CHAPTER 15. IMMUNOASSAYS FOR THE DETECTION AND QUANTITATION OF STAPHYLOCOCCAL ENTEROTOXINS FROM MEAT AND POULTRY PRODUCTS AND/OR BROTH CULTURE FLUIDS

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15.1 Introduction

Some strains of coagulase positive *Staphylococcus aureus* are endowed with the genetic capacity to produce certain extracellular proteins which, when ingested, cause a severe gastrointestinal disturbance. These proteins are known as staphylococcal enterotoxins. There are five distinct, major, serological types of enterotoxins currently recognized as significant and they are designated as serotypes A, B, C (C_1, C_2, C_3), D and E. In 1995 a new serotype, SEH, was identified and reported in the literature, however, it's significance to foodborne illness is still undetermined. When an enterotoxigenic strain of *Staphylococcus aureus* becomes established in a food product, environmental growth conditions may become optimum to allow for high proliferation of the organism and resulting production of the enterotoxin. Ingestion of this food usually results in a foodborne illness. For regulatory and epidemiological purposes in investigating foodborne illnesses it is important to be able to recognize the presence and serotype of staphylococcal enterotoxins in a suspect food product.

Recent advances and refinements in the development of immunoassays and immunological reagents, specifically with regard to the staphylococcal enterotoxins, have allowed the completion and implementation of assays for quantitative detection of these toxins. These new assays provide advantages of increased sensitivity, reduced analysis time, and a capability for greater sample number analyses due to the reduction of high labor intensive operations associated with procedures previously employed. The following provides a detailed description of two immunoassay procedures which are to be used by the Field Service Laboratories for the determination of the major staphylococcal enterotoxins in various meat and poultry product samples and/or broth culture fluids. The procedure described in PART A is to be used only as a presumptive, qualitative screen test. The procedure described in PART B is to be used as the confirmative test which will provide quantitative and qualitative information.

PART A

15.2 (Presumptive) Staphylococcal Enterotoxin Reverse Passive Latex Agglutination Test

15.21 Introduction and Principles

A Staphylococcal Enterotoxin - Reverse Passive Latex Agglutination (SET-RPLA) test for the qualitative determination of enterotoxin serotypes A, B, C and D is commercially available. This test system is available as a complete, stable kit form. The test kit was evaluated by the Immunology Section of the Microbiology Division and was found to be suitable for use as a presumptive, qualitative screen test on meat sample extracts or broth culture filtrates. The SET-RPLA test was found to be specific and capable of detecting each homologous enterotoxin down to at least 1 ng/ml of sample extract fluid.

A latex agglutination test employed for presumptive screen testing of meat and poultry food samples for staphylococcal enterotoxins should meet or exceed the following performance characteristics:

Sensitivity	≥99% *
Specificity	≥99%
False Negative Rate	≤ 1%
False Positive Rate	≤ 1%
Efficiency	≥99%

* All at a toxin concentration level of ≥1 ng/ml of sample extract fluid and/or Protein A concentration level of <50 ng/ml of sample extract fluid.

The test functions on the principle of using individual suspensions of red latex particles which are each sensitized with specific antibody against a particular enterotoxin serotype. The presence of homologous enterotoxin will then cause visible agglutination of the specific antibody sensitized latex particles after an appropriate incubation period. The absence of toxins or the presence of heterologous toxin serotypes will not cause agglutination of the latex particles. The presence or absence of visible agglutination is discerned by observing the characteristic settling pattern of the red latex particles on the bottom of the reaction well.

The following details provide all the necessary instructional information to perform the SET-RPLA. These instructions are to be used in place of the instruction sheet supplied with the test kit.

15.22 Equipment and Supplies

a. Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)
b. Minishaker for microtiter plates, Cat. #002-963-0900 (Dynatech Laboratories, Inc., Alexandria, VA).
c. Microtiter Test Reading Mirror, Cat. #001-010-4900 (Dynatech).
d. Microtiter plates, 96 well, "V" bottom, polystyrene, Cat.#001-010-2602 and lids for above plate, Cat. #001-010-5550 (Dynatech).
e. Eppendorf Repeater Pipette, Cat. #G20551 with accessory of 1.25 ml capacity Combitips, Cat. #G20552B (Daigger Scientific Co.).
f. Waring blender and appropriate blending vessel.
g. Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.
h. Kimwipes®.
i. Glass separatory funnels, with stopper, 125 ml size.

