U.S. FOOD & DRUG ADMINISTRATION

Bacteriological Analytical Manual

Chapter 13B: Staphylococcal Enterotoxins Detection Methods

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Revision History

- September 2022: Section H. Correction for kit minimum enterotoxin detection level
- January 2018: Hyperlink for CDC APHIS/CDC Form 4 corrected
- June 2017: New Chapter 13B was added to the Bacteriological Analytical Manual

Introduction

Staphylococcal food poisoning (SFP) is an intoxication resulting from consumption of food contaminated with adequate levels of pre-formed enterotoxins. Symptoms of SFP are manifested within 2-8 hours of ingestion and include nausea, vomiting, abdominal cramping with or without diarrhea that typically resolve within 24-48 hours. (Argudin *et al*., 2010). The number of people affected by SFP is only an estimate due to misdiagnosis and minor outbreaks that are not reported. Hospitalization is rare but has been noted in people that are immunocompromised particularly the elderly and very young (Scallan *et al*., 2011).

Staphylococci are normally present on human skin and mucous membranes with approximately 20-30% for persistent and 60% for intermittent colonization (Kluytmans *et al*., 2005). Food handlers that are colonized with enterotoxigenic staphylococci are considered to be the main source of food contamination by direct contact with the products or contact surfaces. Animals such as dairy cattle can also carry staphylococci and can provide a source of contamination for milk and milk products. Finally, staphylococci present in the environment can be transferred to food products serving as a potential source of contamination (Gutiérrez *et al*., 2012).

Foods commonly linked to SFP include processed foods, meat, poultry, dairy, and bakery products. Once the food is contaminated, staphylococci growth and enterotoxin production can occur, especially if good manufacturing conditions are not followed that prevent growth such as refrigerated storage conditions or heat kill steps such as pasteurization (Gutiérrez *et al*., 2012). Staphylococcal enterotoxins are heat stable and not denatured unless exposed to high temperatures for long periods, *i.e*., autoclave at 121°C (250°F) at 15 PSI for 60 minutes (CDC, 2007).

Staphylococcus aureus is most commonly linked to staphylococcal food poisoning outbreaks, but other enterotoxigenic coagulase positive staphylococci such as *S. hyicus* and *S. intermedius* have also been implicated in outbreaks (Hennekinne *et al*., 2010). Staphylococcal enterotoxins (SEs) are pyrogenic exotoxins with superantigenic activity. Enterotoxins are globular proteins that are resistant to heat and proteases with molecular size on average about 25 kDa. The estimates for the amount of toxin required to cause disease are very low. One outbreak linked to chocolate milk detected levels of SEA at 0.5ng/ml (Evenson *et al*., 1988) and 0.38ng/ml of SEA was detected in an outbreak linked to powdered milk (Asao, *et al*., 2003).

There are SEs and SE-like toxins (SEI); SEA, SEB, SEC1,2,3, SED, SEE, SEG, SEH, SEI, SEIJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER, SES, SET, SEIU, SEIV, SEIW, SEIX, SEIY (Fisher et al., 2018). The SE group is recognized to exhibit emetic activity, while the SE-like have not been established to cause emesis or gastroenteritis in primates (Fisher *et al*., 2018). All *se* and *sel* genes have been located on mobile genetic elements including bacteriophages, pathogenicity islands, plasmids or transposons (Argudin *et al*., 2012).

Detection of SEs is paramount to food safety and protection of the food supply. SE detection methods rely on commercially available polyvalent enzyme-linked immunoassays (ELISA) or enzyme-linked fluorescent immunoassay (EFLA) with antibodies that detect SEA-SEE. Monovalent methods are specific for SEA-SEE and can be used to distinguish these types. The methods require extraction of enterotoxin from suspected food prior to analysis. The method sensitivity and selectivity is improved with dialysis concentration of the food extract, but time constraints may require preliminary testing without concentration. However, dialysis concentration should be performed on all dairy products prior to analysis (Hennekinne *et al*., 2012).

CAUTION: Staphylococcal enterotoxins are highly toxic and procedures that may create aerosols should be performed in approved biological safety cabinet (BSC). **Staphylococcal enterotoxins (SEA, SEB, SEC, SED, and SEE) are select agents. Scientists must follow guidelines established by CDC**: https://www.selectagents.gov/faq-general.html.

