APPLIED MICROBIOLOGY, Nov. 1967, p. 1382-1387
Vol. 15, No. 6

Serological Identification of Enterotoxigenic Staphylococci from Cheese

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Received for publication 15 June 1967

Single and double gel-diffusion techniques were employed to examine serologically coagulase-positive staphylococci from cheese for enterotoxigenicity. Supernatant fluid from sac cultures was examined for enterotoxins A and B. The results indicated that 9 of 155 cultures from market cheese and 7 of 77 cultures from food-poisoning cheese produced enterotoxin A, and that none of the cultures produced detectable levels of enterotoxin B. Results of serological tests were confirmed by intravenous injection of cats.

Cheese has been implicated in suspected staphylococcal food-poisoning outbreaks for many years. Several investigators have isolated coagulase-positive staphylococci from cheese (1, 12, 14); however, data identifying any of these coagulase-positive staphylococci as enterotoxigenic are limited (1, 12, 14, 21). Production of coagulase by *Staphylococcus aureus* is accepted as an indicator of pathogenicity, and most enterotoxigenic staphylococci produce coagulase, but the literature contains few data to support the view of McDivitt and Topp (17) that "there is much evidence to indicate that coagulase production indicates enterotoxin production, although absolute certainty has not been established." The two most popular bioassays for enterotoxin, the monkey feeding test (11) and the cat injection test (12), have had restricted application because of the expense. The purification of enterotoxin (4) and the demonstration of its antigenicity (5, 6) have led to the application of gel-diffusion tests for the serological detection of enterotoxin in culture preparations (5, 6) and in foods (8, 13, 18).

This report describes the application of the single and double gel-diffusion tube techniques in examing cultures of coagulase-positive staphylococci from cheese for enterotoxins A and B, as well as the application of the micro double diffusion slide technique for identifying enterotoxin A.

MATERIALS AND METHODS

Production of immune serum. Approximately 20% pure enterotoxin A from fermentations of *S. aureus* 196E-2 was used to immunize rabbits by the subcutaneous route. Partially purified enterotoxin A (37 to 40 mg) with Freunds complete adjuvant was injected subcutaneously over a period of 3 months (M. S. Bergdoll, *personal communication*). The schedule of injections, which varied slightly for each rabbit, consisted of an initial dose of 50 or 100 μg followed by doses of 500 μg after 1 week, 2,500 μg after 2 weeks, 8 mg after 6 weeks, and 11 mg after 10 weeks. A terminal dose of 15 mg was given approximately 13 weeks after the initial injection. Three weeks after the 11-mg dose and 3 weeks after the 15-mg dose, the rabbits were bled, and the sera from the bleedings were titered and pooled.

Cultures. Seventy-seven coagulase-positive cultures of *S. aureus* isolated from food-poisoning cheese and 155 from market cheese (12) were examined for enterotoxins A and B. In addition, strains of *S. aureus* known to produce enterotoxin A (196E, 230, and seven others), enterotoxin B (243), or A and B (S-6), as well as four that were negative for enterotoxins A and B, were used to determine the antiserum specificity. The cultures were carried in cooked meat medium held at room temperature and on nutrient agar slants held at 4 C.

Production of enterotoxin. In the selection of a method for growing staphylococci that would yield concentrated enterotoxin, both the cellulose-tube technique (E. P. Casman and R. W. Bennet, Bacteriol. Proc., p. 108, 1961) and the sac-culture method were considered. The cellulose tube was inoculated with 0.5 ml of a heavy suspension of enterotoxigenic staphylococci and was suspended on 100 ml of medium contained in a Roux bottle. In the sac culture, this technique was reversed, and the 100 ml of medium in a cellulose sac was surrounded by a large volume of inoculum. Because staphylococcal enterotoxin will not pass through a dialysis membrane, the cellulose tubing was used in both methods to effect a concentration of enterotoxin, by confining the staphylococcal cells and the enterotoxin to a relatively small volume, yet allowing the cells to be nurtured by a large volume (100 ml) of medium.

