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Highly sensitive fluorogenic enzyme-linked immunosorbent assay: detection of staphylococcal enterotoxin B[1]

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Abstract

A highly sensitive, indirect double sandwich fluorogenic enzyme-linked immunosorbent assay (FELISA), has been developed for the detection of staphylococcal enterotoxin B (SEB). The described FELISA allows a SEB quantification of 0.1 fg/ml for purified toxin and a detection limit of at least 10 pg/g of contaminated food. Given the sensitivity of FELISA elaborate procedures for the extraction and concentration of enterotoxin from food samples became unnecessary. The monoclonal anti-SEB antibody (MCA a-SEB) used in the described method reacted with SEB only. The FELISA is simple to perform and, provided the reaction plates are pre-prepared, the results can be obtained in approximately 3 h.

Key words: Enterotoxin B; Fluorogenic ELISA; Monoclonal antibody; *Staphylococcus aureus*; Toxin

1. Introduction

Among food poisoning, staphylococcal poisoning is one of the most common food borne illnesses. It is caused by a closely related group of enterotoxins, which are water soluble, simple proteins released into the medium during growth by certain strains of the gram-positive bacterium, *Staphylococcus aureus*. These toxins are of small molecular weight (26–29 kDa) and have been differentiated by their immuno-

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[1]In conducting the research described in this report, the investigators adhered to the 'Guide to Care and Use of Experimental Animals' published by Canadian Council on Animal Care.

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logical specificity into seven distinct types designated as staphylococcal enterotoxin SEA, SEB, SEC_1, SEC_2, C_3, SED and SEE [1]. Very recently a new enterotoxin (SEF) has been identified and characterized [2].

The amount of enterotoxin required to cause illness in humans is still not determined. It is however estimated that 1 μg or even less can induce food poisoning symptoms in an average weight individual [3] since sensitive detection methods are necessary to assess the safety of a food for consumption.

Several enzyme-linked immunosorbent assay (ELISA) methods have been described lately for the detection of SEB in culture supernatants and food extracts [4–7]. Nevertheless, because of the variability and complexity of food composition these methods are affected by interferences and as a consequence contaminated food can escape detection. Compared to colorimetric techniques the use of fluorometric enzyme substrates can lead to a 10^3-10^6 fold increase in detection limits [8,9]. In this report we describe a highly sensitive fluorogenic enzyme-linked immunosorbent assay (FELISA) for the quantitation and detection of purified or food extracted SEB down to femtogram (fg) and picogram (pg) values respectively.

2. Materials and Methods

2.1. Animals

Female New Zealand rabbits, age 6 months and female Balb/c mice, age 6 to 7 weeks were purchased from Charles River Ltd. (St-Constant, Qué.)

2.2. Reagents

Staphylococcal enterotoxins (A–E) and toxic shock syndrome toxin-1 (TSST-1) were purchased from Toxin Technology Inc. (Sarasota, FL). Diethanolamine (DEA), 4-methyl umbelliferyl-phosphate (4-MUP), pristane (2,6,10,14-tetramethylpentadecane), crystalline bovine serum albumin (BSA), 2,2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), alkaline phosphatase labelled goat anti rabbit IgG and horseradish peroxidase-labelled sheep anti-mouse IgG antibody [$F(ab')_2$ fragment] and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma Chemical Co. (St. Louis, MO). Phosphate buffered saline (PBS) tablets were purchased from Oxoid Canada Ltd. (Ottawa, Ont.). Dulbecco's Modified Eagle Medium (DMEM), hypoxantine-aminopterin-thymidine (HAT), polyethylene glycol (PEG 4000) and Freund's complete (FCA) and incomplete (FIA) adjuvants were purchased from GIBCO/BRL (Burlington, Ont.). Micro BCA* protein assay reagent was obtained from Pierce Chemical Co. (Rockford, IL). Immulon-4 flat-bottomed plates and Multi Screen-HA filtration plates were obtained from Dyanatech Laboratories (Alexandria, VA) and Millipore (Canada) Ltd. (Mississauga, Ont.). Protein A-Sepharose CL-4B was purchased from Pharmacia (Canada) Inc. (Baie D'Urfé, Qué.). The non-secretor myeloma cell line NS-1 (ATCC TIB 18) was obtained from American Type Culture Collection (Rockville, MD). Anti mouse class, subclass and light chain antibodies were purchased from ICN Biomedicals Canada Ltd. (Mississauga, Ont.). Falcon tissue culture labware was obtained from Fisher Scientific (Montréal, Qué.).

