Reassessment of the Coagulate and Thermostable Nuclease Tests as Means of Identifying *Staphylococcus aureus*

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A total of 91 enterotoxigenic strains of *Staphylococcus aureus* isolated from foods and tested for production of coagulase and thermostable nuclease and the ability to ferment glucose and mannitol showed, with the exception of four strains, a complete correlation among these properties. A similar correlation was observed with 103 cultures of *S. aureus* isolated from clinical material. In all instances, the coagulase reactions were sufficiently strong to be scored at either the 3+ or 4+ levels. Presumptive staphylococcal cultures isolated during routine examination of foods and yielding 2+ coagulase reactions or lower were invariably negative for thermostable nuclease production. It is suggested that the thermostable nuclease test be performed on cultures with doubtful coagulase reactions before classifying them as *S. aureus*.

Laboratories monitoring the microbiological quality of foods routinely conduct analyses for the detection of coagulase-positive staphylococci, yet there appears to be some uncertainty in the interpretation of the coagulase test. The procedure for determining the presence of coagulase-positive staphylococci in a sample involves plating a representative portion of the sample on one or more selective media, followed by testing for coagulase production. Plasma coagulation is regarded as positive identification of *Staphylococcus aureus*, and unless the sample is involved in a food poisoning outbreak, additional tests such as enterotoxin production and phage typing are not routinely performed. The coagulase reactions are scored progressively 1+ through 4+ depending on the extent of clotting of the plasma. Unfortunately, because of the subjective nature of this test, it is at this stage of the analysis that inconsistencies arise. In the procedure of the Association of Analytical Chemists for the isolation of *S. aureus* from foods (1, 12), any degree of clotting of plasma is regarded as positive evidence of coagulase production. In our laboratory, we score the extent of clotting in accordance with the scale outlined by Turner and Schwartz (14), and routinely regard a 2+ or greater reaction as positive evidence of coagulase production. Recently, Sperber and Tatini (Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, E67, p. 12) stated that only a 4+ coagulase reaction can be considered positive identification of *S. aureus*. Cultures yielding 1+ through 3+ reactions warrant additional testing for characters such as anaerobic glucose and mannitol fermentation, thermostable nuclease production, and/or lysostaphin sensitivity before being confirmed as *S. aureus*.

In this study, we attempted to determine the extent to which clotting of plasma should be considered positive evidence of coagulase production.

**MATERIALS AND METHODS**

**Bacterial cultures.** Cultures were isolated from foods and clinical cases. Those from foods were grouped into three categories: (i) confirmed enterotoxigenic strains of *S. aureus* isolated from foods and personnel involved in food poisoning episodes; (ii) staphylococci isolated from foods during routine microbiological analyses and which have been tested for enterotoxin production; and (iii) isolates from foods presumed to be coagulase-positive staphylococci and which have not been tested for enterotoxin production.

The clinical isolates were obtained from the Laboratory Centre for Disease Control, Ottawa, Canada. **Coagulase.** Coagulase production was determined by the tube method. Approximately 0.1 ml of an overnight culture grown in brain heart infusion broth (Difco) was added to 0.5 ml of reconstituted rabbit plasma ethylenediaminetetraacetic acid (EDTA; Difco). Tubes were incubated at 35 C and read both at 4 h and after standing at room temperature for an additional 20 h. The extent of clotting of the plasma, if any, was scored 1+ through 4+ in accordance with the types of reactions outlined by Turner and Schwartz (14).

**Thermostable nuclease.** Thermostable nuclease
activity was determined by the microslide method of Lachica et al. (7). Broth cultures used for the determination of coagulase production were heated in a boiling water bath for 15 min and aliquots of the heated cultures were added to precoat wells in toluidine blue/deoxyribonucleic acid agar layered on microscope slides. The slides were incubated in a moist chamber at 35 C and examined after 4 h for the appearance of bright pink halos which are indicative of nuclease activity. In the event of doubtful reactions, the slides were returned to the incubator and reexamined after an additional 2 to 3 h.

Glucose utilization. The ability of the cultures to utilize glucose under aerobic and anaerobic conditions was determined by the method proposed by the International Subcommittee on Staphylococci and Micrococci (5).

Mannitol utilization. The ability of the cultures to utilize mannitol was determined by a modification of the Hugh and Leifson’s test as described by Mosse (9).

