APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1997, p. 2361–2365 Vol. 63, No. 6
0099-2240/97/$04.00+0

In Vitro Assay of *Staphylococcus aureus* Enterotoxin A Activity in Food

LINDA RASOOLY,[1] NOEL R. ROSE,[1,2] DHIREN B. SHAH,[3] AND AVRAHAM RASOOLY[2,3]*

Department of Molecular Microbiology and Immunology[1] and Department of Pathology,[2] Johns Hopkins University, Baltimore, Maryland, and U.S. Food and Drug Administration, Division of Microbiological Studies, Washington, D.C.[3]

Received 3 February 1997/Accepted 24 March 1997

Staphylococcus aureus **enterotoxin A (SEA) is a leading cause of food poisoning. The current test for functional activity of SEA requires monkeys or kittens. The major drawbacks of animal assays are lack of quantitation, poor reproducibility, low sensitivity, and high cost. In this report we describe and evaluate an alternative assay using T-cell proliferation to measure SEA activity in food. Human and rat lymphocytes proliferate in response to concentrations of SEA as low as 1 pg/ml, well below the pathogenic dose of 100 ng. This proliferation assay is highly sensitive, quantitative, and simple. Nonradioactive assays of T-cell proliferation were also suitable for detecting and measuring SEA, although with a 10-fold lower sensitivity. To evaluate the utility of this assay for food testing, four different food samples were mixed with SEA. In each sample, SEA was detected at a concentration of 1 ng/ml. Heat-inactivated SEA produced no detectable proliferation. These results demonstrate that an in vitro cell proliferation assay is an advantageous alternative to existing animal assays for measuring SEA activity in food.**

Staphylococcus aureus enterotoxin A (SEA) is a leading cause of food intoxication (1). SEA is an extremely potent gastrointestinal toxin; as little as 100 ng is sufficient to cause symptoms of intoxication (5).

The current assay to detect SEA in food is an enzyme-linked immunosorbent assay (ELISA) (8, 9). In this assay, monovalent or polyvalent antibodies to SEA are reacted with a food sample. However, the ELISA does not measure the functional activity of the toxin. This can be a problem in testing heat-processed foods where SEA may no longer be serologically active due to heat denaturation.

The current test for biological activity of SEA involves the use of monkeys or kittens. In the monkey feeding test (3), developed in 1948, the animals are administered a 25- to 100-ml food sample by gavage into the stomach. The activity of SEA in the food sample is assessed by the presence or absence of a vomiting reaction in the experimental animal. At low levels of toxin (5 to 10 mg/animal) only 50% of the animals react. At higher levels of toxin (20 mg/animal) 75 to 83% of animals react. The assay using kittens requires prior inactivation of substances initially present in the food samples that cause similar symptoms to those caused by the enterotoxin (2). The inactivated sample is then injected intravenously into kittens. Both animal assays have serious drawbacks for SEA analysis, including lack of quantitation, poor reproducibility of results, low sensitivity, and high cost associated with maintaining animals.

SEA has at least two separate biological effects. It acts both as an enterotoxin on gastrointestinal cells and as a superantigen, stimulating non-antigen-specific T-cell proliferation (6). There is a high correlation between both biological effects of SEA. In most cases loss of SEA superantigen activity (due to mutations) results in loss of SEA enterotoxic activity (7). High correlation was also demonstrated between loss of mitogenic activity and lack of serologic activity of SEA after thermal inactivation (12). The use of mitogenic activity of SEA as a marker for the detection of SEA in foods has been suggested previously (11).

The goal of this research was to develop an in vitro assay that would exploit the superantigen activity of SEA as a means of evaluating the level of active SEA in processed and unprocessed food. The T-cell proliferation assay described here is highly sensitive, quantitative, and simple to perform. We show here that human as well as rat lymphocytes proliferate in response to low quantities of SEA. We demonstrate the applicability of this assay for detection of low levels of SEA in food and lack of proliferation in response to control samples of food. We also show that nonradioactive assays are suitable for measuring SEA, although the sensitivity was 10-fold lower than the radioactive assay. These results demonstrate that the in vitro proliferation assay is a good alternative to existing in vivo animal assays for measuring SEA activity in food.

