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Simple Solutions to False-Positive Staphylococcal Enterotoxin Assays with Seafood Tested with an Enzyme-Linked Immunosorbent Assay Kit (TECRA)

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The TECRA kit, a commercial staphylococcal enterotoxin visual immunoassay kit, is an enzyme-linked immunosorbent assay system which utilizes polyvalent antisera against staphylococcal enterotoxin types A to E. The test is simple and rapid to perform (4 h) and has therefore been widely used for screening purposes. In this study, the TECRA kit produced a number of false-positive reactions with seafood; 25% of 218 samples of seven types of seafood gave false-positive results, particularly shellfish such as mussels (85%), clams (32%), oysters (23%), winkles (20%), and squid (13%). Some nonshellfish samples also gave false-positive results with the TECRA kit (smelt [20%] and trout [10%]). The substance contributing to the false-positive results differed from true staphylococcal enterotoxins in that it was: (i) heat labile, being completely inactivated by heating for 3 min at 70°C, compared with 5% inactivation of true staphylococcal enterotoxins by the same heat treatment, (ii) in a selective reaction with normal rabbit or calf serum (nonspecific reactions were completely abolished by these sera, whereas staphylococcal enterotoxins were not affected), and (iii) incapable of binding to a copper-chelate Sepharose gel (all of the substance remained in the unbound wash fraction, whereas staphylococcal enterotoxins were quantitatively bound to the gel). The false-positive reactions occurring with seafood were not associated with substances produced by microorganisms, since the bacterial isolates from the samples did not give positive results with the TECRA kit. The problems of nonspecific reactions occurring with the TECRA kit could be resolved by any of the following procedures: (i) metal chelate affinity chromatography (2 h) with copper chelate Sepharose, (ii) treatment with rabbit or calf normal serum (0.1 volume of serum added to food extract and held at room temperature for 2 min), or (iii) heat treatment at 70°C for 3 min. The recovery of staphylococcal enterotoxin following any of these procedures ranged from 90 to 95%.

A 10-year summary of data from 1975 to 1984 (20) indicates that marine products accounted for a total of 586 foodborne illness outbreaks in Canada. The etiological agent in 24 of these outbreaks was attributed to staphylococcal enterotoxin (SE). We examined various types of seafood products for SE with three types of commercially available SE assay kits: a polyvalent enzyme-linked immunosorbent assay (ELISA) kit for SE screening purposes, a monovalent ELISA kit, and a reversed passive latex agglutination (RPLA) kit for determination of individual SE types. The polyvalent ELISA was the TECRA kit (Bioenterprises Pty. Ltd., Roseville, New South Wales, Australia), which utilizes polyvalent antisera for detecting (but not differentiating) SE types A to E in a single test. Of the monovalent SE assay kits, the RIDASCREEN ELISA kit (R-Biopharm GmbH, Darmstadt, Germany) was used for the detection of individual toxins SE types A to E in five separate tests, and the RPLA kit (Denka Seiken Ltd., Tokyo, Japan) was used as an additional test for detection of individual toxins SE types A to D in four separate tests. During the course of the survey, however, a number of nonspecific SE reactions were observed when seafood samples were tested with the TECRA kit.

To date, little information is available on the specificity of the TECRA kit with respect to seafood, although limited reports indicate that certain microorganisms other than *Staphylococcus aureus* in various foods are capable of producing substances giving rise to false-positive results with the kit (11, 22).

The purpose of this study was to evaluate the specificity of the TECRA kit and to find a solution to the problems of nonspecific SE reactions occurring with seafood samples tested with the kit.

MATERIALS AND METHODS

Collection of seafood samples. Prepacked or unpacked fresh or frozen raw seafood, listed in Table 1, were purchased every second week (Tuesday) from six different seafood stores in the Ottawa-Hull area during a 6-month period from August 1992 to January 1993.

Sources of SE assay kits. The TECRA kits produced by Bioenterprises Pty. Ltd. were obtained from International BioProducts Inc., Redmond, Wash. The RIDASCREEN kits produced by R-Biopharm GmbH were obtained from Bioman Products Inc., Mississauga, Ontario, Canada. The RPLA kits manufactured by Denka Seiken Ltd. for Oxoid Ltd. (Basingstoke, Hampshire, England) were obtained from Oxoid Canada Inc. (Nepean, Ontario, Canada).