A. Special Equipment and Materials

1. Temperature controlled room or refrigerator 2-8°C.
2. Blender or homogenizer.
3. Incubator 35-37°C.
4. Analytical balance and weigh boats.
5. Tray for dialysis.
6. pH meter. The pH during extraction and the pH of buffers used in the extraction are important. Make adjustments within ± 0.1 pH unit.
7. Refrigerated centrifuge 2-8°C.
8. Laboratory-ware in glass or polypropylene to avoid toxin adsorption.
9. Filter cloth is used to collect debris after centrifugation. Often, several layers of pre-wetted coarse cheesecloth are used to perform this task.
10. Separatory funnel.
11. Dialysis membrane MWCO 6,000-8,000 Daltons (e.g. Spectra/Por® with closures) flat width 23 ± 2 mm.

12. Vacuum conical tube filtration devices (0.22µm membrane) such as Steriflip (EMD Millipore) recommended for filtering liquid culture supernatants as a safety measure to avoid aerosolization of enterotoxins.

13. Biological safety cabinet.

B. Reagents

Note: kit manufacturers may require different buffers or solvents.

1. Phosphate buffered saline (PBS solution) pH 7.3 + 0.2 ($NaCl/Na_2HPO_4$: 145 mM/10 mM) to prepare 1L PBS dissolve 9 g NaCl and 3.58 g Na_2HPO_4 in 1L distilled water. Adjust pH to 7.2 ± 0.2 using HCl.

2. Sodium chloride (NaCl).

3. Sodium phosphate (Na_2HPO_4).

4. Polyethylene glycol (PEG) (20,000 mol wt) Prepare 30% (w/v) PEG solution by adding 30g PEG for each 70 ml distilled water.

5. 1 N (or 0.1 N) NaOH.

6. 1 N (or 0.1 N) HCl.

C. Preparation of Dialysis Membrane

Cut dialysis membrane long enough to accommodate food extract to be concentrated. Soak tubing in 2 changes of distilled water to remove glycerol coating. Using a membrane closure or tie one end of tubing with 2 knots close together. Fill tube with distilled water and test for leaks by squeezing filled sac while holding open end tightly closed. Empty sac and place it in distilled water until use.

D. Extraction of Enterotoxin from Food Portion

NOTE: this procedure and other procedures that may generate aerosols of pathogenic microorganisms or enterotoxins should be performed in an approved biosafety cabinet.

1. If possible, grind entire food product or portions from representative selections of the product in a blender to homogenize the sample so that any SE present in the food is evenly distributed.

    a. For cheeses with rind, take cheese sample with 10% rind and 90% cheese.

b. For dried products, use equal amounts of water with product to blend or follow manufacturer's instructions.

2. Weigh 25 g of sample in a glass beaker and transfer the test portion to a blender with 40 ml of distilled water and blend at high speed for 3 minutes generating a homogenized slurry. Do not add water to liquid samples but proceed to step 3.

3. Allow the toxin to diffuse by shaking the sample at room temperature for 30 minutes.

4. Acidify the mixture with 0.1N HCl to pH between 3.5 and 4.0. Note: if pH drops below 3.0 another 25 g portion must be prepared.

5. Transfer acidified slurry to 50 ml conical propylene tubes. Centrifuge at 3,130 × g for 20 min at 5°C. Lower speeds with longer centrifuge time can be used but clearing of some foods is not as effective. Separation of fatty materials is ineffective unless food is centrifuged at refrigerated temperature.

6. Decant supernatant fluid into 800 ml beaker through cheesecloth or other suitable filtering material placed in a separatory funnel. If the supernatant is not clear, centrifuge again and decant fluid through the cheesecloth. Test the pH which should be between 3.5 and 4.5. If the pH is correct, neutralize the mixture with 0.1N NaOH to obtain a pH between 7.4 and 7.6.

7. If the pH is >4.5 repeat acidification, but if <3.0 or="">9.0 repeat process with another 25 g food portion.

8. Provided there is sufficient sample available for repeat testing, an aliquot can be removed for screening using a validated assay (see Sec. H). If the screening results are negative, the remaining extract must be concentrated with dialysis and retested.

E. Dialysis Concentration of the Extract

1. Place extract in prepared dialysis sac. Lay the closed sac down in a tray and immerse sac in 30% (w/v) PEG. Hold at 5°C until volume is reduced to 15-20 ml or less. This process may take 24-72 hours, but if volume is not reduced after holding overnight, add powdered PEG to the tray. Remove sac from PEG and wash outside thoroughly with water to remove any PEG adhering to sac. Soak in distilled water for 1-2 min. Pour contents into small beaker.