MATERIALS AND METHODS

Proliferation of lymphocytes. Lymphocytes were isolated from 5 ml of heparinized human or Lewis rat blood (2.5 ml of blood per rat), and purified on Ficoll-Paque (Pharmacia). Blood taken from each rat was sufficient for testing 100 samples and did not require sacrifice of the animals. Lymphocytes were incubated at 0.5×10^5 to 1×10^5 cells per well (corresponding to approximately 25 μl of blood per sample) in 96-well U-bottomed tissue culture plates (Costar) with 100 μl of lymphocytes per well diluted in culture medium (10% fetal calf serum in RPMI medium) and 100 μl of either culture medium with varying amounts of SEA (supplied by the Food and Drug Administration) or food samples spiked with SEA per well. Plates were incubated for 2 to 5 days at 37°C with 5% CO_2. On the last day, 1 μCi of [*methyl*-^{3}H]thymidine (Amersham) (4) or 20 μl of Alamar blue (BioSource) was added per well. After 24 h plates were harvested with a Micromate 196 harvester (Packard Instrument, Groningen, The Netherlands) onto glass fiber filters (Packard Instrument) and dried at room temperature. Radioactivity was read continuously for 5 min on a Matrix 96 Direct Beta Counter (Packard Instrument). Alternatively, the color reaction of Alamar blue was read with a Dynatech MRX plate reader at 570 nm.

Preparation of food samples. Ten grams of potato salad, canned mushrooms, or hot dogs from local stores was homogenized with 10 ml of phosphate-buffered saline (PBS). Samples were centrifuged at 1,000 × *g* for 10 min, and the supernatant was collected. Milk was prepared as 5% (wt/vol) dry milk (Carnation) in PBS. Food samples were tested either directly, with different concentrations of SEA, or after boiling in water for 15 min or autoclaving for 40 min. Food samples

* Corresponding author. Mailing address: Food and Drug Administration, 200 C St. SW, Washington, DC 20204. Phone: (202) 205-4192. Fax: (202) 401-7740.

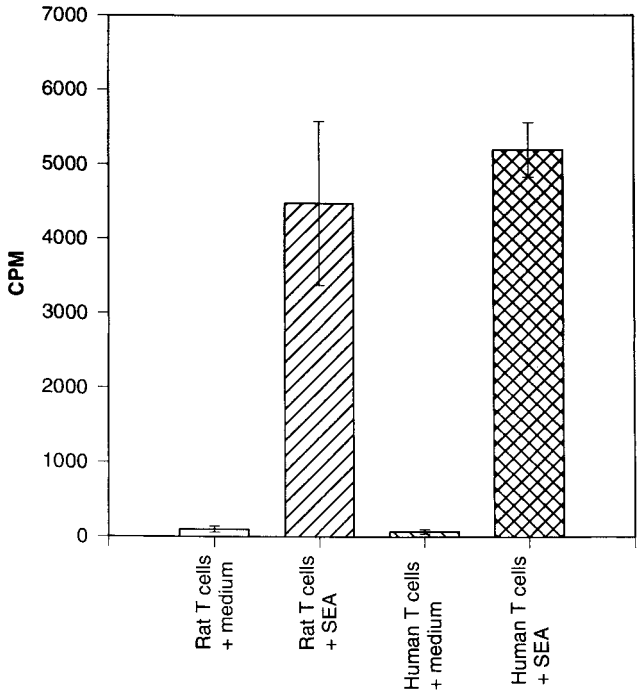

FIG. 1. Comparison between the proliferation of human and rat lymphocytes in response to SEA. Lymphocytes were isolated from heparinized blood and purified on Ficoll-Paque. Lymphocytes were incubated at a concentration of 10^5 cells per well with 10 ng of SEA per well for 4 days. [^{3}H]thymidine was added, and plates were harvested and radioactivity was measured on the fifth day. Data are means ± standard errors.

were diluted 1:10 in PBS and sterilized by gamma-radiation prior to being spiked with SEA.

RESULTS

Comparison of human and rat lymphocyte proliferation in response to SEA. SEA was identified by T-cell proliferation of rat or human lymphocytes as measured by the uptake of radioactive [^{3}H]thymidine (Fig. 1). SEA elicited a strong proliferation response comparable to other mitogen-activated responses (e.g., that elicited by concanavalin A). Upon prolonged incubation of the lymphocytes in the presence of SEA, T-cell death was observed (data not shown). Background proliferation levels were low in both human and rat lymphocyte cultures.

