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DETECTION OF STAPHYLOCOCCAL ENTEROTOXIN A, B, AND C IN MILK BY AN ELISA PROCEDURE

By

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OLSVIK, Ø., S. MYHRE, B. P. BERDAL and K. FOSSUM: *Detection of staphylococcal enterotoxin A, B, and C in milk by an ELISA procedure.* Acta vet. scand. 1982, *23*, 204—210. — Enterotoxigenic reference strains of Staphylococcus aureus were cultivated in sterile whole and skim milk for 18 h at 37°C. Staphylococcal enterotoxin A, B, and C were detected directly in the milk by an enzyme linked immunosorbent assay (ELISA), sensitive down to 1 ng/ml. Enterotoxins in the range of 1 ng—20 μg/ml milk were detected without any concentration or extraction. Skim and whole milk were almost identical as medium for enterotoxin production.

Stapholococcuc aureus; enterotoxin A, B and C; detection; milk; ELISA.

Staphylococcus aureus enterotoxins are the most common causes of human food poisoning. Ingestion of preformed enterotoxin in food have resulted in large-scale outbreaks. The enterotoxins, designated A-F, are heatstable and may therefore resist high temperatures involved in pasteurization and food processing. Dairy products like milk and cheese are involved in most of the large-scale outbreaks of S. aureus food intoxications (*Anderson & Stone* 1955, *Armijo et al.* 1957, *Allen & Stovall* 1960). Milk is easily exposed to staphylococci as these organisms are common in udder, and they may induce mastitis in cows (*Thatcher & Simon* 1956, *Garcia et al.* 1980, *Olsvik et al.* 1981). Milk contaminated with enterotoxigenic staphylococci could thus through storage reach high amounts of enterotoxins (*Donelly et al.* 1968). Cheese and other dairy products made from contaminated milk may therefore induce food poisoning when ingested (*Weed et al.* 1943, *Hendricks et al.* 1959, *Ikram & Luedecke* 1977).

To investigate outbreaks due to staphylococcal enterotoxins, the best diagnosis is performed by direct detection of the poisoning agent, the enterotoxins (*Read et al.* 1965, *Reiser et al.* 1974, *Bennett & McClure* 1980). Enzyme-linked immunosorbent assays (ELISA) for detection of staphylococcal enterotoxins A, B, and C (SEA, SEB and SEC), are well suited for toxin detection in bacterial broth supernatants (*Berdal et al.* 1981, *Olsvik et al.* 1981). In the present study an ELISA method is used for direct detection of these toxins in milk inoculated with enterotoxigenic Staphylococcus aureus strains.

MATERIALS AND METHODS

Bacterial strains

Three enterotoxigenic reference strains of S. aureus were received from the Food Research Institute, University of Wisconsin, Madison, Wisconsin, USA. Strain 2/81 (enterotoxigenic reference-strain No. 722) produced SEA, strains 4/81 (enterotoxigenic reference-strain No. 901) produced SEB, and strain 5/81 (enterotoxigenic reference-strain No. 137) SEC. The strains were all kept on human blood agar plates at 4°C for maximum 1 month.

Cultivation media

The strains were tested for enterotoxin production in 2 milk media (*Olsvik & Kapperud* in press): Whole milk (Fellesmeieriet, Oslo, Norway) was sterilized by autoclaving (120°C for 10 min). According to the producers information, the milk contained 4.8 % (w/w) carbohydrates, 3.9 % fat and 3.3 % protein. No external vitamins were added. The skim milk (Fellesmeieriet) was also sterilized by autoclaving. It contained 5.9 % carbohydrates, 3.4 % protein, 0.1 % fat, and was thus deprived of natural fat soluble vitamins.

Purified toxins

Purified SEA, SEB and SEC (*Berdal et al.* 1981) of various concentrations were mixed in the sterile milk and stored at room temperature overnight before ELISA toxin detection. Enterotoxin quantitation was carried out by using the standard curves obtained from these samples. Sterile milk samples were used as negative controls.