15.23 Chemicals and Reagents

a. NaCl (Fisher, S-271).
b. Chloroform† (Fisher, C-298).
c. SET-RPLA test kit consisting of the following items:

    i. Vials of antibody sensitized latex suspensions of Anti A, Anti B, Anti C, Anti D, and Control latex (unsensitized).

    ii. Vials of enterotoxin† reference standards of A, B, C, and D serotypes.

    iii. Vials of buffered diluent.

NOTE: Store entire kit at 4°C when not in use. DO NOT FREEZE.

15.24 Preparation of Stock Reagent Solutions

0.2 M Sodium chloride solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.25 Sample Preparation for Enterotoxin Analysis

a. Meat Food Products

i. Blend 20 grams of meat sample together with 40 ml of 0.2 M NaCl solution, pH 7.5, at high speed in a Waring blender for 3 minutes.

ii. Centrifuge the resulting slurry at 32,000 X G for 15 minutes in a refrigerated centrifuge.

iii. Pour off the supernatant fluid and adjust the pH to 7.5 with 1 or 0.1 N NaOH solution.

iv. In a separatory funnel, in a chemical fume hood with the exhaust on, extract the supernatant fluid with a 1/3 volume (about 10 ml) of cold chloroform by shaking vigorously and letting stand for 15-30 minutes.

v. Pour the supernatant - chloroform mixture into chloroform resistant centrifuge tubes and centrifuge the mixture at 32,000 X G for 15 minutes in a refrigerated centrifuge.

vi. Pour both the supernatant layers through a double layer of kimwipes® back into a clean separatory funnel (make sure solid particles are retained by the Kimwipes®) and <u>without</u> further shaking allow the two layers to settle and clearly separate.

vii. Discard the chloroform (lower layer), and collect the clear meat extract (upper layer) free of any chloroform into a clean tube and use in the immunoassay. Keep the extract refrigerated until actually used in the performance of the assay.

b. Culture Fluids

i. Occasionally it may be of interest to determine if an isolated, coagulase positive, culture of *S. aureus* is capable of producing one or more enterotoxins (enterotoxigenic). This can be accomplished by first growing the pure culture for 24 h at 37°C in a medium such as Brain Heart Infusion Broth on a shaker at 150 RPM.

ii. Centrifuge the 24 h broth culture at 15,000 X G for 15 minutes and obtain the cell free culture fluid.

iii. Make a 1:100 dilution of the culture fluid in the buffered diluent supplied in the SET-RPLA kit. Use this diluted culture fluid directly in the assay to determine the presence of enterotoxins.

15.26 Performance of the SET-RPLA Test

a. Obtain the SET-RPLA test kit from the refrigerator, allow to equilibrate to room temperature and see that all the necessary kit components are present.

b. For the first time that the kit is used, rehydrate each of the lyophilized enterotoxin standards (A, B, C, and D) with the appropriate volume (given on kit instruction sheet or vial label) of buffered diluent and mix well. They can now be used without any further modifications in all subsequent assay performances.

c. Obtain the meat sample extracts previously prepared and make a 1:2 dilution of each in the buffered diluent in separate tubes. Culture fluids, if any are to be assayed, can be used directly as previously prepared.

d. Obtain a 96 well, "V" bottom, Dynatech microtiter plate and cover from stock supplies.

e. Place 25 µl of buffered diluent in each well of column 1 in rows A, B, C, D, and E using the Pipetman and a disposable tip.

f. Place 25 µl of reference enterotoxin A, B, C, and D into the wells of column 2 in rows A, B, C, and D respectively.

g. Place 25 µl of any one of the reference enterotoxin standards (your choice) in the well of column 2 in row E.