Dose response of rat T-cell proliferation to SEA. In order to compare the sensitivity of the radioactive and nonradioactive proliferation assays, decreasing amounts of SEA were added to rat lymphocytes. The color change of Alamar blue (which measures cell metabolism) was compared with the uptake of [^{3}H]thymidine (a measure of DNA synthesis). The radioactive assay showed a significant level of proliferation ($P < 0.05$), compared with control medium, at levels as low as 0.1 pg of SEA per well (a concentration of 1 pg/ml) (Fig. 2B). The Alamar blue assay detected significant levels of proliferation at a concentration of 1 ng/well, but no response was detectable at lower SEA concentrations (Fig. 2A). However, the results of the Alamar blue assay were less reproducible than the results of the radioactive method (data not shown). These results suggest that the radioactive proliferation assay is 10-fold more sensitive than the Alamar blue detection method, but both methods detect SEA at levels that are well below those which are considered to cause human illness.

Detection of SEA in food. In order to assess the ability of the cell proliferation assay to measure the presence of SEA in foods, we tested food products artificially contaminated with SEA. In previous work, inhibitory factors were removed by dialysis (11). However, this procedure made the assay more complex. We evaluated dilution of food samples as a simpler method of overcoming the possible inhibitory effect of food. Four food products that are frequently contaminated with SEA (milk, potato salad, mushrooms, and hot dogs) were homogenized with PBS (1:1, wt/wt), and the homogenates were diluted 1:10 and spiked with various levels of SEA. Proliferation of rat lymphocytes was assessed in response to food alone, food spiked with SEA, and food spiked with SEA and autoclaved. The diluted foodstuffs without SEA did not induce T-cell proliferation (Fig. 3). With the addition of as little as 100 pg of SEA per well (1 ng/ml) to the food samples, however, statistically significant ($P < 0.05$) proliferation was measured in all foods (Fig. 3).

The T-cell response to SEA in food correlated well with increasing amounts of SEA in the different foods with the exception of milk (Fig. 3D). In milk, proliferation at 10 ng/well was lower than that for the sample with 1 ng/well, but the difference between the two values was not statistically signifi-

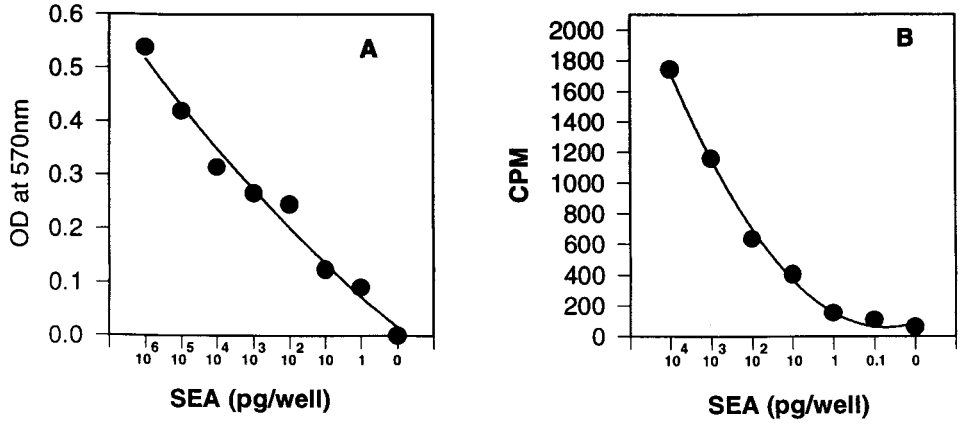

FIG. 2. Comparison of Alamar blue optical density (OD) (A) and thymidine incorporation (B) for measurement of proliferation in response to SEA. Lymphocytes were isolated from heparinized Lewis rat blood and purified on Ficoll-Paque. The assays were performed as described in the legend for Fig. 1 except for the addition of Alamar blue on day 4. OD was read at 570 nm on the fifth day.