Enterotoxin production by bacterial strains

Five ml of each medium were put into 15 ml screw-capped culture tubes. After inoculation of 10^5 bacteria/ml, the caps were only partly closed to allow proper aeration during incubation. This was performed in a roller drum (Bellco, Vineland, New Jersey, USA) with the tubes inclined at nearly horizontal level. The roller drum was placed in a walk-in incubator at 37°C for 18 h, the tubes were then centrifuged for 20 min at 5000×g, and the supernatants stored at —20°C and examined for enterotoxin activity within 1 month.

Enterotoxin assay

The presence of enterotoxin A, B, and C was tested directly in the milk by a 4 layer sandwich ELISA (*Berdal et al.*).

Microtiter plates (Cooke, Alexandria, Virginia, USA) were coated with goat anti-SEA, SEB or SEC, 1:1000 in a 0.06 mol/l carbonate buffer, pH 9.6. The coating was allowed to occur for at least 48 h at 4°C, and the plates were kept in their performance when stored at 4°C up to 3 months when the coating buffer was kept in the wells.

Before use, the plates were washed 6 times with phosphate buffered saline with 0.05 % Tween-20 (Technicon, Tarrytown, New York, USA) (PBS-Tween). To each well was then added 0.05 ml PBS-Tween before an equal volume of the milk test sample was added. Two wells were used for each test. The plates were incubated on desk overnight in a humid atmosphere.

The microtiter plates were then washed 6 times with PBS-Tween before addition of 0.1 ml rabbit anti-SEA, SEB, or SEC, 1:1000 in PBS-Tween with 1 % normal goat serum. The plates were incubated for 2 h at 37°C in the humid atmosphere before another 6 times washing.

The conjugate, alkaline phosphatase-labelled goat-anti rabbit IgG was diluted 1:800 in PBS-Tween with 1 % normal goat serum, and 0.1 ml was put into each well. The incubation was identical to the last step, and after the last 6 times washing, the substrate was added. This consisted of 0.1 ml of 0.1 % *p*-nitrophenylphosphate in diethanolamine buffer and was allowed to react in the wells for 5 min before the enzyme process was stopped by adding 0.025 ml of 4 N NaOH. The yellow colour was quantitated photometrically at 410 nm by a Microelisa Minireader (MR 590, Dynatech, London, England).

Protein A

Prior to enterotoxin testing, the growth supernatants were assayed for protein A by the ELISA method described by *Olsvik & Berdal* (1981). The amount of protein A was so low that it did not influence the staphylococcal enterotoxin ELISA (*Berdal et al.*).

RESULTS

The ELISA recordings of standard curves prepared by mixing different amounts of purified SEA, SEB, and SEC into whole milk and skim milk, are shown in Fig. 1. The detection limit of 1 ng/ml was attained by all the enterotoxins in the milk. There was no significant difference between the sensitivity of the ELISA in whole milk and in skim milk.

The enterotoxin production of the 3 reference strains in the

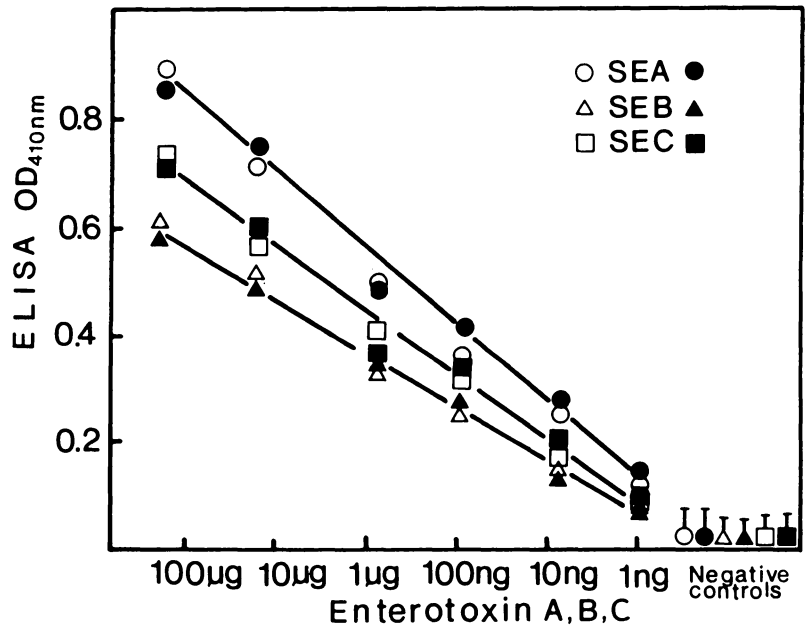

Figure 1. Standard curves showing the ELISA Optical Density (OD) response of purified SEA, SEB, and SEC mixed in whole milk and skim milk. Sterile milk samples were used as negative controls.
○ SEA in whole milk, ● SEA in skim milk
△ SEB in whole milk, ▲ SEB in skim milk
□ SEC in whole milk, ■ SEC in skim milk
Negative controls are indicated with their standard deviations.