h. Place 25 µl of each test sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 3.

i. Obtain the individual vials of latex Anti A, Anti B, Anti C, Anti D, and Control latex suspensions and mix each thoroughly but gently to produce uniform latex suspensions.

j. Using an Eppendorf Repeater Pipette and individual 1.25 ml capacity combitips, dispense 25 µl of latex Anti A, Anti B, Anti C, Anti D, and Control latex into each occupied well of rows A, B, C, D, and E respectively.

k. Mount the plate on the carrier of the Minishaker and carefully shake the plate at a "medium" dial setting for 15 seconds to thoroughly mix, but not spill, the contents of each well.

l. Allow the covered plate to remain undisturbed at normal room temperature for 24 h before the final reading is made.

15.27 Test Reading and Sample Interpretation

a. After the appropriate period of time, remove the cover from the plate, mount it on the Microtiter Test Reading Mirror and observe from the bottom of the plate the pattern of settled red latex particles in each well.

b. The pattern of settled red latex particles determines whether or not agglutination has taken place. Nonagglutination is determined by observing that all of the latex particles have settled into a distinct pile at the bottom of the "V" in a particular well; usually referred to as a "button". Agglutination is determined by observing that all the latex particles in a given well are uniformly spread out over the entire surface of the "V" bottom without any distinct pile or "button". The agglutination patterns illustrated on the SET-RPLA kit instruction sheet may be helpful in regard to understanding this.

c. Observe each well and record whether or not agglutination has taken place.

d. To insure that the test is working properly, the following results should be obtained with regard to the controls employed. All wells of column 1 should be negative (no agglutination) as these are negative controls. All wells of column 2 of rows A, B, C, and D should be positive (agglutination) as these serve as positive homologous controls. The single well in column 2 of row E should be negative.

e. If all of the above controls have reacted properly, proceed to the interpretation of sample results. If any controls did not react properly, the test must be considered invalid and the procedure must be repeated and technical assistance should be sought to determine the nature of the problem.

f. Each sample can be interpreted with regards to the presence or absence of enterotoxins by observing the reactions of that sample column with respect to rows A, B, C, D, and E, which, of course, correspond to Anti A, B, C, D, and Control latex respectively. A positive reaction in any well of Anti A, B, C, or D identifies the presence of that particular toxin serotype. The control latex well (row E) should never show agglutination. If the sample column contains no positive wells, then the sample may be considered to be free of enterotoxins A-D and can be reported out as such.

g. If a sample contains enterotoxin it will usually be of only one serotype. The presence of more than one serotype in a food sample or culture fluid is possible but is rather unusual and one should not normally expect to find this.

h. If a sample should produce a positive reaction in Anti A, B, and C wells simultaneously (but not for the Anti D or Control latex wells) this is usually indicative of the presence of Protein A and the sample must be further treated, as described below, before it can be accurately assessed with regards to the presence of enterotoxins.

i. Add normal rabbit serum to a total concentration of 5% (v/v) to a sample extract suspected of containing significant concentrations of Protein A. Allow the sample to incubate for 30 minutes at 37°C. Centrifuge the sample at 10,000 X G for 15 minutes. Obtain the sample supernatant and perform the SET-RPLA test again to determine the presence of enterotoxins. The normal rabbit serum treatment should effectively neutralize the interfering reactivity of the Protein A.

j. All SET-RPLA positive samples or those with questionable results are to be confirmed by the procedure outlined in PART B.

15.28 Quality Control Procedures

a. Store and maintain the SET-RPLA kit components at refrigerator temperature (4 - 8°C) when not in use. DO NOT ALLOW THEM TO FREEZE.

b. Observe the kit manufacturer's expiration date for all test kit components. Kits should not be used beyond their expiration date.

c. Use only "V" bottom microtiter plates to perform the assay.

d. Allow all test components to equilibrate to room temperature prior to performing an analysis.

e. Thoroughly but gently resuspend the settled latex particle reagents in their vials to produce uniform latex suspensions immediately prior to dispensing this reagent in the test.

f. Always run negative and positive enterotoxin controls and control latex (unsensitized) when performing the analysis.

g. All negative and positive controls must give expected correct results before correct interpretation of test sample results can be made.

h. Do not allow the plate to be disturbed once all reagents have been added and properly mixed. Disturbing the plate may cause the settling pattern of agglutinated or

nonagglutinated latex to form abnormally and thus produce erroneous results.

