Serologic, Biochemical, and Cultural Traits of Enterotoxigenic and Coagulase-Positive Staphylococci Isolated from Food Handlers

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STAPHYLOCOCCAL food poisoning is caused by the ingestion of preformed enterotoxin, which is so designated because of its specificity for the enteric tract. Sugiyama and Hayama (*1*) have demonstrated that the abdominal viscera is the site of emetic action. The filtrates of enterotoxigenic staphylococci remain potent for at least 2 months when stored at 4° C. Elek (*2*) stated that staphylococcal enterotoxins must be boiled for 1 hour before an appreciable loss in potency is noted. Casman (*3*) showed that emetic properties of the enterotoxins are not destroyed by pancreatic or tryptic digestion.

The enterotoxins are simple proteins that have molecular weights ranging from 30,000 to 35,000. They are soluble in water and salt solutions, but insoluble in organic solvents. Casman and associates (*4*) originally designated two immunological types of staphylococcal enterotoxins. More recently, Casman and associates (*5*) identified a fourth type of enterotoxin. According to Casman (*3*), type A is the predominant enterotoxin identified in outbreaks of food poisoning in the United States. Type D is second in significance with respect to food poisoning outbreaks. Enterotoxigenic strains of staphylococci usually elaborate, besides enterotoxin, either alpha or beta hemolysins. However, a direct correlation between enterotoxin formation and other staphylococcal products has not been established.

In cooperation with the Washington County (Ark.) Public Health Department, we conducted a study for two purposes: (*a*) to determine the incidence of enterotoxigenic staphylococci among food handlers in the county and (*b*) to identify the types of enterotoxins, by serologic methods, elaborated by these potential food poisoning staphylococci.

Materials and Methods

An epidemiologic survey was made of food handlers in public restaurants as a source of potential food poisoning staphylococci. The restaurants surveyed were those inspected regularly by the Washington County Public Health Department. Fifty food handlers volunteered for the study. Using sterile cotton swabs, they took specimens from the anterior portion of the right nasal

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cavity and from the inside of the index finger and thumb of the right hand. This was done under the direct supervision of health department personnel.

The swabs were placed aseptically in 10 ml. of nutrient broth and incubated at 37°C. for 24 hours. After incubation, 1 loopful of the broth culture was streaked onto plates of *Staphylococcus* 110 agar medium (Difco Manual, 1971) and incubated for 24 hours. Colonies comprising gram-positive cocci in irregular clusters, as revealed by Gram's staining procedure, were subcultured to brain heart infusion agar slants and kept at 4°C.

Coagulase tests were performed on all isolates by the tube-coagulase method with Bacto-Coagulase Plasma. The content of the ampule was dissolved in 3 ml. of distilled water. Five-tenths ml. of the plasma was pipeted into a Kahn tube, which was immersed partially in a water bath at 37°C. Two drops of an 18-hour brain heart infusion culture of the test organism then were added to each Kahn tube. The tubes were examined after 1 and 3 hours of incubation and any degree of clotting after either interval was considered to be a positive reaction.

The coagulase-positive isolates were characterized further by their ability to produce hemolysins and fermentation of mannitol, both of which are linked, to some degree, with virulence of pathogenic staphylococci. In light of the widespread usage of penicillin as an antibiotic for treating staphylococcal infections, the sensitivity of the isolates to this antibiotic was tested. Fermentation of mannitol was determined using both Bacto-Phenol Red Broth Base containing 1 percent d-mannitol and Bacto-Mannitol Salt Agar. The media were inoculated and incubated at 37°C. for 24 hours. Fermentation of mannitol was determined by production of a typically acid reaction; that is, a changing of the phenol red indicator from a red to a yellow color.

The coagulase-positive isolates were tested for hemolysis on both sheep and rabbit blood agar. The basal medium was prepared by rehydrating Bacto Blood Agar (B45) as directed and adding 5 percent of defibrinated sheep or rabbit blood, respectively. To insure sterility, plates were incubated at 37°C. overnight and then streaked with the particular isolates of staphylococci and reincubated for 24 hours. The plates then were observed for the presence or absence of hemolysis, with results recorded as showing alpha or beta types of hemolysis or negative.

