Enzyme-Linked Immunosorbent Assay for Detection of Staphylococcal Enterotoxins in Incriminated Foods and Clinical Specimens from Outbreaks of Food Poisoning

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(Received 10 January 1990/Accepted 10 March 1990)

Jpn. J. Vet. Sci. 52(4): 847–850, 1990

KEY WORDS: ELISA, food poisoning, staphylococcal enterotoxin.

Staphylococcal food poisoning is caused by enterotoxins (ETs) produced by *Staphylococcus aureus* [2]. ETs are antigenically typed into five, A (ET-A), B (ET-B), C (ET-C), D (ET-D) and E (ET-E). Different methods have been developed for detection of ETs in foods [1, 3–9, 11, 12, 16, 17]. Enzyme-linked immunosorbent assay (ELISA) has recently been used more commonly since, not requiring the use of radioactive materials, it is as quick to perform and sensitive as radioimmunoassay (RIA) [1, 4–8, 16, 17]. ETs produced in different foods have been analyzed by sandwich ELISA with either polystyrene balls or microtiter plates as the solid-phase support [1, 4–8, 16, 17]. The ball system has more commonly been used [4–6] since such equipment as a multichannel pipetter or a micro-ELISA-plate reader is not necessary. The ball system is less efficient but more sensitive than the microtiter plate system. Foods incriminated for outbreaks of staphylococcal food poisoning have usually been analyzed for ETs by ELISA or other methods [5, 6, 8, 9, 11, 16], whereas such clinical specimens as vomitus and feces have seldom been analyzed. Attempts were made, therefore, to detect ETs by sandwich ELISA in clinical specimens obtained from as well as foods incriminated for outbreaks of staphylococcal food poisoning.

Purified ETs and rabbit anti-ET sera were prepared by the methods described previously [13–15]. Anti-ETs were each purified by affinity chromatography on ET-coupled CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden). Horse radish peroxidase (Toyobo Co. Ltd., Osaka) was conjugated to purified anti-ET by the method of Nakane and Kawaoi [10]. After NaBH₄ treatment, the anti-

body-enzyme conjugate was fractionated by gel filtration on Sephadex G-200 equilibrated with 0.01 M phosphate buffered saline, pH 7.2 (PBS). The fractions containing the antibody-enzyme conjugate were pooled and stored at –20°C until used.

Polystyrene balls (0.25 inch, Ono Pharmaceutical Co., Osaka) were washed with deionized water for 4 hr and then with 0.1 M sodium carbonate buffer, pH 8.4. The washed balls (300 balls) were agitated in 45 ml of the carbonate buffer containing a purified anti-ET (10 µg/ml) for 4 hr at 30°C and then for further 12 hr at 4°C [5]. The coated balls were washed three times in 100 ml of PBS, soaked in PBS containing 1% bovine serum albumin (BSA), and stored at 4°C until used.

ETs in incriminated foods, vomitus, and feces were extracted by suspending 10 g of a sample in 10 ml of PBS, pounding to homogeneous slurry with a Colworth 400 Stomacher (Seward Lab., England), and centrifuging at 12,000 rpm for 20 min at 4°C. Rabbit normal serum was added to the supernatant to 5%. The mixture was incubated for an hour at 37°C and used for ELISA. ETs were each added to food to 5 ng/ml before extraction to serve as positive controls.

Twenty-well plates designed for ELISA (Ono Pharmaceutical Co., Osaka) were used. A mixture of 100 µl of a test sample and 200 µl of 4% rabbit normal serum in PBS was added to each well. A polystyrene ball coated with purified anti-ET was added to the mixture in each well. The whole plate was incubated for 60 min at 37°C. Each ball was washed three times in 2 ml of PBS and 250 µl of 100-fold diluted antibody-enzyme conjugate was added. The plate was incubated for 2 hr at 37°C. After washing in a similar way, each ball was transferred to a test tube containing 500 µl of 0.1 M citrate-phosphate buffer, pH 5.7, containing 0.02% H₂O₂ and 3

mg/ml of o-phenylenediamine (Nakarai Chemicals, Ltd., Kyoto). The tubes were kept standing in the dark for 60 min at 37°C to develop color. The reaction was stopped by addition of 3 ml of 1 N H₂SO₄. The developed color was read at 492 nm with a Shimadzu double-beam spectrophotometer UV-150 (Shimadzu Co., Ltd., Kyoto). A buffer blank and negative controls were also assayed for any nonspecific color reaction. Absorbances obtained with three or five determinations were averaged, from which the average blank value was subtracted. The net absorbances were plotted against ET concentrations.

Extracts of foods and clinical specimens were also determined for ETs by reversed passive latex agglutination (RPLA) with RPLA test kits (Denka-Seiken Co., Tokyo). The tests were carried out with 96-well V-bottomed microtiter plates. The extract was diluted twofold serially in PBS containing 0.5% BSA. The reaction mixture contained 25 μ l of a diluted extract and 25 μ l of latex sensitized with purified anti-ET. Agglutination was read after incubation for 18–24 hr at room temperature.

For detection and enumeration of *S. aureus*, a 10 g sample was mixed with 90 ml of sterilized PBS. The mixture was homogenized with the Colworth 400 Stomacher. The homogenate was

diluted to 1:10¹ to 1:10⁵. *S. aureus* in each dilution was counted by the surface plating technique on mannitol-NaCl agar plates supplemented with 3% egg yolk. After incubation for 24–48 hr at 37°C, yellow colonies or colonies surrounded with opaque zones were counted. Some colonies were picked up for testing biochemical properties and ET production.