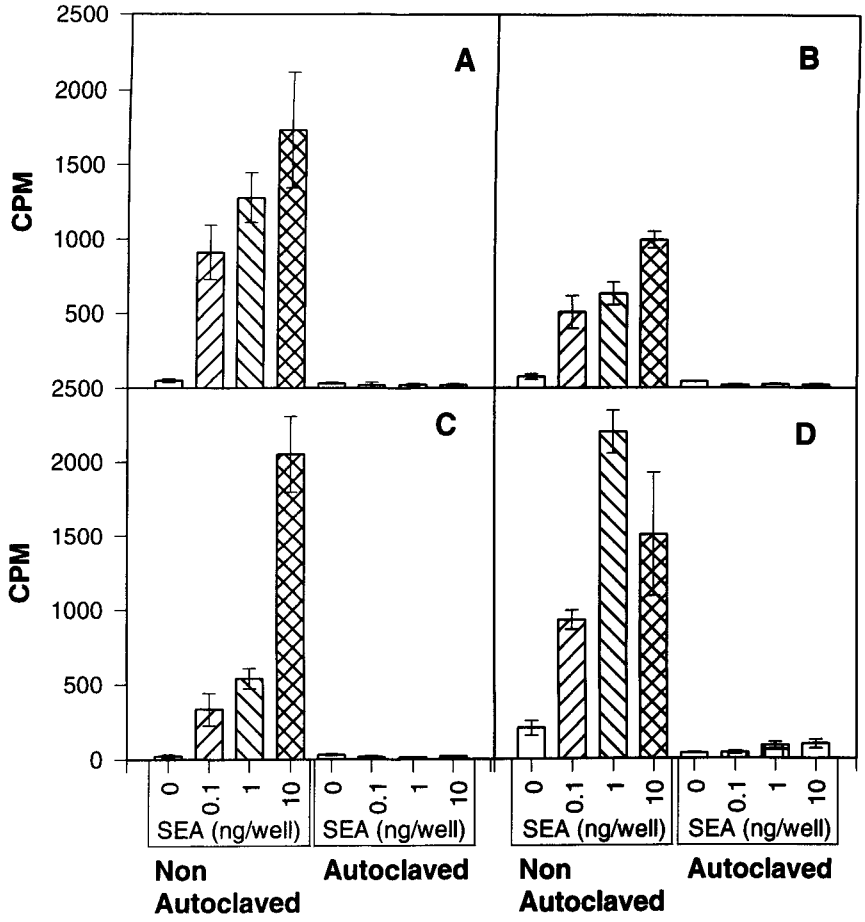

FIG. 3. Detection of SEA in hot dogs (A), potato salad (B), mushrooms (C), and milk (D) with a proliferation assay. Lewis rat lymphocytes were isolated from heparinized blood and purified on Ficoll-Paque. Lymphocytes were incubated at 10^5 cells per well with food samples for 2 days. [^{3}H]thymidine was added, and plates were harvested 24 h later and radioactivity was measured. Food was homogenized, diluted 10-fold in PBS, and tested in the presence or absence of different concentrations of SEA either directly or following autoclaving for 40 min at 121°C. Data are means ± standard errors.

cant. The effect of SEA on proliferation was totally abrogated after autoclave inactivation of SEA in all four foods (Fig. 3). There was some variability in the absolute level of SEA-induced proliferation observed in different foods. For example, SEA in potato salad gave a lower response than the other samples at all concentrations. However, this variability, which is probably due to interactions with food ingredients, does not affect the ability of the assay to detect SEA, although it may hamper precise quantitation of SEA.

These results suggest that T-cell proliferation assays are able to detect even low levels of SEA in various food products.

T-cell proliferation as a tool to measure inactivation of SEA. One of the possible applications of the T-cell proliferation assay is to measure the effect of food processing on inactivation of SEA. We used the assay to evaluate the effects of various treatments on SEA activity. As seen in Fig. 4, SEA is a relatively heat-stable protein, so it retains significant activity even after 15 min of boiling. Autoclaving the SEA sample for 40 min, however, abolished all the mitogenic activity of SEA (Fig. 3). In addition, the presence of milk in the SEA sample increased the efficiency of SEA heat inactivation compared with medium alone (Fig. 4). It appears that properties of the food itself (such as protein content, pH, etc.) affect the heat stability of SEA, as was suggested previously (9).

DISCUSSION

When the immune system encounters an ordinary antigen, it is normally degraded by the antigen-presenting cells (such as B cells, macrophages, or dendritic cells), and antigen fragments are presented to T cells. Only T cells that have the appropriate antigen-specific receptor respond by proliferation. In the case of superantigens such as SEA, activation is not limited by the antigenic specificity of the T cells. Instead, the intact toxin binds and cross-links the T-cell receptor and antigen-presenting cells of the major histocompatibility complex class II. The formation of this bridge between the two molecules causes an activation of the T cell and results in T-cell proliferation. Because the activation is not restricted to certain antigen-specific T-cell receptors, the toxin is able to activate a large portion of the T cells.

