

Detection of Low-Enterotoxin-Producing *Staphylococcus aureus* Strains

NIGHAT P. KOKAN AND MERLIN S. BERGDOLL*

*Department of Food Microbiology and Toxicology, Food Research Institute, University of Wisconsin,
Madison, Wisconsin 53706*

Received 16 June 1987/Accepted 24 August 1987

Culture supernatant fluids from 26 (23.6%) monkey feeding test-positive *Staphylococcus aureus* strains, negative for enterotoxins by gel diffusion, were positive by enzyme-linked immunosorbent assay for one or more of the identified enterotoxins. Staphylococcal enterotoxin D (SED) was produced by 23 (88.5%) strains, SED and SEA were produced in two strains, and SED and SEC were produced in one strain. One strain produced only SEA, and two strains produced only SEC.

The determination of enterotoxin production by the staphylococci is usually done by examining culture supernatant fluids by double gel diffusion methods. The most sensitive of these methods, the microslide method, which can detect enterotoxins at concentrations of about 100 ng/ml (3), is used infrequently because of difficulties often encountered with it. The standard procedure used in the Food Research Institute for detecting enterotoxins is the membrane-over-agar method for production of the enterotoxins, with detection by the optimum sensitivity plate (OSP) method (8). Occasionally, it is necessary to concentrate the culture supernatant fluid severalfold to detect the small amounts of enterotoxin produced by some strains. Although the minimum sensitivity of the OSP method is around 500 ng of enterotoxin per ml of culture supernatant fluid, the overall sensitivity is increased 5- to 10-fold by using the membrane-over-agar method for production of the enterotoxins. Based on the estimated dose of enterotoxin (1 µg) required to produce staphylococcal food poisoning (1), it was assumed that more-sensitive methods for detection of enterotoxin production by staphylococcal strains were not needed. On occasion, however, a staphylococcal isolate from food implicated in staphylococcal food poisoning was found to be negative for all of the identified staphylococcal enterotoxins (A [SEA], B [SEB], C [SEC], D [SED], and E [SEE]), but did produce an emetic reaction in monkeys when the culture supernatant fluid was administered intragastrically (2). It was concluded that the strain produced an unidentified enterotoxin which was responsible for the food poisoning. Such strains appeared to be responsible for about 5% of food poisoning outbreaks.

A total of 110 *Staphylococcus aureus* strains, identified as producers of enterotoxins by the monkey feeding method (9; Table 1) but negative by the OSP method for the production of identified enterotoxins, were examined for production of small amounts of identified enterotoxins by an enzyme-linked immunosorbent assay (ELISA). ELISAs are approximately 500 times more sensitive than the OSP method. These assays were performed because new information showed that a dose of 100 to 200 ng of enterotoxin was sufficient to result in staphylococcal food poisoning (unpublished data). These *S. aureus* strains were isolated over a period of years from a variety of sources: 38 from food poisoning outbreaks, 25 from foods, 11 from uncooked fish,

26 from human nares, 4 from animal nares, and 6 from miscellaneous items. These strains either had been stored in the lyophilized form or were maintained on porcelain beads (6).

The membrane-over-agar method (7) was used for production of the enterotoxin with 3% N-Z Amine NAK (a pancreatic digest of casein; Sheffield Chemical Co., Union, N.J.) plus 1% yeast extract (Difco Laboratories, Detroit, Mich.) in agar as the medium. Incubation was at 37°C for 18 to 24 h. Enterotoxin detection was done by the ELISA microtiter plate method of Freed et al. (4). Normal rabbit serum (final concentration, 5%) was routinely added to the culture supernatants to eliminate possible protein A interference. Standard doubling dilutions of partially purified enterotoxin solutions (0.625 to 10 ng/ml) were included in each assay. The antiserum was prepared in rabbits (8) and partially purified by the method of Herbert et al. (5). All tests were run in triplicate.