† Safety Caution: **Do not dispose of hazardous (chloroform) or biohazardous (enterotoxin) fluids by pouring down the sink drains.**

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.29 Selected References

Bergdoll, M. S. 1980. Staphylococcal food poisoning, p. 108-119. *In* H. D. Graham (ed.), The Safety of Foods, 2nd Edition, AVI Publishing Company, Inc., Westport, CT.

Parks, C. E., and R. Szabo. 1986. Evaluation of reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C and D in foods. Can. J. Microbiol. 32:723-726.

Sanjeev, S., and P. K. Surendran. 1992. Evaluation of reversed passive agglutination test kits for the detection of staphylococcal enterotoxins A, B, C and D in fishery products. J. Food Sci. Technol. 29:311-312.

PART B

15.3 (Confirmative) Biotin-streptavidin Enzyme Linked Immunosorbent Assay for Staphylococcal Enterotoxins

15.31 Introduction and Principles

Enzyme Immunoassay (EIA) provides an alternative approach to the immunological detection of staphylococcal enterotoxins. EIA offers the major advantages of being more reliable in their reactions than latex agglutination and they can also be used for quantitation of the material under analysis. The Immunology Section of the Microbiology Division developed a Biotin-streptavidin Enzyme Linked Immunosorbent Assay (ELISA) for the quantitative detection of staphylococcal enterotoxin serotypes A, B, C, D, and E. This developed assay makes use of a biotin-streptavidin amplification reaction for the indicator portion of the assay.

The biotin-streptavidin ELISA described in this procedure is one of a solid phase, double antibody, "sandwich" type with a final biotinylated antibody-streptavidin peroxidase reaction to provide visual evidence of the degree of reaction upon substrate addition. The brief functional principles of this assay are as follows. Specific antibody (capture) against a particular enterotoxin serotype is bound to the walls of a microtiter plate (solid phase) and is allowed to react with test material which may contain enterotoxin(s). Only the homologous enterotoxin will react and bind to the wall bound antibody. A second antibody (probe) is introduced into the system with the same specificity as the first wall bound antibody and can now react with previously bound homologous enterotoxin. This second antibody is one which has had biotin chemically introduced into the molecule and is referred to as biotinylated antibody. Five "sets" of specific antibody pairs are simultaneously but individually employed in the assay corresponding to each of the five enterotoxin serotypes in question. A commercial preparation of streptavidin-peroxidase conjugate is next generally introduced into the assay system. This reaction makes use of the natural, very high, chemical binding affinity of biotin and streptavidin. Amplification is achieved by the fact that each molecule of streptavidin can bind four molecules of biotin. The streptavidin-peroxidase introduced into the assay will therefore bind to any biotinylated antibody present. With the final addition of the substrate to the system, the visible evidence of a positive reaction is produced from

conversion of the substrate to a colored end product by the enzyme peroxidase. If homologous toxins are not present, biotinylated antibody does not bind and subsequent reactions cannot take place, which therefore results in no colored change in the added substrate.

The following details provide all the necessary information for the performance of the Biotin-streptavidin ELISA for the quantitative determination of staphylococcal enterotoxin serotypes A, B, C, D, and E from meat and poultry products or broth culture fluids. All samples giving positive or questionable results in the SET-RPLA analysis (PART A) must be subjected to this confirmative Biotin-streptavidin ELISA for a final quantitative determination of enterotoxin presence before the final analytical results are reported.