2 milk media after 18 h at 37°C are shown in Table 1. SEA and SEB were produced in amounts of 1—2 and 20 ng/ml, respectively. The SEC producing strain did, however, produce large amounts of toxin, 10 µg—20 µg/ml. There was no difference between the quantity of toxin produced in the 2 milk media by these 3 strains.

Table 1. Enterotoxin A, B and C produced in whole milk and skim milk by 3 enterotoxigenic reference strains of Staphylococcus aureus growing at 37°C for 18 h.

Strains No.	Ref. No.	Toxin	Amount of enterotoxin produced	
			whole milk	skim milk
2/81	(722)	SEA	2 ng/ml	1 ng/ml
4/81	(901)	SEB	20 ng/ml	20 ng/ml
5/81	(137)	SEC	20 µg/ml	10 µg/ml

DISCUSSION

The ELISA method for specific staphylococcal enterotoxin detection reached a detecting level of 1 ng/ml directly in the milk. The method is simple and especially suited for large scale quantitative routine diagnosis. Several tests using immunological methods for direct diagnosis of these enterotoxins in milk have been published, which do not take into consideration the fact that protein A may give false positive results by binding to the Fc part of the antibody molecules in the coating, and then bind either the second antibody layer, or the conjugate directly (*van der Ouderaa & Haas* 1981, *Berdal et al.* 1981). However, in the present study protein A was not produced in sufficient amounts to influence the test.

The amounts of enterotoxin produced by different strains in culture broth were in the range of 1 ng—100 µg/ml (*Olsvik et al.* 1981). In the milk media also different amounts of enterotoxin were found, the SEC producing strain appeared to produce in the range of 1000—10,000 times more toxin than the others. This difference may, however, be under influence of components in the media.

Some organic elements block the repression of the genes encoding for enterotoxin production (*Martinez-Cadena et al.* 1981). An example of this is the presence of carbohydrates like lactose in milk, which blocks the production of the heat-stable enterotoxins of Yersinia enterocolitica and Escherichia coli (*Olsvik &*

Kapperud in press). The question whether the production of SEA and SEB in this study are depressed by components in the milk, or these strains really are low enterotoxin producers, remains open.

In this work a simple direct method for enterotoxin detection in contaminated milk was used successfully, without any extraction or concentration of the material. After bacterial killing by heating, the enterotoxins still remain active. Diagnosis of foodborne staphylococcus enterotoxin disease has so far usually been carried out by looking for the presence of temperature-stable nucleases or by isolation of coagulase positive staphylococci. However, micrococci, S. epidermidis and S. saprophyticus can produce enterotoxins. They produce, however, no coagulase, and usually no heat-stable nuclease (*Breckinridge & Bergdoll* 1971, *Olsvik et al.* submitted). The described ELISA method is therefore well suited by its direct enterotoxin detection in nutrients like milk instead of the indirect indications.

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SAMMENDRAG

Påvisning av staphylococcal enterotoxin A, B og C i melk ved en ELISA metode.

Stafylokokk enterotoxin A, B og C (SEA, SEB, SEC) ble målt i melk direkte ved en firelags enzym-merket immunologisk metode (ELISA). Ved å tilsette renset SEA, SEB eller SEC til skummet og hel melk, var det mulig å bestemme toxinmengder ned til 1 ng/ml. Enterotoksiske Staphylococcus aureus ble inokulert i steril hel og skummet melk og dyrket ved 37°C i 18 timer. Enterotoxin (A, B og C) mengden ble bestemt til 1 ng—20μg per ml, og det var liten forskjell på enterotoxin produksjonen i hel og skummet melk.

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