The results presented here demonstrate that T-cell proliferation is a sensitive measure of SEA activity in food. This assay can detect SEA at levels as low as 1 pg/ml (Fig. 2). These results are at the same sensitivity level as those of previous studies in which human cells were used (11). Although the nonradioactive assay is less sensitive than the radioactive method, it is able to detect SEA at levels that are well below those considered pathogenic (Fig. 2). Nonradioactive assays

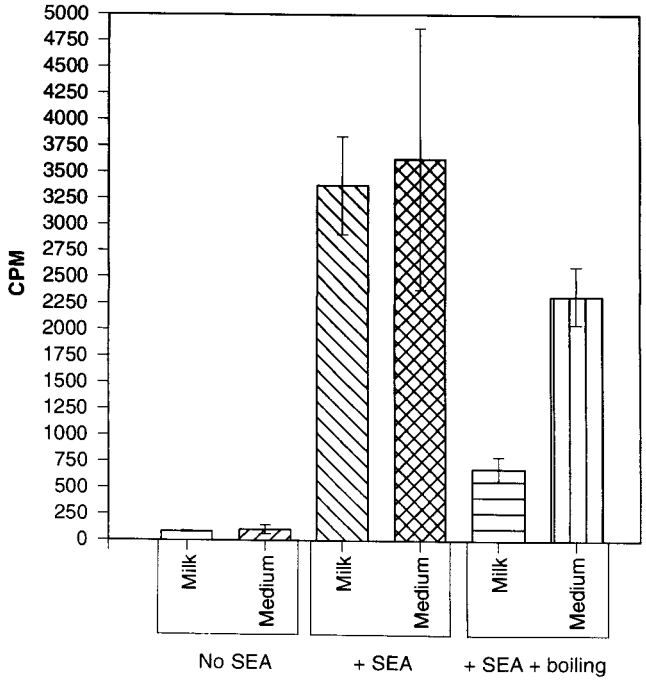

FIG. 4. Effect of boiling on SEA activity. Lewis rat lymphocytes were isolated from heparinized blood and purified on Ficoll-Paque. Milk (5% [wt/vol] dry milk) and culture medium (RPMI medium with 10% fetal calf serum) were tested with and without 10 ng of SEA per well and with 10 ng of SEA per well after boiling for 15 min. Lymphocytes were incubated at a concentration of 10^5 cells per well with the samples for 1 day. [^{3}H]thymidine was added, and plates were harvested 24 h later and radioactivity was measured. Data are means ± standard errors.

have many advantages, including easier handling of materials and lower cost for performing the assay because a cell harvester and radioactivity reader are not required. Thus, the nonradioactive assay is potentially useful for screening for SEA contamination of food samples in many settings.

We demonstrate here that the radioactive proliferation assay detects SEA in four kinds of food products that are common sources of food poisoning (Fig. 3). This finding suggests that T-cell proliferation assays are versatile enough to detect SEA activity in many different types of foods. The application of this assay to food testing requires only that the correct dilution level (level with a low background) be determined for each specific food product (normally in the range of 1:10).

It was reported earlier (12) that after heat treatment of SEA (at 121°C), mitogenic activity was lost more rapidly than serological activity. Because the binding between SEA and T cells requires a functional toxin, the proliferation assay described here also has the advantage of being able to determine the inactivation of SEA during food processing (Fig. 3 and 4) and, as a result, may be used to monitor the effectiveness of food processing. For example, we have shown that inactive toxin (autoclaved) does not induce proliferation. However, as was demonstrated previously (10) and as shown in Fig. 4, the rate of SEA inactivation is dependent on the ingredients of the medium in which the toxin is present.

The proliferation assay described here measures proliferation in response to superantigen. However, it cannot identify the specific superantigen. Such identification will require characterization of the superantigen by Western immunoblotting ELISA, or other identifying methods. In order to rule out proliferation activity because of the presence of food ingredients, a control of food alone is required in the proliferation assay. In order to quantitate the amount of SEA in the samples, control samples with known amounts of SEA need to be run parallel to the test samples.

A simplified in vitro assay was developed to overcome the many limitations of the current in vivo animal tests that measure SEA activity. One of the main limitations of the current animal tests is the lack of SEA quantitation. As seen in Fig. 2, the T-cell proliferation assay shows a dose-response curve to decreasing amounts of SEA, thus allowing accurate quantitation of the amount of SEA in the sample. Even in complex food samples, where precise quantitation may not be possible, the relative amount of SEA activity could be determined among different samples (Fig. 3).