15.32 Equipment and Supplies

a. Flow (ICN) Laboratories Titertek Multiskan MC Plate Reader, Cat. #78-530-00.
b. Flow (ICN) Laboratories Titertek Microplate Washer, Cat. #78-431-00.
c. Flow (ICN) Vacuum Pump for above washer, Cat. #78-426-00.
d. Flow (ICN) Titertek Multichannel Pipette, 8 channel, adjustable 50-200 µl volume, Cat. #77-859-00.
e. Eppendorf Repeater Pipette (Daigger Scientific Co., Cat. # G-20551) with accessory of 2.5 ml capacity Combitips (Daigger, Cat. #G-20552C) and 5.0 ml capacity Combitips (Daigger, Cat. #G-20552D).
f. Dynatech Laboratories Microelisa Plates, Immulon I, flat bottom, 96 wells, Cat. #11-010-3350 and covers.
g. Incubator, 37°C (any properly operating brand).
h. Centrifuge, refrigerated, capable of operation at 32,000 X G and appropriate centrifuge tubes resistant to chloroform.
i. Microtest Manifold, Wheaton, straight, 8 place with Luer Lock connection (Daigger, Cat. #G-20560A).
j. Kimwipes®.
k. Glass separatory funnels, with stopper, 125 ml size.
l. Waring Blender and appropriate blending vessel.
m. Rainin Pipetman, Model P-200 adjustable digital microliter pipette and RC-20 disposable microliter pipette tips. (Rainin Instrument Co., Woburn, MA.)

15.33 Chemicals and Reagents

a. Na_2HPO_4 (Fisher, Cat. #S-374).
b. NaH_2PO_4 (Fisher, Cat. #S-369).
c. NaCl (Fisher, Cat. #S-271).
d. Citric acid, anhydrous (Fisher, Cat. #A-940).
e. Hydrogen peroxide, 30% reagent grade (Fisher, Cat. #H-323).
f. Tween 80 (Fisher, Cat. #T-164).
g. Sodium azide† (NaN_3), purified (Fisher, Cat. #S-227).
h. Bovine Serum Albumin, powder, fraction V (Sigma, Cat. #A-4503), store in refrigerator.
i. Chloroform† (Fisher, Cat. #C-298).
j. ABTS substrate indicator; 2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid), (Sigma, Cat. #A-1888).
k. Streptavidin-peroxidase conjugate, Cat. #43-4323 (Zymed Laboratories, Inc., San Francisco, CA), store in refrigerator.

15.34 Staphylococcal Biochemical Reagents

a. Anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
b. Biotinylated anti-staphylococcal enterotoxin A, B, C, D, and E antibody stock solutions.
c. Staphylococcal enterotoxin† A, B, C, D, E standard reference stock solutions.

NOTE: The above 3 sets of items must be stored in the frozen state at all times to maintain stability.

15.35 Preparation of Stock Reagent Solutions

a. 0.15 M Phosphate Buffered Saline at pH 7.2 (PBS).

Add 10.35 grams of NaH_2PO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "acid" solution. Add 10.65 grams of Na_2HPO_4 and 4.38 grams of NaCl to 1 liter of distilled water and dissolve completely to prepare the "base" solution. While mixing with a magnetic stirrer and monitoring the pH on a pH meter, add a sufficient quantity of the "acid" solution to the "base" solution to achieve a final, stabilized pH of 7.2. Dispense into glass containers, autoclave at 121°C for 15 minutes and store

at room temperature. It is most convenient to make up this buffer in 5 liter quantities at a time.

b. Phosphate Buffered Saline containing 0.5% Tween 80 (PBS-Tween).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2 add 0.5 ml of Tween-80 and mix (not on magnetic stirrer) for several hours at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

c. Phosphate Buffered Saline containing 0.5% Bovine Serum Albumin (PBS-BSA).

To 1 liter of prepared 0.15 M phosphate buffered saline at pH 7.2, add 5 grams of powdered bovine serum albumin and 1 gram of sodium azide (NaN_3) and mix (not on magnetic stirrer) at room temperature until completely dissolved. Store this prepared solution in the refrigerator (4°C).

d. ABTS - H_2O_2 Substrate Buffered Solution.

