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Nonspecific Reactions of a Commercial Enzyme-Linked Immunosorbent Assay Kit (TECRA) for Detection of Staphylococcal Enterotoxins in Foods

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A staphylococcal enterotoxin visual immunoassay kit (TECRA) has recently become commercially available. Since the kit is an enzyme-linked immunosorbent assay system equipped with polyvalent antisera against staphylococcal enterotoxin types A to E (SEA to SEE) and the test is simple and rapid to perform (4 h), it has been widely used for screening purposes. In this study, the sensitivity of the kit for detection of SEA, SEB, and SEC in ham, cheese, and mushrooms was similar to those of kits based on an enzyme immunoassay and reversed passive latex agglutination: 0.75 to 1.0 ng of SEA per ml, 0.5 to 0.75 ng of SEB per ml, and 1.0 to 1.25 ng of SEC per ml. However, the TECRA kit showed nonspecific reactions with food samples contaminated by microorganisms other than *Staphylococcus aureus*, such as *Enterobacter agglomerans*, *Enterobacter cloacae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Serratia marcescens*. The substance contributing to the false-positive results differed from true staphylococcal enterotoxins in that it was (i) heat labile (completely inactivated by heating for 2 min at 100°C, whereas true staphylococcal enterotoxins were inactivated by about 10% with this treatment), (ii) lower in molecular weight than staphylococcal enterotoxins, and (iii) not bound 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 problem of false-positive results with the TECRA kit could be resolved by heat treatment (2 min at 100°C) or by cleanup procedures involving metal chelate affinity chromatography with copper chelate Sepharose for 4 h before use of the TECRA kit.

Staphylococcal food poisoning is caused by the ingestion of enterotoxins produced in foods by some strains of *Staphylococcus aureus*. Growth of enterotoxigenic strains of *S. aureus* to a population of 10^6 or more cells per g of food is generally considered necessary for production of a sufficient amount of enterotoxin to cause intoxication if the food is consumed (20). A number of antigenically different types of staphylococcal enterotoxins (SEs) have been identified: SEA, SEB, SEC_1, SEC_2, SEC_3, SED, and SEE (5). The enterotoxins SEC_1, SEC_2, and SEC_3 are very closely related antigenically and can be identified by their cross-reactions with antibodies prepared against any one of them (5). Of the SEs, SEA is the one most commonly involved in staphylococcal food poisoning outbreaks (4, 16). Studies indicate that as little as 100 to 200 ng of SEA can produce symptoms of intoxication (9).

To detect this low level of enterotoxin in 100 g of food (1 to 2 ng of enterotoxin per g), several sensitive detection methods have been employed: (i) a radioimmunoassay (6, 8, 17, 22); (ii) an enzyme-linked immunosorbent assay (2, 10, 11, 14, 21), and (iii) a reversed passive latex agglutination assay (RPLA) (16, 23, 26, 27). Among these assay techniques, some commercial kits are now available: four enzyme-linked immunosorbent assay kits (an enzyme immunoassay [EIA] kit made in Switzerland, a visual immunoassay [TECRA] kit made in Australia, and an ELISA membrane kit and an ELISA tube kit made in France) and one RPLA kit made in Japan. The TECRA kit employs a polyvalent antisera system against enterotoxin types SEA to SEE and is both simple and rapid to perform; it is therefore widely used for screening purposes. Unfortunately, on several occasions when testing mushrooms from swollen cans and meat products, we have experienced false-positive results with the TECRA kits. To date, little information is available on the specificity of the kit with respect to contaminated foods.

The purpose of this study was to evaluate the specificity and sensitivity of the TECRA kit and to find a solution to the problem of false-positive results when assaying SEs in various foods.

MATERIALS AND METHODS

Sources of SE assay kits. The SE visual immunoassay kits (originally called TECRA, then called REPORT, and now called TECRA again) were produced by Bioenterprises Pty. Ltd., Roseville, New South Wales, Australia, and were distributed by Microbiology Products, 3M Health Care, St. Paul, Minn.; they are now available from International Bioproducts, Inc., Redmond, Wash. Additional SE assay kits used in this study included the Swiss EIA, which was commercially obtained from W. Brommeli A. G., Bern, Switzerland, and the RPLA manufactured by Denka Seiken Ltd., Tokyo, Japan, for Oxoid Ltd., Basingstoke, Hampshire, England, and distributed by Oxoid Canada Inc., Nepean, Ontario, Canada.