Tests for sensitivity of the coagulase-positive isolates to penicillin were conducted in the following way. Baltimore Biological Laboratory Sensi-Discs of penicillin G, having a concentration of 2 units, were used. Plates of Bacto Tryptose Agar (B64) were poured, allowed to solidify, and inoculated with 0.10 ml. of the test micro-organisms. The inoculum was spread evenly over the surface with a sterile glass spreader. A Sensi-Disc was placed in the center of each plate, which then was incubated for 24 hours at 37°C.

Production of enterotoxin was attained by growing the staphylococci on 0.7 percent brain heart infusion agar at pH 5.3 (*6*). Tubes of sterile medium (25 ml.) were poured aseptically into sterile petri dishes. After solidification, the surface of the semisolid medium was inoculated with 4 drops of an aqueous suspension containing approximately 300 million bacteria per ml. The turbidity of the suspension was determined to be equal to that of a No. 1 tube of the McFarland Nephelometer scale. The inoculum was spread evenly over the surface and the plates were incubated for 72 hours at 37°C. The organisms and agar were sedimented and removed by high-speed centrifugation. The supernatant obtained was tested for the presence of an enterotoxin or enterotoxins.

The enterotoxin tests were done serologically by the slide gel diffusion method (*7*). Slides were prepared by placing a double layer of plastic electric insulation tape, 2 cm. apart, on a scrupulously clean glass slide. The top surface of the slide, between the tape layer, was coated with 0.2 percent agar and allowed to dry in a dust-free container. The fluid base for the agar was prepared as follows:

NaCl	0.85 percent
Sodium barbital	0.80 percent
Merthiolate	1:10,000
pH	7.4

Agar (Special (Noble) Difco 0142-01) was added to a concentration of 1.2 percent in the fluid base. This suspension was melted and filtered in the Arnold Sterilizer. Thirty-five one-hundredths ml. of the melted and cooled agar (55°C.) was placed between the tapes on the prepared slide.

The reagents were added separately to individual wells in a special plastic template made according to the specifications of Casman and associates (*7*). A thin film of silicone grease was added to the underside of the template before it was placed on the agar, to aid in its removal. Petri dishes were prepared for incubating slide assemblies by plac-

ing strips of absorbent cotton around the periphery and saturating these with distilled water.

Reference toxins and antitoxins were obtained from Dr. Reginald Bennett, Division of Microbiology, Food and Drug Administration, Public Health Service. The reagents were supplied as lyophylized preparations, which were dissolved following the directions specified.

The antitoxin was placed in the center well with the corresponding reference toxin in the left well. The test supernatant was placed in the top well. All wells were filled to convexity with a Pasteur pipet. Care was taken to remove trapped bubbles of air, since these prevent diffusion of the reagents into the agar medium. Control slides were prepared by using only the reference toxin and its specific antitoxin. The test supernatant was used undiluted and at a 1:20 dilution, which was included because excess enterotoxin will cause lines of precipitation to occur too closely to the well containing specific antitoxin. Consequently, these lines might be obscured or seen only with difficulty. All tests were made in duplicate. The slides were allowed to incubate in a humid atmosphere for 3 days at ambient temperature before they were examined for enterotoxin-antitoxin reactions.

Identification of a line of precipitation was possible through its coalescence with the reference line of precipitation. Care was taken not to disturb the agar gel when the templates were removed. All slides were preserved by staining, a procedure which reveals the presence of lines of precipitation not otherwise visible, thus increasing the sensitivity of the test. A modification of the staining procedure described by Crowle (*8*) was used. Slides were passed through the following baths: distilled water, 20 minutes; staining solution, 10 minutes; and 1 percent acetic acid, 20 minutes. The staining solution consisted of 0.1 percent thiazine red in 1 percent acetic acid containing 1.0 percent glycerol.

Results and Discussion

A survey was made of 15 public restaurants in Washington County to determine the presence of enterotoxigenic staphylococci among food handlers. The selection of these restaurants depended on the schedule of regular monthly inspections made by the Washington County public health officer. All of these establishments met the minimum requirements of the Arkansas State Department of Health, with respect to the preparation and serving of food for human consumption.