Prepare a 0.1 M citric acid stock solution by dissolving 1.92 grams of anhydrous citric acid in 100 ml of distilled water. Prepare a 0.1 M dibasic sodium phosphate stock solution by dissolving 1.42 grams of Na_2HPO_4 in 100 ml distilled water. Add sufficient quantities of these two stock solutions together while mixing with a magnetic stirrer and monitoring the pH on a pH meter to prepare 100 ml of a 0.1 M citrate-phosphate buffer at a final stabilized pH of 4.0.

To 100 ml of the above prepared 0.1 M citrate-phosphate buffer add 22 mg of ABTS [2,2' azino-di-(3-ethyl Benzthiazoline Sulfonic acid)] and 15 µl of stock 30% hydrogen peroxide, mix gently by hand (no magnetic stirrer) until completely dissolved. Pass this substrate solution through a 0.45 µm Millex® filter, place in a sterile glass container, and store in the dark at room temperature until needed. This substrate solution should be prepared 24 h in advance of need and may be used as long as it retains its original light green color. A solution which has deteriorated to the

point where it cannot be used is evidenced by a dark azure-green color formation.

e. 0.2 M Sodium Chloride Solution at pH 7.5.

Add 11.69 grams of NaCl to 1 liter of distilled water. Dissolve the salt completely and adjust pH to 7.5 with use of 0.1 N NaOH solution.

15.36 Sample Preparation for Enterotoxin Analysis

Sample extracts for enterotoxin analysis from meat and poultry products or culture fluids are prepared exactly as described under the similar section (15.25 a. or b.) of PART A for SET-RPLA. These should be prepared in advance of the actual ELISA performance and kept refrigerated until needed.

15.37 Performance of the Biotin-streptavidin ELISA

a. Obtain a flat bottom, 96 well Dynatech Immulon I microtiter plate and cover from stock supplies.

b. Dilute the anti-staphylococcal enterotoxin antibody stock solutions in PBS in individual tubes to contain the following amounts of antibody protein as shown below for each respective serotype.

Anti-SEA antibody = 5 µg/ml
Anti-SEB antibody = 5 µg/ml
Anti-SEC antibody = 1 µg/ml
Anti-SED antibody = 5 µg/ml
Anti-SEE antibody = 5 µg/ml

c. Sensitize wells of the Immulon I microtiter plate with antibody for enterotoxin serotypes A, B, C, D, and E by placing 200 µl of the above concentrations of each antibody protein solution (PBS) in the wells of rows A, B, C, D, and E respectively. Leave all wells of column 2 empty.

d. Incubate the covered plate for 3 h at 37°C.

e. Remove the plate from the incubator, remove the cover and mount on the carrier of a Flow Titertek Microplate Washer which has been primed with PBS-Tween and set to deliver 300 µl fluid to each well.

f. Remove the solution from the wells by aspiration with the washer and wash the wells once with 300 µl fluid to each well.

g. Remove the plate from the washer, invert over a sink, hold the plate tightly in one hand and flick several times to remove any remaining excess liquid from the wells.

h. Tap the plate in an inverted position several times on a soft paper towel (Sorgs Laboratory towels) placed on the surface of the lab bench and allow the plate to remain inverted for 1-2 minutes to complete the draining process. Place the plate right-side up and cover until next reagent addition.

i. Block unwanted reactive sites on the plastic wells by filling all wells (including those in column 2) with 250 µl of PBS-BSA per well, dispensed from an 8 place microtest manifold attached to a Cornwall syringe.

j. Replace the cover on the plate and let stand undisturbed overnight at normal room temperature.

k. Wash the wells once by repeating steps (e thru h).

l. With a Pipetman microliter pipette place 200 µl of PBS-BSA to all wells of column 1 and 2 to serve as negative controls.

m. Obtain previously prepared standard reference enterotoxin solutions of serotypes A, B, C, D, and E at concentrations of 1, 5, 10, 25, and 50 ng/ml in PBS-BSA.

n. Place 200 µl of each of the above concentrations of toxins A, B, C, D, and E to the homologous antibody sensitized wells of rows A, B, C, D, and E respectively, beginning with column 3 wells at the lowest concentration.

o. Place 200 µl of each previously prepared sample extract in each well of a single, respective column in rows A, B, C, D, and E, beginning with column 8.

p. Incubate the covered plate for 2 h at 37°C.

q. Wash the wells twice by repeating steps (e thru h).

r. Prepare the following dilutions of biotinylated anti-staphylococcal enterotoxin antibody stock solutions in PBS-Tween in individual tubes as shown below for each respective serotype.