As a result of preliminary studies, which included modifications of the sac culture (in which the volume

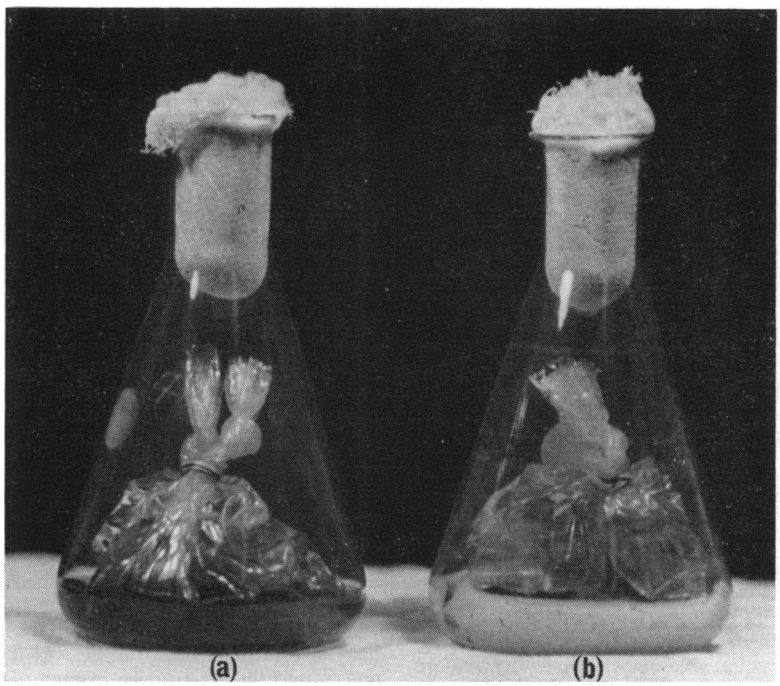

FIG. 1. *Growth of Staphylococcus aureus in sac cultures, incubated on rotary shaker at 35 C for 48 hr. (a) Uninoculated sac culture; (b) inoculated sac culture. Note heavy growth in (b) flask.*

and concentrations of the media were varied) and modifications of the cellulose-tube technique, the sac-culture method of growing staphylococci was selected for the production of enterotoxin. The sac-culture method offered certain advantages over the cellulose-tube technique in that the sac-culture assemblies were easier to prepare and inoculate, and yielded a larger volume of growth and crude enterotoxin that was much easier to recover than the small quantity (approximately 1 ml) produced in the cellulose tubes. Supernatant fluid from the sac cultures contained five to ten times as much enterotoxin as supernatant fluid from conventional flask cultures. The sac-culture assembly was made from 48A cellulose dialyzing tubing, approximately 7.5 cm wide. A 40- to 45-cm piece of this tubing was washed in distilled water, knotted at one end, and inflated to make a sac. The knotted end was placed in a 300-ml Erlenmeyer flask so that it rested on the bottom of the flask. Double strength Brain Heart Infusion (100 ml; BBL) was placed in the sac and the open end was then knotted. The sac was positioned in the flask in a U shape and the two knotted ends, located in the neck of the flask, were secured to each other with a rubber band. The flask was sterilized in the autoclave at 121 C for 15 min, and 18 ml of phophate-buffered dilution water were added to the flask, outside the sac of medium (Fig. 1).

The cultures were transferred to Brain Heart Infusion Agar (BBL) slants and were incubated at 35 C for 18 to 24 hr before they were used to inoculate sac-culture assemblies. The growth from a slant was harvested in 2 ml of phosphate-buffered dilution water and was added to the dilution water surrounding the sac to provide an inoculum volume of 20 ml.

Approximately 40 sac-cultures were prepared and inoculated at one time. They were incubated on a rotary shaker at 35 to 37 C for 48 hr. The growth (Fig. 1) surrounding the sac was removed from the flasks, and the culture fluid was separated from the cells by centrifugation at 23,500 $\times$ *g* for 20 min. The supernatant fluid was preserved with 1:10,000 Merthiolate and stored at 4 C.