Of the 110 strains, 26 (23.6%) were positive by ELISA for one or more of the identified enterotoxins (Table 1). SED was produced by 23 of the strains, either alone (20 strains) or in combination with SEA (2 strains) or SEC (1 strain). One strain produced only SEA, and two strains produced only SEC. Most strains produced 1 to 5 ng of enterotoxin per ml, whereas a few of the strains produced 10 to 20 ng of enterotoxin per ml. The maximum sensitivity of the OSP method, about 100 ng/ml when the supernatant fluids are concentrated fivefold, is adequate to detect these small amounts of enterotoxin. The combination of the membrane-over-agar production method with the OSP method for detection of the enterotoxins is equivalent in sensitivity to other production and gel diffusion methods, including the microslide method, which has a sensitivity of about 100 ng/ml when performed by experienced operators.

The specificity of the results obtained with ELISA was confirmed by examining culture supernatant fluids from five representative SED-producing strains after separate treatment with polyclonal antibodies to both SED and SEB. The culture supernatant fluids were mixed with an equal volume of a 1:20 dilution of polyclonal antiserum or with brain heart infusion as a control. The mixture was incubated either at room temperature for 2 h or at 37°C for 30 min before testing by ELISA. The reactions of five representative SED-producing strains with polyclonal antibodies are shown in Table 2. Essentially complete neutralization occurred if the reaction was due to SED. Although a partial neutralization

* Corresponding author.

TABLE 1. Emetic reaction of monkeys to *S. aureus* strains negative for identified enterotoxins by OSP

ELISA reaction	Source of strain	No. of cultures producing emetic reaction at dosage of:		
		50 ml	300 ml	1,000 ml
Positive	Food poisoning outbreak	3	5	2
	Foods	2	2	0
	Fish (raw)	6	0	0
	Nares (human)	1	1	2
	Nares (horse)	0	1	0
	Miscellaneous	1	0	0
Negative	Food poisoning outbreak	16	8	4
	Foods	8	7	6
	Fish (raw)	5	0	0
	Nares (human)	11	4	7
	Nares (horse)	0	3	0
	Miscellaneous	1	4	0

occurred with polyclonal SEB antisera, this may have been due to neutralization of protein A interference in the ELISA. This type of neutralization did not occur with a monoclonal antibody to SEB.

SED-producing strains have been the cause of a number of staphylococcal food poisoning outbreaks, particularly those related to dairy products. The amount of SED produced by most SED-positive strains is about one-fifth the amount of SEA produced by SEA-positive strains (7), which may account for the difficulty in detecting low-producing SED strains by the OSP method. The low SED producers are

TABLE 2. Reaction of SEB and SED polyclonal antisera with culture supernatant fluids from SED-producing *S. aureus* strains as determined by ELISA^a

Supernatant source (strain no.)	<i>A</i> ₄₁₀ when reacted with:		
	Brain heart infusion	Anti-SEB	Anti-SED
355	0.938 ± 0.072	0.806 ± 0.030	0.082 ± 0.040
382	0.194 ± 0.012	0.120 ± 0.020	0.023 ± 0.007
475	0.224 ± 0.039	0.155 ± 0.010	0.087 ± 0.081
547	1.025 ± 0.109	0.462 ± 0.070	0.096 ± 0.016
663	0.143 ± 0.022	0.103 ± 0.009	0.018 ± 0.008

^a The absorbance readings (*A*₄₁₀ ± standard deviation) for standard amounts of SED per ml were as follows: 0.625 ng, 0.203 ± 0.023; 1.25 ng, 0.488 ± 0.049; and 2.50 ng, 0.922 ± 0.127. The supernatants were diluted 1:1 with either brain heart infusion or the polyclonal antisera. The SED standards were dissolved in brain heart infusion.

significant because very little enterotoxin (100 to 200 ng) is required to make an individual sick, especially among children and the elderly. Although not all strains found to produce enterotoxins were isolated from foods implicated in food poisoning outbreaks, several of them were, including strains from several types of food. This is significant because when strains isolated from such foods are not found to produce an identified enterotoxin, it is usually considered unproductive to examine the food for the presence of enterotoxin.

Many of the staphylococcal strains that were negative for any of the identified enterotoxins by ELISA produced emesis in monkeys with as little as 50 ml (Table 1) of culture supernatant fluids with enterotoxin levels equivalent to those produced by enterotoxigenic strains easily detectable by OSP. The conclusion is that these strains produce an unidentified enterotoxin, because any substance produced by the staphylococci that elicits an emetic reaction in monkeys is considered an enterotoxin.

This research was supported in part by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by various companies and associations of the food industries.

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