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Evaluation of Three Test Procedures for Identification of *Staphylococcus aureus* from Clinical Sources

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A total of 520 clinical and environmental isolates of the family *Micrococcaceae* that fermented glucose anaerobically were tested for their ability to produce coagulase, thermostable nuclease, and deoxyribonuclease. Of these, 450 isolates coagulated rabbit plasma, produced thermostable nuclease, and were identified as *Staphylococcus aureus*, 447 of which produced a 3+ to 4+ clot. The remaining three isolates produced a 2+ clot, deoxyribonuclease, and thermostable nuclease. It was found that three of the *S. aureus* isolates failed to produce deoxyribonuclease. A total of 70 isolates which did not coagulate rabbit plasma and which were thermostable nuclease negative were identified as *S. epidermidis*. Three of them produced deoxyribonuclease. It is suggested that the thermostable nuclease test be performed on all isolates producing a 2+ (or 1+) clot in the coagulase test before identifying them as *S. aureus*.

The tube coagulase test is the most widely used procedure for identification of *Staphylococcus aureus* (1, 12). Several factors have been reported which may affect the results of the coagulase reaction, including the type of plasma, the nature of anticoagulant used, and variation from one lot to another (9, 15). Confusion and uncertainty were also reported recently regarding interpretation of this test (10, 11). This controversy was attributed to a disagreement on the degree of clotting that should be considered as positive evidence of coagulase production. The coagulase test is usually read within 4 h of incubation; however, occasionally it might be necessary to continue the incubation overnight where weak coagulase producers may become more pronounced.

The deoxyribonuclease (DNase) test is a routine diagnostic procedure used by some laboratories to distinguish between *S. aureus* and other *Micrococcaceae* (3, 7). This test requires 18 to 24 h to complete.

The thermostable nuclease test was reported recently as a useful confirmatory test for the identification of *S. aureus* (10, 11, 15). This test is generally read within 4 h.

All these test procedures rely on the ability of *S. aureus* to produce the respective enzymes. It is known that certain related saprophytic *Staphylococcus* species may produce DNAse enzyme (7), and some pathogenic *S. aureus* may lose their ability to produce coagulase (8); however, thermostable nuclease appears to be a constant property produced by *S. aureus* (2, 14).

The purpose of this investigation was to evaluate the coagulase, DNase, and thermostable nuclease tests used for the identification of *S. aureus* isolated from clinical sources.

MATERIALS AND METHODS

Bacterial isolates. A total of 520 clinical isolates were collected from a variety of routine specimens submitted to the Public Health Laboratory by various hospitals located in the city of Hamilton and surrounding area for routine *S. aureus* phage typing. All isolates were primarily identified as gram-positive, catalase-positive cocci. These came from clinical and environmental sources (Table 1).

Coagulase test. The coagulase test was performed by adding 0.2 ml of the overnight brain heart infusion broth culture of the organism to 0.5 ml of reconstituted rabbit plasma with ethylenediaminetetraacetic acid purchased from Baltimore Biological Laboratories (BBL). After gentle mixing, the tube (10 by 75 mm) was incubated in an air incubator at 35 to 37°C and examined at intervals of 2, 4, and 24 h. The degree of clot formation was rated 1+ through 4+ according to Turner and Schwartz (13).

Thermostable nuclease test. Thermostable nuclease activity was determined by the method of Lachica et al. (5). The medium was prepared by dissolving 0.3 g of Difco deoxyribonucleic acid, 10.0 g of Difco agar, 1.0 ml of 0.01 M anhydrous $CaCl_2$, and 10.0 g of NaCl in 1 liter of 0.05 M tris(hydroxymethyl)-aminomethane buffer (pH 9.0). This mixture was boiled until all the ingredients were completely dissolved. It was cooled to about 45°C, and 3.0 ml of 0.1 M toluidine blue O was added and completely mixed (5). Approximately 15 to 20 ml of the medium was poured into petri plates (15 by 100 mm), allowed to cool, and stored at 4°C in a refrigerator. Prior to use, the plates were incubated for 1 h at 35°C. Fifteen equally spaced 3-mm wells were cut into the agar.