Immunodiffusion tests. The single and double gel-diffusion tube techniques and the micro-slide test (10) were used in this study. The single diffusion technique was used to screen the cheese cultures for enterotoxins A and B. Enterotoxin-antiserum agar, 0.4 ml (13), in clean agar-coated test tubes (5 mm inside diameter by 50 mm long), was overlaid with 0.4 ml of the supernatant fluid (antigen) from the sac cultures of the cheese isolates. The tubes were sealed with Parafilm and were incubated at 30 C. After 24 hr, they were examined by obliquely transmitted light, and three types of reactions were observed (Fig. 2). The first of these was a positive test that appeared as a zone of precipitated antigen-antibody originating at the antigen-antiserum agar interface, which either remained there as a narrow band (equivalence reaction) or extended down into the serum agar and ended in a sharply defined flat front edge. The length of these zones was measured in millimeters with a hand caliper. The second and third types of reactions were atypical, and were either short, hazy zones with rounded front edges at the antigen-antiserum agar interface, or rings

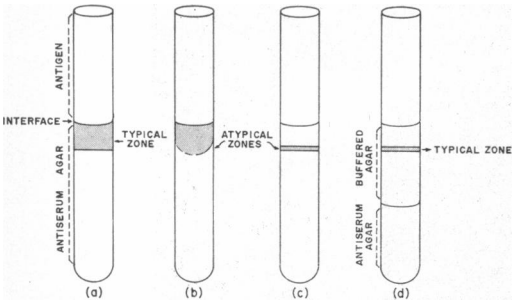

FIG. 2. *Single and double diffusion tube tests for enterotoxin A. (a) Single diffusion test showing typical positive zone; (b) and (c) single diffusion tests showing atypical negative zones; and (d) double diffusion tube test showing typical positive zone.*

occurring at about the midpoint of the serum agar layer. Cultures which produced atypical reactions only, as well as those that produced no reaction, were regarded as negative for enterotoxin.

The double gel-diffusion tube test was used to confirm the results of the single diffusion test. In this test (13), only 0.3 ml of serum agar was used, and was overlaid with an equal volume of buffered agar before the antigen (0.3 ml) was added. The double diffusion tests, also incubated at 30 C, were examined after 3, 7, and 14 days, and only two types of reactions were observed (Fig. 2). The first was a positive test and ranged in size from a narrow band of precipitated antigen-antibody in the buffered agar layer to a wider zone that originated in the buffered agar, but extended down into the serum agar. The second reaction was the atypical, short, hazy zone that has been described. The micro double diffusion slide test was used to verify cheese cultures that were positive for enterotoxin A by the single and double diffusion tube techniques.

The micro-slide test, described in detail by Crowle (10), was made on clean, agar-coated, 7.5 by 2.5 cm slides. A delimited area, 2 cm long, was flooded with buffered agar and was immediately covered with a plastic template with four funnel-shaped holes surrounding a fifth center hole. When the agar had solidified, three adacent corner holes were filled with the reference antigen, the fourth corner hole was filled with supernatant fluid from the *S. aureus* culture being examined, and the center hole was filled with antiserum. The slides were incubated in a moist chamber at 30 C for 72 hr and were then examined. The tests in which the lines of precipitation from the unknown coalesced, or joined with the reference line of precipitation formed by the reaction of the antiserum with the reference antigen, were considered positive for enterotoxin A (8). Tests in which the lines from the cultures curved away from the reference line, or in which no lines were produced, were considered negative (Fig. 3).

Cat-injection tests. Selected cultures were examined for enterotoxin by a modification of the intravenous cat-injection test (12). These cultures were grown by the sac-culture method, were centrifuged, and the supernatant fluid was passed through an ultra-fine fritted glass filter. The filtrates were digested with 0.2% trypsin (Difco 1:250) at 37 C for 2 hr to inactivate α-and β-hemolysins, and were then examined for these hemolysins by a conventional twofold serial dilution test before they were injected into cats.

The filtrates were diluted 1:5 in 0.85% NaCl solution, and 5 ml of each filtrate was injected intravenously into the left rear leg of each of four cats. The cats were observed for 3 hr for emetic responses. When three or more of the four cats vomited between 10 min and 3 hr after injection, the test was considered positive. Vomiting by two cats within this period was considered a questionable reaction. A response by only one or none of four cats was considered negative.

RESULTS

Titer and specificity of immune sera. Several rabbits were immunized with partially purified enterotoxin A, but only three survived the schedule of injections. Of these three, only one produced an antiserum that was specific for enterotoxin A.