Biotinylated Anti-SEA antibody = 1:5000
Biotinylated Anti-SEB antibody = 1:5000
Biotinylated Anti-SEC antibody = 1:2500
Biotinylated Anti-SED antibody = 1:5000
Biotinylated Anti-SEE antibody = 1:1500

s. Place 200 µl of the above dilutions (PBS-Tween) of each biotinylated antibody serotype to all wells in a respective row of homologous, primary antibody sensitized wells (i.e., Anti-A in row A, Anti-B in row B, etc.).

t. Incubate the covered plate for 2 h at 37°C.

u. Wash the wells three times by repeating steps (e thru h).

v. Prepare a 1:5000 dilution of the commercial Streptavidin-peroxidase conjugate in PBS-Tween in a separate tube.

w. Add 200 µl of the 1:5000 dilution (PBS-Tween) of Streptavidin-peroxidase conjugate to all wells of the plate with the use of an Eppendorf Repeater pipette and a 5 ml capacity combitip.

x. Incubate the covered plate for 30 minutes at 37°C.

y. Wash the wells three times by repeating steps (e thru h).

z. With the use of the Flow 8 channel pipette, add 200 µl of ABTS-H_2O_2 substrate buffered solution to all wells.

aa. Place the cover on the plate and incubate for 30 minutes at 37°C.

bb. Twenty minutes prior to the end of the above incubation period turn on the power to the Flow Titertek Multiskan MC plate reader and allow it to warm up.

cc. After the 30 minutes incubation period of step (aa) is complete, remove the plate from the incubator, remove the cover, and place the plate on the carrier of the Multiskan MC plate reader.

dd. Program the reader for the current date, Mode 1 (single wavelength absorbance), Wavelength Filter #2 (414 nm), push the carrier and plate into the measuring head and blank the instrument (zero O.D. point set) on column 1.

ee. Press the START button and obtain a printed paper strip of the Optical Density (O.D.) values for all of the reaction wells on the plate.

ff. Remove the plate from the reader and visually examine the plate to see that the obvious colored reaction intensities generally correspond to the numerical values on the printed data sheet to assure that the plate has been properly read in the instrument.

gg. Turn off the power to the Multiskan MC plate reader and discard the plate (save the cover for reuse) after completion of the Data Analysis Plotting and Sample Interpretation Section described below.

15.38 Data Analysis, Plotting, and Sample Interpretation

a. All wells in column 1, which serve as the zero-blank negative control, should have no color reaction, indicating a proper lack of non-specific attachment of biotinylated antibody or Streptavidin-peroxidase to the antibody sensitized wells. Under these conditions these wells are excellent controls to blank in (zero point set) the O.D. reading instrument.

b. All wells in column 2 serve as BSA negative controls to assess non-specific attachment of biotinylated antibody and also Streptavidin-peroxidase. Since the wells originally were never sensitized with anti-enterotoxin antibodies but only blocked with BSA, no positive reactions (high O.D. values) should ever be observed.

c. Wells in columns 3, 4, 5, 6 and 7 of rows A, B, C, D and E represent the standard quantitative dose response values of the reaction with regard to enterotoxin serotypes A, B, C, D, and E respectively. The response (O.D.) observed in this ELISA should be one of a direct linear relationship to increased dose concentration of enterotoxin.

d. The remaining wells of individual columns 8-12 for rows A, B, C, D, and E represent reaction values for individual test sample extracts with regards to the presence or absence of enterotoxins A-E respectively.

e. Obtain a piece of 4 cycle semi-logarithmic graph paper. Label the ordinate (10 division to the inch) with O.D. values from 0-2.0 in increments of 0.05. Label the abscissa (4 cycle logs) with enterotoxin concentrations of 0, 1, 5, 10, 25, and 50 ng/ml.