TABLE 1. *Source and number of isolates of aerobic Micrococcaceae examined*

Source of isolates	No. of isolates
Lesions	98
Sputum	56
Wounds	41
Environmental	32
Nose swabs	32
Catheterized urine	29
Armpits	26
Gastric	23
Drain site	20
Eye swab	20
Burns	20
Stool (food poisoning)	17
Umbilical cord	17
Throat swab	14
Tracheostomy	14
Vaginal swab	11
Breast milk	11
Episiotomy	11
Chest stab	8
Amniotic fluid	8
Hernia	6
Blood	6

Tubes containing the overnight brain heart infusion broth culture of each test organism were placed into a boiling-water bath for 15 min, removed, and allowed to cool to room temperature. Each of the wells was filled with a boiled broth culture by using disposable Pasteur pipettes. The plates were incubated at 35 to 37°C and observed at 1, 2, 4, and 24 h for the formation of a typical pink halo, 1 to 3 mm wide, surrounding each test well, indicating the presence of thermostable nuclease.

DNase activity. DNase test agar with methyl green (Difco) was prepared according to the manufacturer's instructions. Approximately 15 to 20 ml of the medium was poured into sterile plastic petri dishes (100 by 15 mm), cooled, and then stored at 4°C until used. The test was performed by inoculating plates with up to eight cultures by making streaks about 3 cm long with an inoculating needle, followed by incubation of plates at 35 to 37°C for 18 to 24 h. DNase activity was indicated by a clearing zone surrounding each streaked culture.

Carbohydrate fermentation. The ability of isolates to ferment glucose under anaerobic conditions was determined by the method recommended by the Subcommittee on Taxonomy of Staphylococci and Micrococci (1, 12).

RESULTS

A total of 520 clinical isolates of the family *Micrococcaceae* were tested by three test procedures (Table 2). Of these, 450 were identified as *S. aureus* because they coagulated rabbit plasma and also produced thermostable nuclease. Three of the 450 *S. aureus* strains failed to produce DNase.

Seventy strains did not coagulate plasma or produce thermostable nuclease. These were classified as *S. epidermidis*. Three strains from this group produced DNase. All 520 isolates fermented glucose anaerobically.

The thermostable nuclease test was observed for color change after 1, 2, 4, and 24 h of incubation. With over 99% of the strains, the change of color accompanying a positive reaction occurred within 2 h and became quite distinct within 4 h.

Table 3 shows the number of *S. aureus* strains and the degree of clot formed in the tube coagulase test. A total of 442 strains produced a 4+ clot. An additional five strains produced a 3+ clot. The remaining three strains produced a 2+ clot, but were also characterized as *S. aureus* in that they produced thermostable nuclease as well as DNase.

Table 4 shows the number of *S. aureus* strains, the degree of clot formation, and the incubation

TABLE 2. *Number of isolates of aerobic Micrococcaceae identified by production of coagulase, thermostable nuclease, and DNase and utilization of glucose*

Test	No. of strains identified			
	S. aureus		*S. epidermidis*	
	447	3	67	3
Coagulase	+	+	−	−
Thermostable nuclease	+	+	−	−
DNase	+	−	−	+
Glucose (anaerobic)	+	+	+	+

TABLE 3. *Degree of clot formation with rabbit plasma*

No. of strains of *S. aureus*	Degree of clot formation
0	1+
3	2+
5	3+
442	4+

TABLE 4. *Relationship between degree of clot formation and time required for completion of coagulase test*

Time required (h)	Degree of clot	No. of *S. aureus* strains
4	1+	0
	2+	0
	3+	4
	4+	440
18–24	1+	0
	2+	3
	3+	1
	4+	2

time required for completing the coagulase test. The coagulase reactions of 444 *S. aureus* strains were completed within 4 h. All of these strains produced a 3+ or 4+ clot. Six strains required further incubation overnight. These included three strains which produced a 2+ clot, one strain that produced a 3+ clot, and two strains that produced a 4+ clot. All six strains produced thermostable nuclease and DNase.