Regarding the 50 food handlers who volunteered to have the tests made, it should be emphasized that each was in good health when their nose and hand swab specimens were taken. Good health was interpreted in light of section D of Arkansas Statutes of 1947, 82–110 (*9*). This section specifies that "no person while afflicted with any disease in a communicable stage or while a carrier of such disease, or while afflicted with boils, infected wounds, sores, or an acute respiratory infection shall work in any area of a food service establishment in any capacity in which there is a likelihood of such person contaminating food or food-contact surfaces with pathogenic organisms, or transmitting disease to other individuals."

Of the 50 persons tested, 40 harbored staphylococci. Seventeen isolates were obtained from hands and 35 from nasal cavities. Staphylococci were isolated from the nasal cavities only of 21 (42 percent) of the 50 persons tested, from the hands only of five (10 percent), and from both nasal cavities and hands of 14. Two isolates were obtained from some persons. The incidence of staphylococci among the 40 food handlers was as follows:

Source	*Total*
Hand isolates	17
Nasal isolates	35
Both hand and nasal isolates	14
Total isolates	66

These results were consistent with those of Leedom and associates (*10*), who reported that 82 percent of the persons tested harbored staphylococci, and with the work of Williams (*11*), who isolated staphylococci from the noses of 44 percent of the persons tested.

Chromogenicity and production of coagulase are utilized commonly as criteria for the determination of staphylococcal pathogenicity. An effort was made in this work to ascertain whether these criteria might be correlated with enterotoxigenicity. All three of the enterotoxin-producing isolates of staphylococci were both chromogenic and coagulase positive. However, not all coagulase-positive and chromogenic cultures were enterotoxigenic. These results agree with those of Casman (*12*), who suggested that, generally, only coagulase-positive staphylococci are enterotoxigenic.

The coagulase-positive isolates were characterized further to ascertain what, if any, other biochemical properties might be useful in provisionally identifying enterotoxigenic staphylococci. The 19 cultures of staphylococci were

tested for their ability to ferment mannitol and cause lysis of either rabbit or sheep erythrocytes by growth of the organisms on blood agar plates.

The three isolates of enterotoxigenic staphylococci fermented mannitol and produced beta hemolysis on rabbit and sheep red blood cells. Only two of the 16 isolates of nonenterotoxigenic staphylococci failed to ferment mannitol. Fifteen of these isolates also produced beta hemolysis on rabbit and sheep red blood cells.

These results agree with data summarized by Burrows (*13*), who reported that in some studies as low as 1 percent of coagulase-positive strains are mannitol-negative. Burrows also emphasized a correlation between production of coagulase and the hemolysin-alpha-lysin. In one study, all alpha-lysin-positive strains were coagulase positive and only 4 percent of coagulase-positive strains were alpha-lysin-negative. Alpha-lysin produces beta hemolysis on rabbit and sheep red blood cells whereas beta-lysin causes lysis of sheep, but not rabbit, erythrocytes.

The sensitivity of the 16 coagulase-positive isolates of staphylococci to penicillin was determined. Zones of inhibition of bacterial growth greater than 15 mm. were interpreted as marked sensitivity of the organisms to penicillin, and they were recorded as positive. Zones of inhibition varying from 10 to 15 mm. in diameter were interpreted as moderate sensitivity of the organisms and recorded as equivocal. Zones of inhibition less than 10 mm. were interpreted as definite resistance of the micro-organisms to this antibiotic and were recorded as negative (*14*). Two of the three isolates of enterotoxigenic staphylococci were sensitive to pencillin while the third was moderately sensitive.

All 66 staphylococcal isolates were tested for production of enterotoxins. Sixteen of the 41 nasal isolates were coagulase positive. The three enterotoxigenic isolates found in this study were isolated from the nasal region only and were coagulase positive. Three of the 25 hand isolates were coagulase positive. However, none was enterotoxigenic. The following summarizes these results.

Staphylococcal isolates	*Total*
Nose	41
Coagulase positive	16
Enterotoxin A	2
Enterotoxin B	0
Enterotoxin C	0
Enterotoxin D	1
Hand	25
Coagulase positive	3
Enterotoxin A	0
Enterotoxin B	0
Enterotoxin C	0
Enterotoxin D	0

For a more comprehensive study, nose and hand swab specimens should be obtained daily from the food handlers surveyed to insure that the intermittent carrier of enterotoxigenic staphylococci is not overlooked. Also, it is reasonable to assume that these enterotoxigenic strains of staphylococci, which are part of the residential microbial flora of the nasal region, might be transferred inadvertently to the hands of food handlers and, thus, increase the number of potential cases of staphylococcal food poisoning. Since the three isolates of enterotoxigenic staphylococci were obtained from personnel employed in three different public restaurants, the potential number of food poisoning cases is increased.