2.3. Equipment

Millititer filtration system was obtained from Millipore (Canada) Ltd. (Mississauga, Ont.). The MicroFLUOR reader was purchased from Dynatech Laboratories (Alexandria, VA) and the microplate ELISA reader, model EL-309 was obtained from Bio-Tek Instruments Inc. (Winooski, VT).

2.4. Protein estimation

Protein concentrations were determined using the sensitive micro BCA* protein assay reagent according to manufacturer's instructions. Crystaline BSA and purified mouse immunoglobulin G were used as standards for SEB and antibody quantitation.

2.5. Buffers and solutions

PBS, pH 7.4 was prepared according to manufacturer's indication. The coating buffer was comprised of 50 mM carbonate-bicarbonate, pH 9.6 containing 0.01% sodium azide. The blocking buffer was prepared by adding 2% BSA and 0.1% (v/v) Tween-20 to PBS, pH 7.4. The washing buffer consisted of PBS, pH 7.4 containing 0.1% (v/v) Tween-20 (PBS-T). 1 M DEA was prepared in water containing 20 mM $MgCl_2$ and the pH was adjusted to 9.8 by addition of 1 N HCl. The enzyme substrate 4-MUP, 0.1 mM was prepared in 1 M DEA buffer immediately prior to use. Triple distilled de-ionized water was used in all buffer and solution preparations.

2.6. Preparation of polyclonal anti-SEB antibodies

Six months old white New Zealand rabbits were injected subcutaneously with 10 μg of pure SEB in FCA. Fourteen days later, rabbits were injected subcutaneously with 25 μg of SEB in FIA. After one month, rabbits were given a third injection with 100 μg of SEB in FIA. One week after the last injection, rabbits were bled and the immunoglobulin G (IgG) fraction was obtained by affinity column chromatography. Protein A Sepharose column and serum were equilibrated using 0.1 M phosphate buffer, pH 8.0. After washing the column, the polyclonal anti-SEB (PCA anti-SEB) IgG fraction was eluted by using 0.1 M acetate buffer pH 4.0 and OD_{280} recorded. Fractions containing IgG were pooled and immediately neutralized to pH 7.2 with 2 M Tris and stored at –20°C until used.

2.7. Preparation of monoclonal anti-SEB antibodies

Balb/C mice were immunized intraperitoneally, at 1 week intervals, with 5, 10 and 20 μg of purified SEB emulsified in an equal volume of FCA. A volume of 0.2 ml was given to each mouse. After the first immunization protocol, the mice were allowed to 'rest' for one month then boosted with 25 μg of SEB in Freund's incomplete adjuvant. After an additional month of 'rest' the mice were given intravenously 30 μg of SEB in 0.2 ml sterile PBS. Three days later, the mice were killed and spleens removed aseptically.

The basic procedure of Galfré et al. [10] was used for hybridoma cell lines production. Briefly, spleens from two immunized mice were teased apart and the resultant cell suspension was passed through a stainless steel mesh. Red blood cells were

lysed by osmotic shock and washed with DMEM. The fusion was carried out with a 10:1 ratio of splenic lymphocytes to NS-1 tumour line. After centrifugation, the supernatant liquid was removed and 0.8 ml of 50% (w/v) PEG 4000 pre-warmed at 40°C, was added to the cells drop-wise over a period of 1 min with gentle stirring. The cells suspension was further diluted with DMEM over a period of about 10 min. The cells were again pelleted, washed 3 × with DMEM and resuspended in 40 ml of DMEM containing 20% fetal bovine serum. This suspension was distributed in 96-well tissue culture plates containing 10^5 cells/well rat hepathocytes as feeder cells.

The next day, and at intervals thereafter, the culture medium was changed and supplemented with HAT-medium. Hybridomas formed after 12–14 days of incubation at 39°C under 10% CO_2 and 95% humidity. The content of each well was tested by ELISA for reaction with SEB. Cells from positive wells were further cloned in soft agarose by the method of Coffino et al. [11]. After about 8–10 days, the clones were picked with a micropipette and placed in DMEM containing 20% fetal bovine serum in 96-cell culture plates. After 3–5 days, the culture fluid was tested for antibody content.