DISCUSSION

The tube coagulase test is the most widely used test for identifying *S. aureus* (1, 12). Disagreement was reported recently on the degree of clotting that should be considered as positive evidence of coagulase production (10, 11). Results obtained in this study suggest that *S. aureus* can be positively identified by the coagulase test alone if the degree of clotting is at the 3+ or 4+ level. Below that level (2+ clot), the test is not conclusive, and other tests may be performed. This is in agreement with the findings of Rayman et al. (10) and Yrios (15), but is at variance with those of Sperber and Tatini (11), who consider only a 4+ clot as a positive reaction in the identification of *S. aureus* when only the coagulase test is used.

In the present investigation, three strains showed a questionable coagulase reaction (2+ clot), but were classified as *S. aureus* by using other criteria such as thermostable nuclease and DNase production. Yrios (15) also encountered clinical isolates which formed a 2+ clot with rabbit plasma with ethylenediaminetetraacetic acid (Difco) and which also produced thermostable nuclease. These he identified as *S. aureus*.

It was found that over 98% (444) of the *S. aureus* strains coagulated plasma within 4 h, whereas the remaining six strains required a longer incubation period. Since staphylococci produce staphylokinase and Müller factor that may dissolve the fibrin clot (9, 15), the coagulase test should be observed periodically so that false-negative coagulase reactions are avoided. The absence of a 1+ or 2+ clot in the 70 *S. epidermidis* strains may be attributed to the type of plasma used in this investigation. Sperber and Tatini (11) found that 55 non-*S. aureus* strains produced a 2+ to 3+ clot with Difco rabbit plasma but showed no clotting with BBL plasma. The variation between the degree of clot and type of plasma used was also demonstrated by Yrios (15).

In this study, 447 (99.3%) of 450 *S. aureus* strains and 3 of 70 isolates of *S. epidermidis* were DNase positive on DNase agar (Difco). Morton and Cohn (7) demonstrated that 98% of 304 coagulase-positive cultures and 13.5% of 200 coagulase-negative cultures produced DNase. It would appear from these results that a small percentage of *S. epidermidis* strains may be DNase positive, whereas some *S. aureus* strains may not produce DNase. In the present investigation, the three *S. aureus* strains which were DNase negative on Difco test medium may have produced the enzyme, but not in sufficient amounts to be detected by this test procedure. This assumption is made because these strains were thermostable DNase positive when tested by Lachica's method (5). It is concluded from these findings and those of Jarvis and Wynn (3) that the DNase test cannot be relied on as the only criterion for the identification of *S. aureus*. This test can be useful, however, in the case of strains that give doubtful reactions (2+ or 1+ clot) in the coagulase test where a positive DNase would confirm *S. aureus*.

All 450 clinical isolates identified as *S. aureus* yielded thermostable nuclease (Table 2). These results support the findings of other investigators. Rayman et al. (10) reported that all 103 clinical isolates of *S. aureus* tested were positive for thermostable nuclease. It was shown that only one enterotoxigenic food isolate of *S. aureus* failed to produce thermostable nuclease. Sperber and Tatini (11) also found that all 439 strains identified as *S. aureus* yielded thermostable nuclease. Yrios (15) found that all 416 clinical isolates identified as *S. aureus* produced thermostable nuclease. In addition, 4 out of 177 *S. epidermidis* strains were found to be thermostable nuclease positive. A definite identification of these strains was not carried out, but they were considered to be atypical *S. epidermidis*. None of the 70 *S. epidermidis* strains tested in the present investigation showed any thermostable nuclease activity. Thermostable nuclease production appears to be a consistent property of *S. aureus* (2, 14). The test is easy to perform, is rapid, and is not influenced by as many factors and variations as the coagulase test. With over 99% of the strains, the color change occurred within 2 h and became quite distinct within 4 h. There was no color reversion even after 24 h of incubation.

From these results, it is suggested that the thermostable nuclease test be performed on all clinical isolates yielding a 2+ (or 1+) clot in the coagulase test to confirm and identify any weak coagulase producers or doubtful strains of *S. aureus*. This procedure may reduce the possibility of error in the identification of *S. aureus* and will speed up reporting of results.

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