Enterotoxin production in foods. Microorganisms other than *S. aureus*, as well as *S. aureus* strains (SEA producers), used in this study were from our culture collection. The cultures were individually grown in tryptic soy broth (Difco) for 48 h at room temperature; then each culture (50 ml) was washed twice with sterile saline (0.85% NaCl) by centrifugation at 10,000 × *g* for 20 min. The pellet was resuspended

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in 50 ml of saline, and 5 ml of the cell suspension was inoculated into each of SE-free and sterile food samples consisting of ham, salami, cheese, mushrooms, and pasta (300 g per sample). These food samples were left at room temperature for 7 to 10 days to simulate natural conditions. As SE-negative controls, SE-free and sterile food samples were incubated under the same conditions. For SE-positive controls, purified SEs were added to the SE-negative control food extracts. The purified SEA was a gift from M. S. Bergdoll (Food Research Institute, University of Wisconsin, Madison); SEB was purchased from Makor Chemical Co. Ltd., Jerusalem; SEC_2 was prepared in our laboratory (25).

Extraction of SEs from foods. Equal, double, and triple volumes of SE extraction medium were added to 50 to 100 g of high-moisture, semidry, and dry food samples, respectively, in blender jars, and then the preparations were blended at high speed for 2 to 3 min. The extraction media 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 7.4]) for the RPLA and Swiss EIA kits. The homogenates were centrifuged at 16,300 × *g* for 20 min, and the supernatants were filtered through a membrane (pore size, 0.2 μm).

Procedures for concentrating food extracts. To increase the concentration of SE in food extracts, the following two procedures were used. (i) As described in the procedures accompanying the TECRA kit, the food extract (100 ml) was concentrated to 1 ml or less by dialysis (normally overnight) against 30% polyethylene glycol (molecular weight, 20,000) with a porous membrane tubing (molecular weight cutoff point, 12,000 to 14,000; Spectrum Medical Industries Inc., Los Angeles, Calif.). The outside of the tubing was washed thoroughly with cold water then the tubing was soaked in extraction medium until the concentrated extract was reconstituted to about 5 ml. The extract was then centrifuged at 16,300 × *g* for 20 min, the supernatant fluid was collected, and the pH of the fluid was adjusted to 7 to 8 if necessary. (ii) Metal chelate affinity chromatography was carried out with copper chelate Sepharose gel as previously described (7) with 100-ml samples of food extracts. Eluted SEs were normally found in the 12th to 16th tubes of 1-ml fractions.

SE analysis. The enterotoxin assay was carried out as recommended by the manufacturers of the kits used unless otherwise stated. Color reactions developed by the TECRA and Swiss EIA kits were measured by optical densities (OD) at 414 and 409 nm, respectively, with a microtiter reader (Minireader II; Dynatech Laboratories Inc., Alexandria, Va.).

Detection of peroxidase. The presence of peroxidase was determined by adding 50 μl of the substrate reagent (2,2 azinobis-3-ethylbenzthiazoline-sulfonic acid) provided with the TECRA kit to an equal volume of food extract. Formation of a blue color within 1 min indicated a positive test for peroxidase. As a positive standard, 50 μl of horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) solution (0.0026 purpurogallin unit per ml) was added to the substrate reagent.

RESULTS AND DISCUSSION

Sensitivity of the TECRA kit for detecting SE in food extracts. The minimum detectable amounts of SE in three different food extracts were determined by the TECRA kit and compared with those obtained by the Swiss EIA and RPLA kits. The values from the TECRA kit with ham, cheese, and mushroom samples were 0.75 to 1.00 ng of SEA per ml, 0.50 to 0.75 ng of SEB per ml, and 1.00 to 1.25 ng of SEC per ml, respectively. These values were approximately one-half of those previously reported with the TECRA kit (2) and were similar to those of the Swiss EIA and RPLA kits (Table 1). The data with the Swiss EIA and RPLA kits were comparable to those reported previously (12, 23, 28). The sensitivities of these kits were remarkably higher than those of immunodiffusion methods: about 100 times that of the 6-h microslide immunodiffusion assay (19) and 500 times that of the normal microslide immunodiffusion assay (11) or optimum-sensitivity plate method (5). Since the TECRA kit utilizes polyvalent antisera for detecting (but not differentiating) SEA to SEE in a single test with high sensitivity and the assay time is short (4 h), it is convenient for screening SE in foods. However, if individual toxin types are to be identified, a monovalent serological system, such as the Swiss EIA, RPLA, microslide immunodiffusion assay, or optimum-sensitivity plate method, would have to be used. It should be noted that, in the case of food samples containing low levels of SE (1 to 2 ng/ml of food extract), the detection limits of 100 and 500 ng of SE per ml with the microslide and optimum-sensitivity plate methods, respectively (5, 11, 19), may not be reached even though the food extracts are concentrated 20 to 50 times.