2.8. ELISA assay

Immulon-4, ninety-six-well plates were coated with 50 μl of different enterotoxins (A–E) at a concentration of 2 μg/ml of each toxin suspended in coating buffer and incubated at 4°C overnight. The plates were washed with PBS-T, the hybridoma supernatants (50 μl/well) were added and plates further incubated for 1 h at 37°C. The plates were then washed with PBS-T and 50 μl/well of sheep peroxidase labelled anti-mouse IgG was added to each well at a dilution of 1:1000 in PBS containing 10 mg/ml of BSA. After an additional incubation at 37°C for 1 h the plates were washed again with PBS-T and the reaction developed by adding 50 μl/well of ABTS. The OD_{405} was measured, after 20 min incubation at room temperature, with an ELISA reader. Positive results were considered if the sample OD exceeded two times the mean and standard deviation of control samples which contained culture medium as substitute for hybridoma supernatants.

2.9. Class and subclass determination of monoclonals anti-SEB

Four stable clones were obtained and clone E3B9A7 was selected for production of ascites fluid. Previously to ascites production the monoclonals were characterized for their class and subclass immunoglobulin type by immunodiffusion with specific antisera to mouse immunoglobulins. The selected hybridoma produced immunoglobulins of IgG_1 κ subclass.

2.10. Induction of ascites fluid and purification of anti-SEB monoclonal antibody

Three month old Balb/C mice were injected intraperitoneally three times with 0.5 ml pristane at weekly intervals. After a rest period of 10 days, 5×10^6 log-phase E3B9A7 hybridoma cells were injected into each mouse. Ascites fluid was collected from the abdominal cavity between 7 and 10 days following injection of hybridoma. Collected ascites fluid was centrifuged at 10,000 × g, precipitated with 50% saturated $(NH_4)_2SO_4$, resuspended in distilled water at 25% of initial volume and dialysed for

72 h against 0.1 M phosphate buffer, pH 8.4 containing 0.02% sodium azide. The monoclonal anti-SEB antibody (MCA anti-SEB) was then purified on Protein A-Sepharose column, equilibrated with the dialysis buffer. The MCA anti-SEB was eluted with 0.1 M citrate buffer pH 5.0. Fractions with OD_{280} reading more than 0.3 were pooled, neutralized to pH 7.2 with TRIS 2 M, dialysed against 0.1 M ammonium hydrogen carbonate and lyophilized.

2.11. Standardization of immmunoreagents

All the reagents used in the FELISA were titrated against each other to determine the optimum concentration to be used in final assays according to previously established procedures [12]. The concentration of PCA anti-SEB used at a third layer was determined by checkerboard titration. Blocking steps and incubation time were evaluated throughly and the experimental conditions that gave the highest ratio between the test and background values were used in this study.

2.12. FELISA procedure

The indirect double sandwich FELISA [9] was used with following modifications: MultiScreen-HA filtration plates were coated with 50 μl/well with MCA anti-SEB at a concentration of 1 μg/ml diluted in coating buffer and incubated overnight at 4°C. The plates were washed three times with PBS using the Millititer™ filtration system and the remaining binding sites were blocked by adding 200 μl of blocking buffer for 1 h at 37°C. After additional washing with PBS, 50 μl/well of SEB or food extract, at different dilutions in blocking buffer were added and plates incubated 1 h at 37°C. The plates were again washed and wells filled with 50 μl of PCA anti-SEB at a concentration of 10 μg/ml in blocking buffer. After incubation for 30 min at 37°C and washing with PBS 50 μl/well of goat anti rabbit IgG labelled with alkaline phosphatase (1:1000 diluted in blocking buffer) was added to wells and plates incubated for another 30 min at 37°C. Plates were finally washed 6 times with PBS-T. After blotting the plate bottom dry, enzyme substrate 4-MUP in a volume of 200 μl was added. Immunoassay plates were incubated at room temperature in the dark and the relative fluorescence due to the release of 4-methylumbelliferone (4 MU) was measured in a MicroFLUOR reader at excitation wave length of 365 nm and emmission at 450 nm after 15 min. For the analysis of MCA anti-SEB specificity an indirect FELISA was used. Wells were coated with 50 μl of individual enterotoxins (A–E) and TSST-1 at a concentration of 5 μg/ml in coating buffer. After blocking and washing 50 μl/well of MCA anti-SEB (1 μg/ml in blocking buffer) and plates were incubated for 1 h at 37°C. Plates were then washed and 50 μl/well of sheep anti-mouse IgG antibody labelled with alkaline phosphatase at 1:1000 dilution in blocking buffer was added. All subsequent steps were performed as described above.