The titer of this antiserum for identifying en-

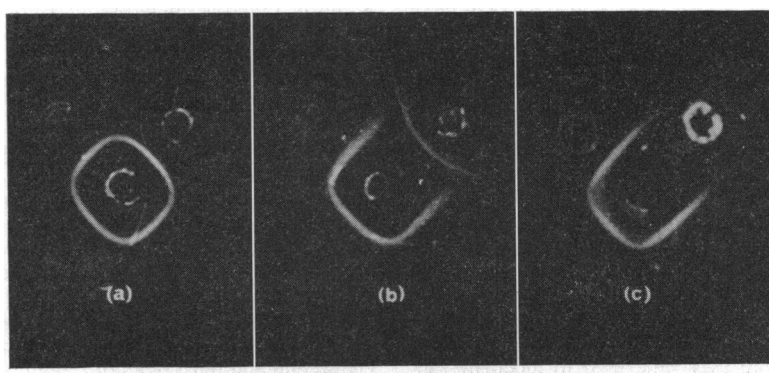

FIG. 3. *Micro-slide test with enterotoxin A as reference antigen. Unknown in upper right-hand corner. (a) Unknown, identity with enterotoxin A; (b) unknown, nonspecific reaction, negative for enterotoxin A; (c) unknown negative, or enterotoxin A level too small to detect.*

terotoxin A was determined in single diffusion tests. Zones of precipitated antigen-antibody were observed at antiserum dilutions greater than 1:100, but they were better defined at higher antiserum concentrations. A 1:60 dilution was used in examining the cheese cultures for enterotoxin A. In the single diffusion test, this dilution produced well-defined, typically positive zones with known enterotoxin A-producing staphylococci. With the 1:60 dilution, strains of staphylococci that were negative for enterotoxin A, and an enterotoxin B-positive strain produced either no reaction or atypical reactions. The enterotoxin B antiserum was also used at a 1:60 dilution and was specific at this dilution (13).

Sensitivity of gel-diffusion tests. Because migration distance of precipitated antigen-antibody complex may be affected by antigen diluent (13), sensitivities of the single and double gel-diffusion tests for detecting enterotoxin in sac-cultures were determined by assaying varying concentrations of enterotoxins A and B in supernatant fluid from sac-cultures of nonenterotoxigenic staphylococci. With a 1:60 dilution of enterotoxin A antiserum, concentrations as low as approximately 0.4 and 0.2 μg of enterotoxin A per ml of sac-culture fluid could be detected by the single and double diffusion techniques, respectively; with enterotoxin B antiserum at the same dilution, 0.6 μg of enterotoxin B could be detected by single diffusion procedure, and 0.2 μg could be detected by double diffusion. The micro-slide test had a sensitivity of approximately 1.0 μg per ml (8).

Quantitation of enterotoxin. Enterotoxin A was quantitated by single diffusion assay procedure through the use of a standard curve. It was assumed (M. S. Bergdoll, *personal communication*) that the enterotoxin A preparation was 20% pure, and that under standard conditions, enterotoxin concentration was an exponential function of migration distance. The standard curve was prepared by relating varying concentrations of enterotoxin A in micrograms, dissolved in sac-culture supernatant fluid, to millimeters of migration of the antigen-antibody complex, and then calculating regression lines. With the resulting straight-line curve, quantities of enterotoxin A produced in sac-culture fermentations were estimated.

Examination of cheese cultures by agar gel-diffusion tests. The supernatant fluids from sac-cultures of 232 cultures isolated from cheese were screened for enterotoxins A and B by the single diffusion test. When examined with enterotoxin A antiserum, 16 of the 232 cultures were confirmed as positive for enterotoxin A by single and double gel-diffusion tests. On reexamination with enterotoxin A antiserum, 25 cultures which initially produced atypical zones or rings or questionable reactions were found to be negative. On reexamination with enterotoxin A antiserum, 13 cultures produced weak or atypical reactions by single diffusion and weak or delayed positive reactions by double diffusion tests. The delayed reactions were not observed until the 7th or 14th day, whereas typical enterotoxin A-positive strains produced positive zones within 72 hr. Wherever possible, the original sac-culture supernatant fluid was used for the re-examination.