TABLE 1. Minimum detectable limits of three commercial kits for testing SEs in ham, cheese, and mushroom extracts

Food extract[a]	SE	Minimum amt[b] (ng/ml) of SE detected by:		
		TECRA	Swiss EIA	RPLA
Ham	SEA	1.00	0.75	0.75
	SEB	0.75	0.50	0.50
	SEC	1.25	1.00	1.00
Cheese	SEA	1.00	1.00	1.25
	SEB	0.75	0.75	1.00
	SEC	1.25	1.25	1.25
Mushroom	SEA	0.75	0.75	0.50
	SEB	0.50	0.75	0.50
	SEC	1.00	1.00	1.00

[a] Ham and cheese were extracted with 2 volumes of extraction medium, and mushrooms were extracted with 1 volume of extraction medium. Cheese extracts were acidified (pH 4.6) with 4 N HCl and centrifuged at 16,300 × *g* for 20 min, and the supernatant fluids were neutralized with 4 N NaOH. If the extracts were still turbid, they were recentrifuged. Before SEs were added to the food extracts, all extracts were tested with the three kits to confirm whether they were SE free. Purified SEs were added to individual food extracts at final concentrations of 0.5, 1.0, and 1.5 ng/ml.

[b] Averages of duplicate experiments.

False-positive results with the TECRA kit for SE testing. SEs are produced exclusively by the staphylococci, primarily *S. aureus*, although new species (*Staphyococcus intermedius* and *S. hyicus*) have been reported to be enterotoxigenic (1, 15). However, the TECRA kit gave false-positive results with unheated and unconcentrated extracts of mushroom samples in which nonstaphylococcal microorganisms had grown (Table 2). This was particularly evident in samples involving growth of *Enterobacter cloacae*, *Proteus mirabilis*, and *Serratia marcescens*. In these samples, the very strong positive results (calculated at an OD of >3.00) exceeded the OD values of samples containing SEA-producing strains of *S. aureus* or samples containing more than 10 ng of purified SEA per ml. This was in contrast to the results with the RPLA and Swiss EIA kits, which gave negative

TABLE 2. Comparison of TECRA results[a] with mushrooms contaminated with non-*S. aureus* microorganisms and *S. aureus* and mushroom extracts to which purified SEs were added

Prepn	OD at 414 nm of extract			
	Unconcentrated		Concentrated (20×)	
	Unheated	Heated[b]	Dialysis[c]	MCAC[d]
Mushrooms contaminated by:				
Enterobacter agglomerans (HPB-EA-M1)	0.68	0.02	0.32	0.02
Enterobacter cloacae (HPB-EC-M2)	>3.00	0.04	1.30	0.03
Proteus mirabilis (HPB-PM-M2)	>3.00	0.05	1.27	0.03
Pseudomonas aeruginosa (HPB-PM-M1)	0.84	0.01	0.48	0.01
Serratia marcescens (HPB-SM-M1)	>4.00	0.03	1.39	0.03
Staphylococcus sciuri (HPB-SS-M1)	0.31	0.01	0.21	0.01
Staphylococcus aureus (HPB-SEA-PI)	1.69	1.52	>20.00	>30.00
Staphylococcus aureus (HPB-SEA-P3)	1.38	1.23	>20.00	>20.00
Mushroom extracts spiked with:				
SEA (1 ng/ml)	0.27	0.23	>4.00	>4.00
SEB (1 ng/ml)	0.33	0.30	>5.00	>6.00
SEC (1 ng/ml)	0.25	0.22	>4.00	>4.00
SE negative controls	0.01	0.02	0.03	0.01

[a] Results are averages of triplicate tests. An OD of ≥0.20 is considered positive. ODs above 2.00 are calculated values based on dilution factors.
[b] Two milliliters of extract was heated in a boiling water bath for 2 min and then cooled immediately.
[c] One hundred milliliters of extract was concentrated to 5 ml by dialysis against 30% polyethylene glycol in dialysis tubing with an exclusion of 12,000 to 14,000 molecular weight.
[d] One hundred milliliters of extract was concentrated to 5 ml by metal chelate affinity chromatography (MCAC) with copper chelate Sepharose.

tests with all food extracts from salami, ham, and pasta associated with nonstaphylococcal contamination and positive tests only with samples harboring growth of *S. aureus* and samples containing aliquots of purified SE (Table 3). Naturally occurring peroxidase can cause false-positive results with the TECRA kit (13). However, the false-positive results with the TECRA kit observed in our experiments were not related to peroxidase, since the enzyme was not detectable in the food extracts tested (data not shown).