Standard curves for ELISA were generated by assaying each of ET-A, ET-B, and ET-C at 0, 0.5, 1, 2.5, 5, and 10 ng/ml. From the standard curves, ETs at 1 ng/ml or higher concentrations were detectable (Fig. 1). With culture supernatants of *S. aureus*, much higher A_{492} was obtained than with PBS, brain heart infusion or 4% NZ-amine used as negative controls. Similar results have been reported by other workers [5, 12, 16]. Since a high absorbance of culture supernatant is due to protein A produced by *S. aureus* [5, 12, 16], rabbit normal IgG was used to adsorb it. Experiments were performed to determine the optimum concentration of rabbit normal IgG to reduce nonspecific color reaction in ELISA with culture supernatants. Rabbit normal IgG was added to culture supernatant to final concentrations of 1, 5 and 10%. A_{492} of culture supernatant was significantly reduced with rabbit normal IgG in 5 and 10% and not in 1%.

Fig. 1. Dose response of purified enterotoxin in ELISA

Symbols: —●—; ET-A
—▲—; ET-B
—■—; ET-C

Table 1. Detection of enterotoxins in incriminated foods and clinical specimens by ELISA and RPLA test

Outbreak No.	Sample	Sample size (gram)	<i>S. aureus</i> population (per gram)	Toxin type of isolates	Type and quantity of ET in sample measured by	
					ELISA	RPLA
1	Rice balls	60	1.6×10^8	A	A(3.6) ^a	A(2-4)
2	Creamed puff	80	4.8×10^7	A+B	A(2.4), B(0.7)	A(2-4), B(0.5-1)
	Vomit	50	2.3×10^6	A+B	A(1.6), B(0.6)	A(1-2), B(0.5-1)
3	Rice balls	110	6.4×10^6	A	A(1.6)	A(1-2)
	Vomit	40	7.2×10^5	A	A(0.7)	A(0.5-1)
4	Box lunch	90	2.5×10^6	A	A(1.2)	A(1-2)
5	Omelet	70	8.1×10^7	A+C	A(1.8), C(1.2)	A(1-2), C(0.5-1)
	Vomit	50	3.6×10^6	A+C	A(0.6), C(0.6)	A(0.5-1), C(0.5-1)
	Feces	10	2.7×10^5	A+C	A(-), C(-)	A(-), C(-)
6	"Sushi"	120	4.1×10^7	A	A(2.6)	A(2-4)
	Feces	10	7.2×10^4	A	A(-)	A(-)

a) (): Amount of enterotoxin ($\mu\text{g}/\text{sample}$). (-): Undetectable amount of enterotoxin.

Therefore, culture supernatant was treated with 5% of rabbit normal IgG before analysis for ET by ELISA in this study. By such a pretreatment of culture supernatant, ET at 1 ng/ml or higher concentrations in culture supernatant was detectable. The sensitivity of the present method is on the same level as those reported previously [4-6, 17].

To test for the applicability of the present ELISA system for detection of ETs in food, different foods added with known amounts of ETs were analyzed. Food extracts were treated with 5% rabbit normal IgG before analysis. When ETs (ET-A, ET-B and ET-C) were added to extracts of creamed puff, cooked rice or omelet to 5 ng/ml, more than 92 to 96% of these ETs were recovered, confirming the usefulness of sandwich ELISA to analyze different food extracts for ETs [4-6, 17]. Furthermore, attempts were made to analyze different foods implicated in outbreaks of staphylococcal food poisoning and in such clinical specimens as vomitus and feces for ETs. In the extracts of specimens involved in six outbreaks, 1.6-3.6 μg of ETs were detected in food extracts from six outbreaks (Table 1). ET-A + ET-B and ET-A + ET-C were detected in extracts of creamed puff and omelet, respectively (Table 1). ETs in extracts of clinical specimens were also detected in extracts of vomitus by ELISA, whereas no ET was detectable in feces (Table 1). Similar results were obtained by RPLA with the same samples (Table 1). *S. aureus* populations of test samples are also

presented in Table 1. The results show that the sandwich ELISA is as sensitive to detect ETs in foods and clinical specimens as RPLA. Analysis of incriminated foods and clinical specimens for ETs by the sandwich ELISA can be completed within 4.5-5.0 hr. From the present results, therefore, we recommend sandwich ELISA with polystyrene balls as a fast, reliable, and sensitive method for detection of ETs in clinical specimens as well as various incriminated food.

ACKNOWLEDGEMENTS. The authors thank Dr. M. Ando and Mr. T. Hanyu, Tsuruga Enzyme Plant of Toyobo Co. Ltd. for their assistance in carrying out the experiments. We also thank Sendai Municipal, Saitama Prefectural, and Iwate Prefectural Institutes of Public Health for their providing test specimens involved in outbreaks of staphylococcal food poisoning.

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要 約

ELISA 法による食中毒原因食品および患者材料中のブドウ球菌エンテロトキシンの検出(短報): 品川邦汎・渡辺浩二¹⁾, 松坂尚典・小沼博隆²⁾・杉井俊二³⁾(岩手大学, ¹⁾仙台市食肉検査所, ²⁾国立衛生試験所, ³⁾北里大学)——精製抗エンテロトキシン-IgG を吸着したポリスチレンボールを用いるサンドウィッチ ELISA によるブドウ球菌エンテロトキシン検出法を検討し, 微量(1ng/ml)の毒素を特異的に, かつ5時間以内に検出できた。さらに, 本方法により食中毒原因食品および吐物からエンテロトキシン0.6-3.6 μ g を直接検出することができた。