Because of the uncertain status of these 13 cultures, they, together with the 16 enterotoxin A-positive cultures and 10 negative controls (selected as being negative for enterotoxin A and B on the basis of gel-diffusion tube and cat tests) were examined for enterotoxin A production by the micro-slide test. For this test, one of the 16 enterotoxin A-positive cultures isolated from cheese was selected as the enterotoxin A reference antigen. The enterotoxigenicity of this culture (*S. aureus* MF 71) had been demonstrated repeatedly by cat tests. It produced no reaction with enterotoxin B antiserum and gave typically positive reactions in single and double diffusion tube tests with enterotoxin A antiserum. The characterization of *S. aureus* MF 71 as an enterotoxin A-positive culture was further established by the fact that it produced lines of identity (Fig. 3) in micro-slide tests with known enterotoxin A-positive staphylococci 196E and 230, (6), and 20% purified enterotoxin A. For examination of the cheese cultures, the reference antigen was diluted to contain approximately 4 or 2 μg of enterotoxin A per ml; these concentrations were used with 1:20 and 1:30 dilutions of enterotoxin A antiserum, respectively.

All 16 cheese cultures that were positive for enterotoxin A by single and double diffusion tube tests produced lines of identity with *S. aureus* MF 71, the reference antigen. None of the other cultures (the 13 questionable ones or the 10 negative controls) produced lines of identity with the reference antigen (Table 1).

None of the 232 coagulase-positive cultures from cheese produced detectable levels of enterotoxin B when tested by the single diffusion tube test.

TABLE 1. *Verification by micro-slide test of enterotoxin A-positive gel-diffusion tube tests*

No. of cultures	Tube diffusion tests (enterotoxin A)	Micro-slide tests (enterotoxin A reference antigen)
16	+	+
13	±	−
10[a]	−	−

[a] Negative controls

TABLE 2. *Comparison of single diffusion assay and cat test for enterotoxin A produced in sac-culture fermentations of staphylococci isolated from cheese*

Culture no. and source	Enterotoxin A		Cat bioassay (emesis per no. injected)
	Filtrate	TD[a] filtrate	
	μg/ml	μg/ml	
Food-poisoning cheese			
31	13.5	8.0	4/4
36	8.4	6.2	4/4
65	8.9	6.2	4/4
70	19.5	19.5	4/4
71	12.0	8.9	4/4
73	19.5	13.6	4/4
Market cheese			
224	12.0	4.1	3/4
224-2	8.4	4.4	4/4
240	8.4	2.4	4/4
240-2	11.2	8.4	4/4
244-2	2.2	1.4	4/4
245	3.4	2.5	4/4
296	2.9	1.8	4/4

[a] Trypsin digested filtrate.

Examination of cheese cultures by cat test. To compare the results by the single diffusion tube test with bioassays for enterotoxin, 13 of the strains that were positive for enterotoxin A and 14 that were negative for enterotoxins A and B were examined by the cat injection test. Table 2 shows that trypsinized filtrates of the 13 strains of staphylococci were positive for enterotoxin A by both single diffusion tests and cat bioassays. The reduced quantities of enterotoxin A in trypsinized filtrates of the 13 strains, compared with that in their respective untreated filtrates, may indicate destruction of enterotoxin A by trypsin, or the effect of the inclusion of trypsin in the immuno-diffusion system.

Of 14 cultures that were negative for enterotoxins A and B by gel-diffusion tube tests, ten were negative for enterotoxin by the cat test. Two cultures produced vomiting in two out of four cats, which is considered a questionable reaction. Two cultures produced positive cat tests, but this may have been due to types of enterotoxin other than A or B.

The trypsinized filtrates of enterotoxin A-positive cultures injected into cats were negative for β-hemolysin, but several of them had low titers for α-hemolysin (e.g., 1:4, 1:8, 1:32±). At these low titers, however, α-hemolysin was not considered to be a cause of emesis in the cats, because a trypsinized filtrate from an enterotoxin-negative strain of staphylococci, which had a titer of 1:32± for α-hemolysin, produced no emetic reaction.