Enterotoxin. Enterotoxin production was determined by the microslide-gel double diffusion test of Casman et al. (3). In our laboratory, with the exception of enterotoxin E, the lower limit of detection of enterotoxin by this method is 0.5 µg/ml. Because of the much lower sensitivity in detecting enterotoxin E, this test was not performed and the unavailability of suitable reagents precluded the testing for enterotoxin F. For these reasons, the micro-slide test was used only for the assay of enterotoxins A, B, C, and D.

RESULTS

The relationship between thermostable nuclease and coagulase production by enterotoxigenic strains of S. aureus isolated from foods and personnel involved in food poisoning outbreaks is shown in Table 1.

Of the 63 isolates tested, 62 were positive for both thermostable nuclease and coagulase production, and in every instance clotting of the plasma was sufficiently complete to be assigned a 4+ rating according to the scheme of Turner and Schwartz (14). The single enterotoxin A-producing strain of S. aureus, which was negative for thermostable nuclease production, was retested twice for enterotoxin, thermostable nuclease, and coagulase production with no change in the original results. Lachica et al. (8) also found that four of their 232 coagulase-positive enterotoxigenic strains of S. aureus failed to produce a thermostable nuclease.

All isolates were capable of utilizing glucose and mannitol under anaerobic conditions.

The cultures isolated from foods during routine microbiological analyses were subjected to similar tests (Table 2).

Apart from three strains which failed to ferment mannitol, the enterotoxin-producing cultures showed complete correlation among thermostable nuclease and coagulase production and the ability to utilize glucose and mannitol under anaerobic conditions. Of the 25 cultures which were negative for enterotoxin production, as determined by the method of Casman et al. (3), nine were positive for both thermostable nuclease and coagulase production, nine were negative for thermostable nuclease but positive for coagulase production, and seven were negative for both thermostable nuclease and coagulase production. Among the nine thermostable nuclease-negative cultures which were coagulase positive, five were incapable of utilizing either glucose or mannitol under anaerobic conditions, and three produced gas from glucose and mannitol. These nine cultures were the only ones yielding 2+ coagulase reactions. The remaining coagulase-positive isolates listed in Table 2 were rated mainly 4+; about 10% yielded 3+ coagulase reactions.

A total of 103 clinical isolates of staphylococci were positive for both thermostable nuclease and coagulase production. The coagulase reactions were at either the 3+ or 4+ levels and all isolates were capable of utilizing glucose under aerobic and anaerobic conditions.

Since the correlation between thermostable nuclease and coagulase production was weak when the coagulase activity was 2+, albeit only nine isolates fell into this category (Table 2), this phenomenon was further investigated using

| No. of isolates tested | Enterotoxin produced | Thermostable nuclease production | Utilization of glucose | Anaerobic utilization of mannitol | Coagulase production |
|------------------------|-----------------------|---------------------------------|-----------------------|----------------------------------|---------------------|
| 34                     | A                     | +                               | +                     | +                                | +                   |
| 3                      | B                     | +                               | +                     | +                                | +                   |
| 5                      | C                     | +                               | +                     | +                                | +                   |
| 20                     | D                     | +                               | +                     | +                                | +                   |
| 1                      | A                     | -                               | +                     | +                                | +                   |

* Clotting of plasma rated 4+.
TABLE 2. Production of thermostable nuclease and coagulase and utilization of glucose and mannitol by staphylococci isolated from foods during routine analysis

| No. of isolates tested | Enterotoxin produced | Thermostable nuclease production | Utilization of glucose | Anaerobic coagulase production | Anaerobic utilization of mannitol | Coagulase production* |
|------------------------|-----------------------|---------------------------------|------------------------|-------------------------------|----------------------------------|-----------------------|
| 17                     | A                     | +                               | + +                    | +                             | +                                | +                     |
| 3                      | C                     | +                               | + +                    | +                             | +                                | +                     |
| 5                      | D                     | +                               | + +                    | +                             | +                                | +                     |
| 1                      | C                     | +                               | + +                    | +                             | +                                | +                     |
| 2                      | D                     | +                               | + +                    | +                             | +                                | +                     |
| 9                      |                       | +                               | + +                    | +                             | +                                | +                     |
| 4                      |                       | +                               | + +                    | +                             | +                                | +                     |
| 5                      |                       | +                               | -                      | -                             | -                                | -                     |
| 7                      |                       | NTc                             | NTc                    | NTc                           | NTc                             | NTc                   |

* Clotting of plasma rated mainly 4+; approximately 10% rated 3+.
* Three of four isolates produced gas.
* NT, Not tested.

presumptive staphylococcal isolates from naturally contaminated foods plated on selective media. Thermostable nuclease production and extent of clotting of plasma by these isolates are recorded in Table 3. Cultures yielding 1+ activity for coagulase were all negative for thermostable nuclease. Of the cultures yielding 2+ coagulase reactions, only 10 of 261 were positive for thermostable nuclease, representing approximately 4% of the 2+ coagulase-producing isolates. Of the 85 cultures yielding 3+ and 4+ levels of coagulase activity, 84 were positive for thermostable nuclease production.