Microbiological analysis. Shellfish samples were cleaned by scrubbing the shells with a sterile brush under running potable water and appropriate quantities of internal contents were collected (10). Nonshellfish samples were used without scrubbing. Each sample (25 g) was transferred to a blender jar containing 225 ml of 0.1% peptone water and blended at low speed for 2 min and then at high speed for 1 min. Serial decimal dilutions of the homogenates were analyzed for total

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aerobic microorganisms (aerobic plate count [APC]) and CFU of *S. aureus*. The total APC was determined by surface plating 0.2-ml portions of serial dilutions onto tryptic soy agar (16), and the CFU of *S. aureus* was determined by plating a total of 1.0 ml of appropriate dilutions onto three plates of Baird-Parker agar; the plates were incubated at 35°C for 48 h (8). Presumptive *S. aureus* colonies were confirmed by microscopic examination, catalase activity, production of both thermonuclease (TNase) and coagulase, susceptibility to lysostaphin, and anaerobic utilization of glucose and mannitol (8). To determine whether *S. aureus* might have been present in the food samples and subsequently killed, all samples were tested for TNase by the procedures described previously (7, 12). All bacteriological media used were products of Difco Laboratories (Detroit, Mich.) unless otherwise stated.

Extraction of SEs from food. Equal, double, and triple volumes of SE extraction medium were added to 50-g samples of shellfish meat with high (oysters, clams, mussels, etc.) and medium (conch, winkles, etc.) moisture content and to 50-g samples of seafood with low-moisture content (shrimp, squid, fish, etc.), respectively. The SE extraction media used were 0.25 M Tris buffer (pH 8.0) for the TECRA kit and phosphate-buffered saline (0.05 M phosphate in 0.15 M NaCl containing 0.05% NaN_3; pH 7.5) for the RIDASCREEN and RPLA kits. The food homogenates were prepared by blending at high speed for 2 min, the homogenates were then centrifuged at 16,300 × *g* for 20 min, and the supernatants were filter sterilized (0.45-μm-pore-size filter) to obtain *S. aureus*-free food extracts for the SE assays.

SE analysis. All food extracts were first screened by the TECRA kit. The TECRA-positive samples were then tested to identify individual SE types with the RIDASCREEN and RPLA kits. The procedures for SE assays were those recommended by the manufacturers of the assay kits unless otherwise stated. Color reactions developed by the TECRA and RIDASCREEN kits were measured by optical densities (OD) at 405 and 450 nm, respectively, using a microtiter reader (Bio-Kinetics Reader [EL 312]; Bio-Tek Instruments, Inc., Winooski, Vt.).

Procedures for elimination of nonspecific SE reactions with seafood samples tested with the TECRA kit. The following procedures were used to eliminate nonspecific SE reactions with seafood samples tested with the TECRA kit: (i) serum treatment (to 1 volume of food extract, 0.1 volume of rabbit or calf normal serum was added, mixed thoroughly, and then held at room temperature for 2 min), (ii) heat treatment (a tube containing 1.0 to 2.0 ml of food extract [held at room temperature before heating] was placed in a water bath at 70°C for 3 min and then cooled immediately), (iii) metal chelate affinity chromatography (MCAC) (the MCAC using copper-chelate Sepharose gel [Pharmacia LKB, Uppsala, Sweden] was carried out as previously described [2]).

Detection of peroxidase. The presence of peroxidase in the food extracts was determined as previously described (11).

Experiments with food isolates to trace the nonspecific SE reactions with the TECRA kit. To determine whether the source of the nonspecific SE reactions with seafood with the TECRA kit was of bacteriological origin, microorganisms were isolated from mussels yielding false-positive results with the kit. These bacteria were subcultured in 50 ml of tryptic soy broth (TSB) for 2 days at room temperature, pooled by centrifugation (13,000 × *g* at 4°C), and then washed twice with saline. The cells were resuspended in TSB (5 ml), and an aliquot (1 ml) of the cell suspension was inoculated into sterile mussel meat and into TSB, followed by incubation at room temperature for 3 to 5 days. Preparation of food extracts and SE assays were carried out by the procedures described above.

Bioassay of SE in mussels using human volunteers. A total of 12 healthy human volunteers (19 to 65 years with equal representation by both sexes) were selected, and each was fed 50 g of mussel meat (boiled for 2 min) as a part of breakfast prepared by the investigators. This sample food showed ODs of >2.00 with the TECRA kit and with 15-fold dilutions of the raw mussel extracts. After consumption of the sample, the volunteers resumed normal activities but were restricted to eating SE-free meals prepared by the investigators. The participants were asked to record the presence or absence of the following symptoms every hour for the first 10-h period and then every 4 h for the remainder of the 24-h period: body temperature, vomiting, nausea, stomach cramps, sweating, prostration, heart rate, breathing difficulty, diarrhea, and others.