DISCUSSION

It is beyond the scope of this paper to explain the atypical zones observed in some of the single and double diffusion tests or the rings observed in single diffusion tests. The atypical zones were apparently not serological reations, because they were observed in plain buffered agar controls of certain cultures. The atypical rings, observed in single diffusion tests with enterotoxin A antiserum, as well as with enterotoxin B antiserum and normal rabbit serum, probably represented an antigen-antibody reaction that did not involve enterotoxins A or B. The positive-appearing reactions that occurred in single and double diffusion tube tests of certain cultures, which were typical except that the reactions in double diffusion tests were not observed until after 72 hr, may have been due to antigens that were closely related but not identical to enterotoxin A.

Of the four types of enterotoxin that have been serologically identified and designated as A, B, C, and D (3, 9; E. P. Casman, R. W. Bennet, and R. E. Kephart, Bacteriol. Proc., p. 13, 1966), A has been most frequently associated with food poisoning in this country. Our study shows that seven of 77 (9.1%) of the coagulase-positive staphylococci isolated from food-poisoning cheese and nine out of 155 (5.8%) isolated from market cheese, produced enterotoxin A. None of the cultures produced enterotoxin B. Casman (7) reported that approximately 4.4% of 245 strains of staphylococci isolated from frozen foods produced enterotoxin A and 4.0% produced enterotoxin B. Among 190 strains isolated from raw milk, he found that 3.2% produced enterotoxin A and 1.0% produced enterotoxin B. He also reported that of 212 strains isolated from the noses of apparently healthy individuals, 40.2% produced enterotoxin A, 4.2% produced enterotoxin B, and 5.5% produced both A and B. He reported that 23.3% of 449 strains from clinical specimens produced enterotoxin A, 4.5% produced enterotoxin B, and 3.8% produced A and B. These data indicate that the incidence of strains producing enterotoxin B is much smaller than the incidence of strains producing enterotoxin A.

In a previous study (12), it was reported that 68 out of 343 market cheese samples contained coagulase-positive staphylococci. The present study shows that seven of these 343 samples (2.0%) contained enterotoxin A-producing staphylococci. The incidence of enterotoxigenic strains would probably have been higher if sera had been available for examining the cultures for other types of enterotoxin. This view is supported by the observation that two cultures that were negative for enterotoxins A or B by the gel-diffusion tests were positive for enterotoxin by the cat test.

Enterotoxigenic stahylococci in cheese have been reported by other investigators (1, 14, 21). The presence of these organisms in small numbers in the finished product does not necessarily mean that they were originally present in numbers too small to produce significant amounts of enterotoxin in improperly handled milk or during cheesemaking. A marked reduction of staphylococci may occur in cheddar and colby cheeses during storage and curing (16, 19). Thus the number present in market cheese probably represents only a small fraction of the number present when the cheese was made. That staphylococci can produce significant levels of enterotoxin in cheese or cheese milk is illustrated by the cheese-borne food-poisoning outbreak in Iowa (15). Most raw milk contains coagulase-positive staphylococci, some of which are enterotoxigenic (2, 7), and therefore enterotoxin could be produced in improperly handled milk by the multiplication of staphylococci (20) before the actual cheesemaking begins (C. B. Donnelly, J. E. Leslie, and L. A. Black, Bacteriol. Proc., p. 13, 1966.). Enterotoxin production is more likely during cheesemaking, when the sequence of temperatures may be from approximately 31 to 38 to 25 C (room temperature) for as long as 24 hr. Jezeski et al. (16) have shown that staphylococci will increase from thousands per milliliter of milk to millions per gram of cheese during this period. Apparently the maximal staphylococcal count obtained in either milk (20) or cheese (16) is proportional to the inoculum level, so that staphylococci in low numbers would have less chance of reaching a cell density that could produce significant levels of enterotoxin than would staphylococci in high numbers.

Three steps that will reduce the public health hazard of enterotoxigenic staphylococci in cheese or cheese milk are: (i) rapid cooling and continuous refrigeration of milk from time of milking until it is used in making cheese, (ii) heating of milk to destroy enterotoxigenic staphylococci, and (iii) prevention of recontamination of heated milk or cheese with staphylococci.

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