Another important limitation of the animal SEA activity assays is that very few samples and replicates can be tested because the assays are time-consuming and cumbersome and require the use of a single animal per sample. The in vitro assay allows multiple samples and replicates to be tested, because only 25 μl of blood is required to test one sample (Fig. 3).

We found that human and rat T cells did not differ significantly in their response to SEA (Fig. 1). Because rat blood is readily available and presents a lower risk of disease transmission, it appears to be a good source of T cells for SEA measurement. Thus, up to 100 samples can be tested with one bleeding of one rat, without necessitating the sacrifice of the animal. For better standardization and convenience, development of SEA-reactive T-cell clones may be desirable.

The proliferation assay takes more time than the animal assay (3 to 5 days versus 4 h). Upon optimization, 3 days were found to be sufficient to show the proliferative response of lymphocytes to SEA. This delay normally does not present a major problem in terms of public health. However, the actual set-up of the test for 33 samples takes merely 2.5 h, and the rest of the time is utilized for incubation of the lymphocytes with SEA. In comparison, the animal assay requires administration of the toxin to the animals and constant monitoring of the vomiting reaction. Although the length of time required to perform the proliferation assay is longer, other considerations, such as the requirement of purchasing kittens of the same age and the necessary maintenance of the animals, make the proliferation assay a more practical assay for SEA testing. Development of techniques to measure other parameters of cellular proliferation (such as cytokine production) may shorten the time required for assay performance to several hours.

In summary, we have developed a highly sensitive method for SEA activity detection and quantitation in food that does not require the sacrifice of animals and will specifically detect only biologically active SEA. The advantages of this assay system are that it is relatively straightforward and the costs are low. This alternative to the current animal assay can be performed in any laboratory, and it has potential application to the monitoring of quality control during food processing.

REFERENCES

1. **Archer, D. L., and F. E. Young.** 1988. Contemporary issues: disease with a food vector. Clin. Microbiol. Rev. **1:**377–398.
2. **Bergdoll, M. S.** 1972. The enterotoxin, p. 301–331. *In* J. Cohen (ed.), The staphylococci. Wiley Interscience, New York, N.Y.
3. **Bergdoll, M. S.** 1988. Monkey feeding test for staphylococcal enterotoxin. Methods Enzymol. **165:**324–333.
4. **Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober (ed.).** 1990. Current protocols in immunology, p. 3.15.5–3.15.10. Wiley Interscience, New York, N.Y.
5. **Evenson, M. I., M. W. Hinds, R. S. Bernstein, and M. S. Bergdoll.** 1988. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. Int. J. Food Microbiol. **7:**311–316.
6. **Frieman, S. M., J. R. Tumang, and M. K. Crow.** 1993. Microbial superanti-

gens as etiopathogenic agents in autoimmunity. Rheum. Dis. Clin. North Am. **19:**207–222.
7. **Harris, T. O., D. Grossman, J. W. Kappler, P. Marrack, R. R. Rich, and M. J. Betley.** 1993. Lack of complete correlation between emetic and T-cell-stimulatory activities of staphylococcal enterotoxins. Infect. Immun. **61:**3175–3183.
8. **Park, C. E., M. Akhtar, and M. K. Rayman.** 1993. Simple solutions to false-positive staphylococcal enterotoxin assays with seafood tested with an enzyme-linked immunosorbent assay kit (TECRA). Appl. Environ. Microbiol. **59:**2210–2213.
9. **Park, C. E., M. Akhtar, and M. K. Rayman.** 1994. Evaluation of a commercial enzyme immunoassay kit (RIDASCREEN) for detection of staphylococcal enterotoxins A, B, C, D, and E in foods. Appl. Environ. Microbiol. **60:**677–681.
10. **Schwabe, M., S. Notermans, R. Boot, S. R. Tatini, and J. Kramer.** 1990. Inactivation of staphylococcal enterotoxins by heat and reactivation by high pH treatment. Int. J. Food Microbiol. **10:**33–42.
11. **Stelma, G. N., and D. L. Archer.** 1980. Detection of mitogenic activity of staphylococcal enterotoxin A in foods. IRCS Med. Sci. **8:**347.
12. **Stelma, G. N., J. G. Bradshaw, P. E. Kauffman, and D. L. Archer.** 1980. Thermal inactivation of mitogenic and serological activities of staphylococcal enterotoxin A at 121°C. IRCS Med. Sci. **8:**629.