RESULTS AND DISCUSSION

Microbiological quality of food samples. A total of 218 samples of 13 types of fresh raw seafood were analyzed for total APC and viable counts of *S. aureus* as well as TNase activity (12, 17, 18). Table 1 indicates that the total APC of all samples, except for lobster meat and crabmeat, were less than 1×10^4 CFU/g, considerably lower than those given in the guidelines (5×10^5 CFU/g) for adherence to good manufacturing practices as recommended by the National Shellfish Sanitation Program (5). All samples showed viable counts of *S. aureus* of <10 CFU/g, except for some samples of lobster meat and crabmeat, which ranged from <10 to 160 CFU/g. TNase activity was not detected in any of the samples, indicating that contamination of the foods by *S. aureus* which could have produced detectable levels of SE prior to dying off, was unlikely. The guidelines for fish and fisheries products (4) indicate that 10^4 CFU/g is an acceptable level for viable counts of coagulase-positive *S. aureus*. Our data suggest that all samples tested were bacteriologically sound and acceptable for consumption and should be considered SE-free food commodities.

False-positive results with the TECRA kit for SE assay. Of the 218 samples, 54 (24.8%) of seven food types gave positive results for SE with the TECRA kit, particularly among shellfish samples such as mussels (85%), clams (32%), oysters (23%), winkles (20%), and squid (13%); however, some nonshellfish samples also yielded positive results with the kit. All seafood samples yielding positive results with the TECRA kits gave negative results with the RIDASCREEN and RPLA kits (Table 2). These results indicate poor specificity of the TECRA kit with certain types of seafoods, since the sensitivities of the three kits for SE assay are similar, ranging from 0.5 to 1.0 ng/ml (1, 11, 13, 15, 21) or as claimed by the manufacturers. To examine the reason for poor correlation between the kits, the seafood extracts which yielded positive results with TECRA kits were treated with either normal serum, mild heat, or copper-chelate Sepharose chromatography. After these treatments, all extracts except positive controls were negative with the TECRA kits (Table 3).

As shown in Table 2, the OD values with most mussel extracts were >2.00 at the 1:15 dilution. If these OD values had resulted from SE, one could assume that the mussels contained at least 150 ng of SE per ml of food extract or 300 ng of SE per g of food, and the food would have caused SE intoxication even if less than 1 g was ingested (3). However,

TABLE 1. Microbiological analyses of raw seafood sold at retail outlets[a]

Seafood	No. of samples tested	APC (CFU/g)		Viable *S. aureus* (CFU/g)
		Range	Average	
Mussels	34	2×10^2–7.3×10^3	3.1×10^3	<10
Clams	28	4×10^2–1.7×10^3	7.5×10^2	<10
Oysters	26	7×10^2–2.2×10^3	1.3×10^3	<10
Winkles	15	2×10^2–9.3×10^3	4.1×10^3	<10
Conch	12	4×10^2–1.4×10^3	7.2×10^2	<10
Shrimp	20	5×10^2–3.0×10^3	1.5×10^3	<10
Lobster meat	13	8×10^2–5.3×10^4	1.2×10^4	<10–130
Crabmeat	10	4×10^3–6.0×10^4	2.5×10^4	<10–160
Squid	15	<10^2–5.0×10^2	1.3×10^2	<10
Smelt	15	3×10^2–6.5×10^3	2.2×10^3	<10
Cod	10	4×10^2–7.1×10^3	1.8×10^3	<10
Trout	10	6×10^2–7.3×10^3	2.2×10^3	<10
Salmon	10	3×10^2–3.6×10^3	1.5×10^3	<10
Total	218	<10^2–6.0×10^4	4.3×10^3	

[a] To estimate probable populations of nonviable *S. aureus* which could produce detectable amounts of enterotoxins in foods, TNase activity was measured in the food extracts according to the reported procedures (8, 11) and negative results were found for all samples tested.

there were no SE-associated food poisoning outbreaks during the period of these studies, either in the Ottawa-Hull area or in the whole of Canada. In a study involving 12 volunteers, no one showed any symptoms of SE intoxication after consuming 50 g of mussels (the extracts had OD readings of >2.00 with the TECRA kit when diluted 1:15 [data not shown]). Since approximately 10 to 15% of SE could be inactivated by the short heat treatment of 2 min at 100°C (11, 19), the consumed sample should have contained about 13 μg of SE per 50 g, which is 65 times the recognized emetic dose (3). From these results, it is evident that the TECRA kits yielded false-positive SE reactions with certain seafood samples, particularly with shellfish such as mussels, clams, oysters, etc.