Casman and associates (*5*) demonstrated that 31 percent of the normal nasal specimens in their study were enterotoxin-producing strains of staphylococci. In our investigation, 19 percent of the coagulase-positive nasal isolates were enterotoxigenic staphylococci. The work of Casman (*5*) showed also that greater than 7 percent of the nasal strains of staphylococci elaborated both enterotoxins A and D. These investigators determined that 3.8 percent of staphylococci isolated from known outbreaks of staphylococcal food poisoning were negative with respect to producing any one of the four types of known enterotoxins. This finding raises the possibility that other types of enterotoxins might be formed by food-poisoning staphylococci, which have not been identified and typed serologically.

REFERENCES

(*1*) Sugiyama, H., and Hayama, T.: Abdominal viscera as site of emetic action for staphylococcal enterotoxin in the monkey. J Infect Dis 115: 330–336 (1965).

(*2*) Elek, S. D.: *Staphylococcus pyogenes* and its relation to disease. E. and S. Livingston Ltd., Edinburgh and London, 1959.

(*3*) Casman, E. P.: Staphylococcal food poisoning. Health Lab Sci 4: 199–206 (1967).

(*4*) Casman, E. P., Bergdoll, M. S., and Robinson, J.: Designation of staphylococcal enterotoxins. J Bacteriol 85: 715–716 (1963).

(*5*) Casman, E. P., Bennett, R. W., Dorsey, A. E., and Issa, J. A.: Identification of a fourth staphylococcal enterotoxin, enterotoxin D. J Bacteriol 94: 1875–1882 (1967).

(*6*) Casman, E. P., and Bennett, R. W.: Culture medium for the production of staphylococcal enterotoxin A. J Bacteriol 86: 18–23 (1963).

(*7*) Casman, E. P., Bennett, R. W., Dorsey, A. E., and Stone, J. E.: The micro-slide gel double diffusion test for the detection

and assay of staphylococcal enterotoxins. Health Lab Sci 6: 185–198, October 1969.

(*8*) Crowle, A. J.: Immunodiffusion. Academic Press, New York, 1961.

(*9*) Rules and Regulations pertaining to Food Service Establishments Acts 1913, No. 96 Arkansas Statutes of 1947, paragraphs 82–110. Arkansas State Board of Health, Little Rock.

(*10*) Leedom, J. M., et al.: Observations of the staphylococcal, nasal carrier state. Ann NY Acad Sci 128: 381–403, July 1965.

(*11*) Williams, R. E. O.: Skin and nose carriage of bacteriophage types of *Staph aureus*. J Pathol 58: 259–268, April 1946.

(*12*) Casman, E. P.: Staphylococcal enterotoxins. Ann NY Acad Sci 120: 124–131, July 1965.

(*13*) Burrows, W.: Textbook of microbiology. W. B. Saunders Company, Philadelphia, 1968.

(*14*) BBL manual of products and laboratory procedures. Division of Becton, Dickinson and Co., Cockeysville, Md., 1969.

MOORE, JAMES M., Jr. (U.S. Army), and BOWER, RAYMOND K.: *Serologic, biochemical, and cultural traits of enterotoxigenic and coagulase-positive staphylococci isolated from food handlers. HSMHA Health Reports, Vol. 86, September 1971, pp. 815–819.*

An epidemiologic survey of food handlers in public restaurants in Washington County, Ark., was conducted to determine the number of these persons who harbored enterotoxigenic staphylococci and thus were potentially capable of causing food poisoning.

Staphylococcal isolations were made from 40 of the 50 persons tested, and enterotoxigenic staphylococci were isolated from three of the 40. An attempt was made to determine whether selected cultural and biochemical characteristics could be useful or might correlate with serologic methods in identifying enterotoxigenic staphylococci. It was determined that the three isolates of enterotoxigenic staphylococci were chromogenic, coagulase positive, mannitol positive, beta-hemolytic, and sensitive to penicillin. However, 14 other isolates which also possessed these characteristics were not enterotoxigenic.