2.13. Extraction of SEB from artificially contaminated food

Various amounts of purified SEB (12.5–125 ng) were added to 25 g of minced beef or cheddar cheese and homogenized in a blender for 30 s. The enterotoxin was then extracted according to a previously described method [4] from which the last chloroform step was omitted. Calculation of SEB in extracts of contaminated foods

was estimated by FELISA from a calibration curve using as diluent an enterotoxin-free food extract.

2.14. Data analysis

Samples of SEB were tested in six replicate wells and the fluorescence counts from each of the 6 values were averaged and standard deviation calculated. At least 4 separate samples were tested. Background (negative control) values were also averaged and two standard deviations were added to determine the cut-off value. Fluorescent counts equal to or above this cut-off value defined the lower limit of a positive result.

3. Results and discussion

In this investigation we describe a highly sensitive FELISA method for detection of staphylococcal enterotoxins using as an example SEB. The outline of the procedure is schematically shown in Fig. 1. The success of this method is based on the use

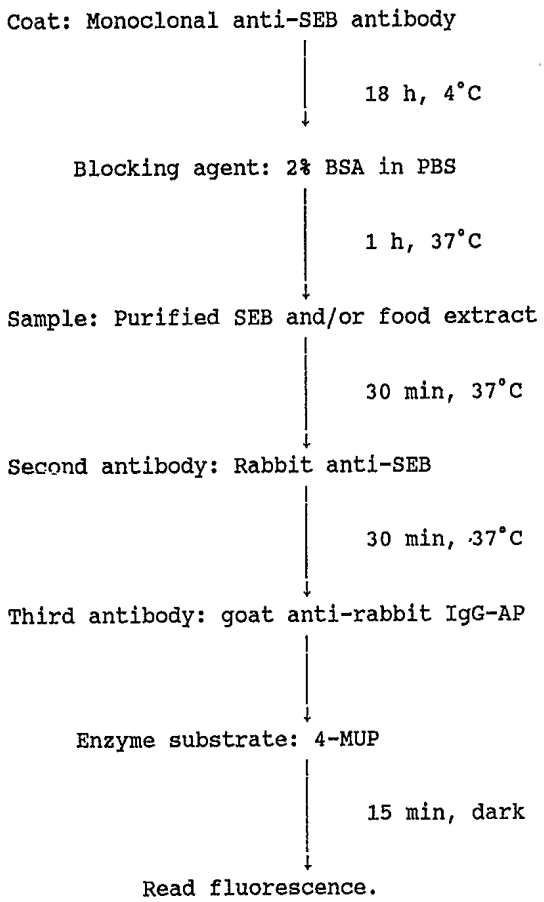

Fig. 1. Schematic outline of indirect double sandwich FELISA.

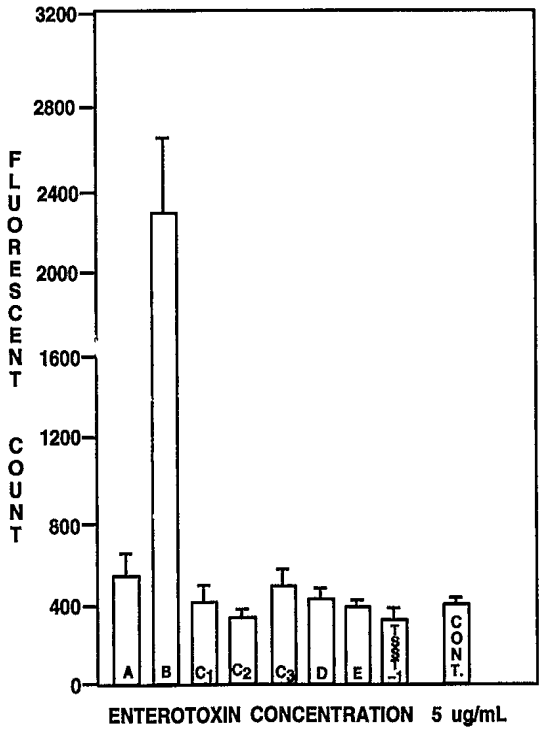

Fig. 2. Specificity of MCA E3B9A7 for staphylococcal enterotoxins.

