” MPC would permit the simultaneous identification and measurement of all seven enterotoxins, including positive and negative controls, from a single sample.

Acknowledgments

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References