**DISCUSSION**

The ability of a culture to produce coagulase is usually regarded as positive identification of *S. aureus*. With the exception of one culture, we have shown a direct correlation among thermostable nuclease production, anaerobic dissimilation of glucose, and coagulase production scored at the 3+ and 4+ levels. In addition, we have shown that all strains of *S. aureus* capable of producing enterotoxin as determined by the method of Casman et al. (3) yielded either 3+ or 4+ coagulase reactions. Three of these strains were repeatedly negative for anaerobic utilization of mannitol, but were capable of fermenting glucose.

Eight of nine cultures which yielded 2+ coagulase reactions and were negative for thermostable nuclease production were atypical of *S. aureus* in that they either produced gas while utilizing glucose and mannitol under anaerobic conditions, or were incapable of anaerobic utilization of these compounds. Of the presumptive staphylococcal isolates, only 10 of 261 cultures yielding 2+ coagulase reactions were positive for thermostable nuclease production, and only one of 52 cultures yielding 3+ coagulase reactions was negative for thermostable nuclease production.

Our results are based on the use of rabbit coagulase plasma EDTA (Difco). Other sources of this plasma were not tested, and this limitation must be borne in mind. We have deliberately avoided the use of citrated plasma because of the experience of others (2, 11) who reported false positive tests by this method. Porcine coagulase plasma EDTA was not tested because it is not routinely used. However, Sperber and Tatini (Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, E67, p. 12) recently reported that this plasma or a mixture of it and rabbit coagulase plasma EDTA is more suitable for use in the coagulase test than rabbit coagulase plasma EDTA alone. Nevertheless, they considered only a 4+ coagulase reaction as positive identification of *S. aureus*, without recourse to further testing.
Our results indicate that _S. aureus_ can be positively identified by the coagulate test if the extent of clotting of plasma is at the 3+ or 4+ level. On the other hand, cultures yielding coagulate reactions scored as 2+ must be interpreted, at best, as only presumptive evidence of coagulate production, and identification of these organisms as strains of _S. aureus_ must await the demonstration of a thermostable nuclease and/or anaerobic dissimilation of glucose and mannitol by the cultures. A 1+ reaction or absence of clotting is usually sufficient evidence to exclude the possibility of an organism being _S. aureus_. There are reports of coagulate-negative enterotoxin-producing strains of _S. aureus_ by Bergdoll, Weiss, and Munster (Bacteriol. Proc. p. 12, 1967) and by others (10, 13); however, these are rare.

The results of Sperber and Tatini (Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, E67, p. 12) and ours are at variance with respect to the degree of clotting of plasma which should be considered positive evidence of coagulate production by a culture. Because of the subjective nature of the coagulate test, it is possible that this disagreement is in the actual scoring of the extent of clotting rather than in the interpretation of the results. We assign a 4+ score only to those reactions in which coagulation is complete, and the clot is not displaced when the tube is inverted.

Unlike the coagulate test which is semi-quantitative, we have employed the thermostable nuclease test on a purely qualitative basis. This test has a number of advantages, chiefly, the lack of ambiguity of the reaction. The likelihood of misreading the thermostable nuclease test is quite remote due to the distinct color change accompanying a positive reaction. Secondly, we have seen no report of other bacteria possessing a thermostable nuclease. Although no pretence is made of a complete search of the literature, it would appear that the thermostable nuclease is specific for _S. aureus_ (4, 6, 7). Third, the test is relatively inexpensive, rapid and simple to perform. Based on these advantages and our results, it is tempting to suggest that the coagulate test be replaced by the thermostable nuclease test in the routine examination of foods for _S. aureus_. Such a suggestion however, would be premature at the present time, especially since we have shown that at least one of a limited number of entero-

toxin-producing strains of _S. aureus_ is negative for thermostable nuclease production. Before relegating the coagulate test to a lesser position in the identification of _S. aureus_, a larger number of strains of _S. aureus_ and a wider range of bacterial strains must be tested for thermostable nuclease production to determine whether the test is indeed specific for _S. aureus_. In the meantime we would recommend that the thermostable nuclease test be performed on all cultures yielding 2+ coagulate reactions before classifying them as _S. aureus_.

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