of a specific and high affinity MCA to SEB. The specificity of the E3B9A7 MCA was tested in indirect FELISA by reacting the antibody with other available enterotoxins and TSST-1. As shown in Fig. 2 the MCA anti-SEB specifically reacted with SEB and no significant cross-reactivity was observed with other toxins. The sensi-

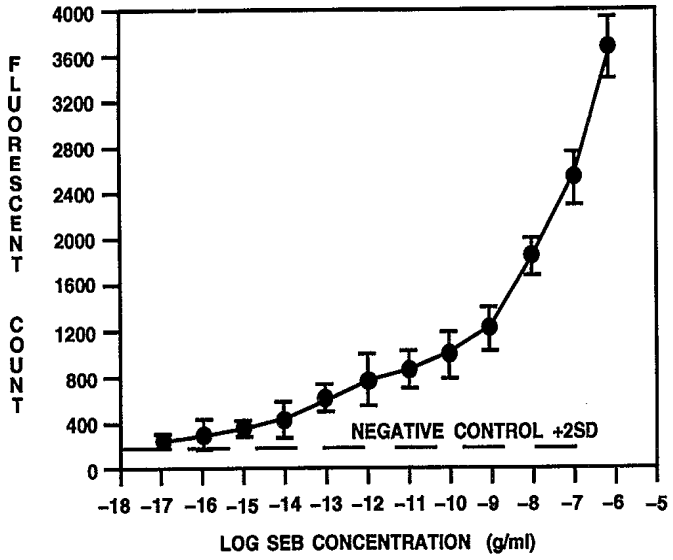

Fig. 3. Sensitivity of FELISA in detection of purified SEB.

Table 1
Detection and recovery of SEB from artificially contaminated food

Type of food	SEB added (ng/g)	SEB detected[a] (ng/g)	Recovery[b] (%)
Minced beef	0.5	0.24	48
	1.0	0.62	62
	5.0	3.90	78
Cheddar cheese	0.5	0.14	28
	1.0	0.46	46
	5.0	3.05	61

[a]Results were calculated considering the dilutions of extract and expressed as ng/g of food.
[b]Percentage rounded to the nearest decimal.

tivity of FELISA was determined by titrating varying concentrations of purified SEB and the results presented in Fig. 3 show that sub-femtogram/ml quantities of toxin can be easily detected. The sensitivity obtained by the described method is comparable with other reports in which similar detection limits have been reported [9,13,14]. The FELISA method can be applied for detection of enterotoxins in foods. We have artificially contaminated minced beef and cheddar cheese with increasing concentrations of SEB. The SEB was then extracted using only aqueous solvents and the results for quantitative measurements and recovery are summarized in Table 1. By omitting the chloroform extraction, our results for recovery are somewhat lower, especially in the case of cheese, if compared to other reports. Furthermore, it has been shown that the yield of recovery is dependent of type of enterotoxin and food texture [15,16]. Even in these conditions the hereby proposed FELISA has a detection sensitivity of pg SEB/g food and can be applied for the detection of other enterotoxins provided a highly specific and high affinity MCA to a given toxin is available. Several sensitive methods, with capacity to detect ng of toxin/g food has been reported [4–7,15,16], however, food poisoning outbreaks do occur in which the amount of enterotoxin is less than 1 ng/g [3]. In such situations, the enterotoxin can be detected only by very sensitive methods. On the other hand, it is essential to use these methods in determining the safety of food for consumption and to assess that no enterotoxin is present.

One problem encountered in staphylococcal enterotoxins detection by either radio- or enzyme-linked immunoassays is the interference of protein A, often secreted by *S. aureus* together with the enterotoxins. Protein A attaches to the Fc fragment of reactive antibody, hence false positive reactions could be recorded. Protein A interference could be prevented by removing it from samples by adsorption on rabbit IgG affinity column [17]. In this case, care must be taken for using only immunoglobulins devoided of anti-enterotoxin activity. Another simple method is the addition of normal rabbit serum to the food extract prior to enterotoxin determination [6].

In summary, we have developed a highly sensitive FELISA for detection of SEB. The presented method is easy to perform and can be completed in less than 3 h provided the assay plates are coated with antibody and blocked in advance.

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