f. Plot the O.D. values against standard enterotoxin concentrations for each individual serotype together on the same piece of graph paper. Draw straight lines from point to point for each homologous set of enterotoxin concentrations. You will now have 5 individual standard curves for enterotoxin serotypes A-E respectively, which will have similar appearances to each other but still be distinctly different. The curves should illustrate the direct linear dose-response relationship in regards to increasing toxin concentration for each serotype.

g. To determine if a test sample contains enterotoxin and its' quantity if present, proceed as follows:

   i. Obtain the O.D. values of individual sample column wells with regards to rows A, B, C, D, and E (which correspond to Anti A, B, C, D, and E antibodies respectively) and determine if any sample O.D. values exceed the 1 ng enterotoxin standard O.D. value for each individual serotype.

   ii. Any sample O.D. value exceeding the 1 ng enterotoxin standard of a given serotype is to be considered as a positive identification reaction for the presence of that enterotoxin serotype in the sample.

iii. Determine the quantitative amount of an enterotoxin which is present by interpolating the O.D. value with regards to concentration from the standard curve for that particular serotype identified and multiply by 3 (food sample) or 100 (culture fluid).

iv. If the sample O.D. value does not fall within the more linear portion (1-25 ng/ml) of the standard curve of a given serotype, then the sample analysis should be repeated using standard dilutions of the original extract in PBS. The dilution factor which produces readable results would then need to be included in the final quantitative calculations.

v. If sample O.D. values are less than those of the 1 ng standards of each serotype, the sample should be considered free of enterotoxins A-E and reported out as such.

h. If a sample is found to contain an enterotoxin, it will usually be of only one serotype. The presence of more than one serotype toxin in a given sample is possible but rather unusual.

i. If a sample is found to produce a strong positive reaction in all the serotype wells, except Anti-D, this usually indicates that the sample contains a significant amount of Protein A and the sample must be treated as described in PART A, 15.27 step i, before a repeat ELISA analysis can be performed to accurately determine the presence of enterotoxins.

j. All enterotoxin positive samples should be reported out by using a statement such as the following. "This food sample was found to contain Staphylococcal enterotoxin serotype _____, at a concentration of _____ ng/g as confirmed by an ELISA procedure." The serotype and quantitative values would, of course, be filled in from your analytical data.

15.39 Quality Control Procedures

a. The assay reagents have been standardized for use only with Dynatech Immulon I microtiter plates. No other plates should be used.

b. All stock reagent solutions must be properly prepared and maintained free of contamination or chemical breakdown.

c. The stock ABTS-H_2O_2 substrate buffered solution should not be used if it has turned to a significantly darker shade of green from that of the original preparation.

d. Be sure the stock, commercial Streptavidin-peroxidase reagent has not deteriorated to the point of producing abnormally low final O.D. readings. Use only an unexpired lot of this reagent.

e. All standard negative and positive enterotoxin control values must be in the correct range before proper interpretation of test sample results can be reliably made.

f. The standard curves generated from the standard enterotoxin concentrations for each serotype should always be of the same general shape and value from run to run. Drastic changes in the shape of these curves usually indicate critical reagent deterioration (or misuse).

g. Standardized reference enterotoxin concentrations must always be carefully and properly prepared from higher concentrated stock solutions to assure reliability of the generated standard curves.

† Safety Caution: Do not dispose of hazardous (chloroform, sodium azide) or biohazardous fluids (enterotoxin) by pouring down sink drains.

Accumulation of sodium azide in lead drains may result in an explosion.

Collect these liquid wastes in separate containers and dispose of according to standard waste management procedures for your laboratory.

Do not allow human exposure to chloroform vapors.

15.4 Selected References

Freed, R. C., M. L. Evenson, R. F. Reiser, and M. S. Bergdoll. 1982. Enzyme-linked immunosorbent assay for detection of staphylococcal enterotoxins in foods. Appl. Environ. Microbiol. 44:1349-1355.