In this study, we have ruled out the possibility that the false-positive reactions with the TECRA kits were caused by substances produced by microorganisms other than *S. aureus*, as previously observed with other foods (11, 22). The evidence for this is that isolates from the seafood did not produce the TECRA-positive substances (data not shown). In addition, TECRA-positive seafoods did not show naturally occurring peroxidase activity, which can cause false-positive results with the TECRA kit (6) (data not shown).

TABLE 3. Effects of three treatments (serum, heat, and MCAC) on elimination of false-positive SE reactions occurring with seafood samples tested with the TECRA kit

Sample	ODs[a] yielded by TECRA kit			
	Before treatment	After treatment[b] with:		
		Serum	Heat	MCAC
Mussels (1:15 dilution)	>2.00	0.03	0.07	0.02
Clams	0.48	0.03	0.06	0.01
Oysters	0.33	0.04	0.05	0.02
Winkles	0.64	0.04	0.09	0.03
Squid	0.31	0.02	0.05	0.01
Smelt	0.68	0.03	0.06	0.02
Trout	0.31	0.03	0.06	0.02
SE-positive controls[c]	0.32	0.30	0.30	>2.00
SE-negative controls[d]	0.05	0.02	0.04	0.01

[a] Values are the average of duplicate experiments. An OD of ≥0.20 is considered positive.

[b] The treatments used were serum (0.1 volume of rabbit or calf normal serum was added to 1 volume of food extract, and the mixture was then held at room temperature for 2 min), heat (food extracts (1 to 2 ml) were heated to 70°C for 3 min), and MCAC (by a clean-up procedure of MCAC using copper-chelate Sepharose gel [2], food extracts were concentrated from 50 to 5 ml [10 times]).

[c] Purified SE types A and B at a final concentration of 1.0 ng/ml were added to known TECRA-negative SE-containing mussel, squid, and trout extracts. These samples served as positive controls.

[d] Mussel, squid, and trout extracts which yielded negative SE reactions with the TECRA kit.

Elimination of nonspecific SE reactions with the TECRA kit. The following procedures, listed in Table 3, were found to be satisfactory to eliminate nonspecific SE reactions with seafood samples tested with the TECRA kit. (i) A 0.1 volume of rabbit or calf normal serum was sufficient to remove the TECRA nonspecific SE reactions from 1 volume of seafood

TABLE 2. Comparison of three commercial kits for the detection of SEs in raw seafood

Seafood	No. of samples tested	TECRA kit			No. positive by RIDASCREEN and RPLA kits
		No. of positive samples (%)	OD or OD range for positive samples[a]	Average	
Mussels	34	29 (85)	0.23–>2.00	>2.00	0
Clams	28	9 (32)	0.25–0.48	0.33	0
Oysters	26	6 (23)	0.21–0.33	0.25	0
Winkles	15	3 (20)	0.32–0.64	0.45	0
Conch	12	0 (0)	<0.19		NT[b]
Shrimp	20	0 (0)	<0.19		NT
Lobster meat	13	0 (0)	<0.19		NT
Crabmeat	10	0 (0)	<0.19		NT
Squid	15	3 (13)	0.25–0.31	0.25	0
Smelt	15	3 (20)	0.31–0.68	0.48	0
Cod	10	0 (0)	<0.19		NT
Trout	10	1 (10)	0.31		0
Salmon	10	0 (0)	<0.19		NT
Total	218	54 (24.8)			

[a] An OD of ≥0.20 is considered positive.

[b] NT, not tested.

extracts when the mixture was allowed to react for 2 min at room temperature. The procedure did not affect the serological activity of standard SEs. Similar results were observed with the false-positive broth obtained by culturing *Serratia rubidaea* (22). (ii) A mild heat treatment of the seafood extracts (70°C for 3 min) eliminated false-positive SE reactions. The recovery of SE from the treatment was approximately 95%. (iii) MCAC with a copper-chelate Sepharose gel (2-h procedure) gave excellent results in the elimination of false-positive SE reactions by the TECRA kits. Additional advantages of this procedure include partial purification of SE from food components (9, 14) and a 20-fold SE concentration while still maintaining quantitative recovery of the toxins (2, 11).

In conclusion, the TECRA kit which has a polyvalent capture antibody against SE types A to E is a convenient, economical, rapid, and sensitive assay for screening staphylococcal enterotoxins in foods. However, as we have shown in these results, the TECRA kit would lead to a significant number of false-positive results if used for assaying certain seafood extracts, particularly mussel extracts, without treatment with rabbit or calf normal serum, mild heat, or copper-chelate-Sepharose gel chromatography. When identification of the enterotoxin type is essential, either the RIDASCREEN or RPLA assay must be used. However, we could not routinely use either of these assays for screening large numbers of samples, because of their prohibitive cost.

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