Differences between the substance causing false-positive TECRA results and SEs. The following are major differences between the substances giving rise to false-positive results and SEs. (i) The substance causing false-positive results in the non-*S. aureus*-contaminated food extracts were completely inactivated by heating for 2 min in boiling water, whereas the *S. aureus*-contaminated food extracts and the solutions of purified SE were little affected (Tables 2 and 3); with mushroom extracts, heating reduced the OD of SEs by about 10% (Table 2). (ii) The molecular weight of the substance contributing to false-positive TECRA results with non-*S. aureus*-associated food extracts was probably less than 12,000, because approximately 95% of the substance was able to pass through the dialysis tubing (12,000-molecular-weight exclusion). This was in contrast to the samples associated with *S. aureus* and standard SE solutions (Tables 2 and 3). The molecular weights of SEs are in the range of 27,000 to 29,000 (5). (iii) When food extracts associated with non-*S. aureus* cultures were allowed to interact with copper chelate Sepharose, the substance contributing to false-positive results with TECRA kits remained in the unbound wash fraction. This phenomenon suggested that these substances were not typical proteins, since the imidazole groups of their

TABLE 3. Comparison of TECRA, RPLA, and Swiss EIA kits for the detection of SEs in salami, ham, and pasta samples contaminated by *S. aureus* (SEA producers) and non-*S. aureus* microorganisms[a]

Microorganism inoculated	Detection of SEs with:											
	TECRA				RPLA				Swiss EIA			
	UH	H	D	M	UH	H	D	M	UH	H	D	M
E. agglomerans (HPB-EA-M1)	+	−	+	−	−	−	−	−	−	−	−	−
E. cloacae (HPB-EC-M2)	+	−	+	−	−	−	−	−	−	−	−	−
P. mirabilis (HPB-PM-M2)	+	−	+	−	−	−	−	−	−	−	−	−
P. aeruginosa (HPB-PA-M1)	+	−	+	−	−	−	−	−	−	−	−	−
S. marcescens (HPB-SM-M1)	+	−	+	−	−	−	−	−	−	−	−	−
S. sciuri (HPB-SS-M1)	V	−	V	−	−	−	−	−	−	−	−	−
S. aureus (HPB-SEA-P1)	+	+	+	+	+	+	+	+	+	+	+	+
S. aureus (HPB-SEA-P3)	+	+	+	+	+	+	+	+	+	+	+	+
SE-positive controls[b]	+	+	+	+	+	+	+	+	+	+	+	+
SE-negative controls[c]	−	−	−	−	−	−	−	−	−	−	−	−

[a] UH, unheated food extracts; H, heat-treated food extracts (see footnote *b* of Table 2); D, food extracts concentrated (20×) by dialysis; M, food extracts concentrated (20×) by metal chelate affinity chromatography. +, positive results; −, negative results; V, assay results varied with the type of foods (e.g., positive tests with salami and negative with other foods).
[b] Purified SEA and SEB at a final concentration of 1.0 ng/ml were added to each food extract used for SE-negative controls.
[c] Extracts of food samples to which neither microorganisms nor SEs were added.

histidines and the thiol groups of their cysteines would have bound to the gel (18, 24). This was in contrast to the samples containing SE-producing strains of *S. aureus* or purified SE (Tables 2 and 3), in which SE was quantitatively recovered from the gel in a concentrated form (Table 2). These results support the data previously reported by Dickie and Akhtar (7). The physicochemical nature of the substance causing the nonspecific reaction with the TECRA kit remains to be studied.

In conclusion, the TECRA kit is a convenient and sensitive tool for screening staphylococcal enterotoxins in foods. However, the kit suffers from a lack of specificity, as shown by a number of false-positive results obtained with foods in which non-*S. aureus* microorganisms had grown. The problem of false-positive results with the TECRA kit can be resolved by heating the samples in boiling water for 2 min or by a cleanup procedure with copper chelate Sepharose chromatography for 4 h before use of the TECRA kit.

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