2. Rinse inside of sac with 2-3 ml distilled water (PBS is used for milk and dairy products) by running fingers up and down outside of sac to remove material adhering to sides of tubing. Repeat rinsing until rinsate is clear. Keep volume as small as possible.

3. Adjust pH of extract to 7.4-7.6.

4. Concentrated extracts should be analyzed within 48 hours and stored at 3-5°C, otherwise, freeze the extracts at 18-20°C and thaw completely at 3-5°C prior to testing. Some assays such as the Vidas SET2 require immediate analysis.

F. Testing SE from Bacterial Cultures

Staphylococcal strains suspected of producing enterotoxins can be pre-enriched in nutrient broth such as TSB or BHI.

1. Transfer two or three morphologically similar colonies to 10 mL of nutrient broth.
2. Incubate 35-37°C overnight on an orbital shaker.
3. Centrifuge cultures 5 minutes 3500 × g at 10°C
4. Filter the supernatant using a Steri-Flip vacuum filter device with a 0.22µm filter or other closed system to avoid aerosolization of enterotoxins. This procedure must be performed in a biological safety cabinet to ensure safety of the scientist.
5. Culture supernatants may have high levels of SE that are outside the linear range of the kit may require dilution with PBS.

G. Reporting Results

The presence of staphylococcal enterotoxin detected in food products shall be reported to the Federal Select Agent Program managed by the Center for Disease Control and Protection (CDC): https://www.selectagents.gov/form4.html by completing APHIS/CDC Form 4.

Positive results shall be reported as Staphylococcal enterotoxoin detected in × g (ml) of product. Negative results shall be reported as Staphylococcal enterotoxin not detected in × g (ml) of product.

H. Notes

The kit must be able to detect enterotoxin at the minimum level of 0.5ng/g with high levels of relative sensitivity (>90%) and relative specificity (>90%).

Mention of trade names or commercial products in the method is solely for the purpose of scientific information and does not imply recommendation or endorsement by the U. S. Food and Drug Administration. Two methods that are commonly used for SE detection include the AOAC approved Vidas SET2 (bioMerieux, Inc) which is an automated polyvalent enzyme linked fluorescent assay (EFLA) that detects SEA-SEE (AOAC Official Method 2007.06 VIDAS SET2 for Detection of Staphylococcal Enterotoxin in Select Foods, Final Action, 2010). The current catalog number for Vidas Set2 is Ref. 30705. The Ridascreen SET Total (R-Biopharm AG) is a manual enzyme linked immunoassay performed in wells coated with polyvalent antibodies. The polyvalent Ridascreen kit detects staphylococcal enterotoxins SEA-SEE and has been validated and verified thorough ring trials and third party validation studies led by European Union Reference Laboratory for Coagulase Positive Staphylococci for the European Committee for Standardization (CEN) mandate proposed ISO Standard 19020. A monovalent kit Ridascreen Set A,B,C,D,E (R-Biopharm AG) is available, but this kit has not been validated through a third

party. The catalog number for the Set Total polyvalent kit is R4105 (96 tests) or R4106 (48 tests) . The catalog number for the SET A,B,C,D,E monovalent kit is R4101.

Interferences

Non-specific reactions may occur in food products that have endogenous enzymes such as lactoperoxidase or alkaline phosphatase and interfere with kits such as Vidas SET2 that uses alkaline phosphatase as the detection enzyme (Vernozy-Rozand, *et al*., 2005). It is recommended that all positive results are confirmed with an alternative method that uses a different detection enzyme. In the case of the Vidas SET2, one alternative method is the Ridascreen SET Total that uses lactoperoxidase.

A heat treatment may be applied to remove endogenous alkaline phosphatase from the sample as follows:

- Transfer 600 µL of concentrated extract into a tube
- Heat 80°C for 2 minutes
- Repeat the Vidas SET2 assay with the cooled concentrate. Some loss of enterotoxin may occur.

If inaccurate result is suspected using the Ridascreen or other kit using lactoperoxidase, transfer 100 µL of concentrated extract to a tube. Add 50µL each of substrate and chromagen kit solutions. Mix and observe for a blue-green color. If blue-green color appears, it is indicative that intrinsic lactoperoxidase is present in the sample and interfered with the assay. A different detection method must therefore be used.

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