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Microtube Coagulase Test for Detection of Coagulase-Positive Staphylococci

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Studies were performed to determine the sensitivity and specificity of a new microtube method for the detection of coagulase production by *Staphylococcus aureus*. Rabbit plasma containing EDTA was added to and lyophilized in API microtubes. Two standard coagulase plasmas containing EDTA were used in the conventional macrotube test and served as a basis for comparison. No false-positive or false-negative reactions were encountered with the microtube system. With this system, 53% of the coagulase-positive strains tested were detected within 1 h after inoculation, 82% were detected after 2 h, 97% were detected after 3 h, and 99% were detected after 4 h. With the first conventional method, 45, 81, 96, and 98% of the positive strains were detected in 1, 2, 3, and 4 h, respectively, whereas with the second conventional method, only 6% of the positive strains were detected in 1 h, 24% in 2 h, 66% in 3 h, and 81% in 4 h. With the microtube method, 5 of the 139 coagulase-producing strains studied reverted to negative between 5 and 24 h after inoculation, whereas 9 reverted with the more rapid conventional method, and no reversions occurred with the second conventional method. All reversions involved strains which caused gelation of plasma within 1 h after inoculation. The data obtained showed that 99% of the coagulase-positive strains tested could be detected within 4 h by the microtube method. In addition, the microtube method offers a more convenient and economical format for the performance of the coagulase tube test.

The ability to clot plasma is generally accepted as the most reliable criterion for the identification of staphylococci (1). The tube test, which demonstrates the production of free coagulase (7), is considered the definitive test for coagulase production (10). Bound coagulase, or "clumping factor," is antigenically distinct from free coagulase (7) and is demonstrated by the slide procedure, which is considered a screening technique. The exact mechanism of clot production in plasma by staphylocoagulase has not been fully elucidated, but recent evidence suggests that staphylocoagulase reacts with a substance in plasma called coagulase-releasing factor to form a stable complex called staphylothrombin (9). This reaction results in the conversion of fibrinogen to fibrin in a fashion similar to that observed physiologically with thrombin (9).

Several factors have been demonstrated to affect coagulase production. Among these are the content of fibrinolysin and coagulase-releasing factor in the plasma and the nature of the anticoagulant used. Rabbit plasma has been shown to clot more rapidly than plasma from other species in the coagulase test (5), although it has also been shown to contain the highest concentration of fibrinolysin (plasmin) (12). Human plasma has been reported to contain the highest concentration of coagulase-releasing factor, followed by porcine, rabbit, and horse plasmas (12). The use of rabbit plasma has been designated as superior for detecting *Staphylococcus aureus* (14) because of the rapidity of the reaction and the lack of the inhibitors of coagulase production that are sometimes found in human serum. The type of anticoagulant used in coagulase plasma has a marked effect on results obtained. Staphylococcal coagulase coagulates plasma in the presence of a variety of anticoagulants, such as citrate and EDTA (11). Since citrated plasma may be coagulated by species other than *S. aureus*, such as gram-negative bacilli and enterococci that have the capacity to metabolize the anticoagulant (2), the use of EDTA-containing plasma has been recommended (8, 10).

The standard method used to demonstrate coagulase activity is the macrotube method (13, 14). In this technique, reconstituted lyophilized coagulase plasma is dispensed into a test tube which is inoculated with an isolated colony of bacteria grown on a noninhibitory agar plate (3, 8, 10, 13). The tube is placed in an incubator or

water bath at 35 to 37°C, and results are read at specified times. At present, some controversy exists regarding the optimum time of incubation. Several investigators have recommended that readings be taken at 4 h and, in the absence of a clot, that the tubes be reexamined after 24 h of incubation (8, 11, 13). Most recently, incubation for only 4 h has been recommended (10).

We undertook the present investigation to determine the performance characteristics of API coagulase plasma, containing EDTA, dispensed and lyophilized in API microtubes for the detection of coagulase production by *S. aureus*. The sensitivity and specificity of the system were determined on the basis of results obtained by the conventional test for coagulase detection. In addition, the optimum times needed to demonstrate a positive reaction and a negative reaction were examined.

MATERIALS AND METHODS

Bacteria. The test organisms consisted of 339 strains of staphylococci isolated from a variety of patient specimens submitted to API Diagnostic Laboratories from numerous laboratories throughout the United States. In addition, 10 strains of enterococci and 3 *Pseudomonas aeruginosa*, 3 *Escherichia coli*, 2 *Enterobacter cloacae* and 2 *Enterobacter agglomerans* isolates were included in the sensitivity and specificity studies. One strain each, obtained from the American Type Culture Collection (ATCC), of *Staphylococcus hyicus* (ATCC 11249) and *Staphylococcus intermedius* (ATCC 29663) were also tested. All isolates were stored at −70°C in 15% glycerol broth. Before being used, the isolates were subcultured onto Trypticase soy agar plates containing 5% sheep blood (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 35 to 37°C for 18 h. Organisms were passaged on the plates twice more before analysis. All isolates were tested simultaneously for the production of coagulase with the three coagulase tests listed below and were identified with conventional media according to the schema of Kloos and Smith (10).

Conventional macrotube coagulase test. The conventional standard coagulase test was performed as described by the U.S. Food and Drug Administration (14) and the Subcommittee on Taxonomy of Staphylococci and Micrococci (13). Two commercially available lyophilized coagulase plasmas containing EDTA (from BBL Microbiology Systems for conventional test 1 and from Difco Laboratories, Detroit, Mich., for conventional test 2) were reconstituted with sterile distilled water per specifications of the manufacturers and were dispensed aseptically in 0.5-ml aliquots into sterile plastic tubes (12 by 75 mm). One colony from an 18-h culture on a Trypticase soy agar plate containing 5% sheep blood was transferred to the plasma-containing tube with a sterile applicator stick and emulsified into the plasma. The tubes were then placed in an incubator at 35 to 37°C.

API microtube system. The API microtube system (Analytab Products, Plainview, N.Y.) consisted of rabbit plasma with 2.5% EDTA lyophilized in API microtubes on API strips. The microtubes were stored in a desiccator at room temperature until just before they were used. Each microtube was reconstituted with two drops of sterile distilled water. One colony from an 18-h culture on a Trypticase soy agar plate was transferred with a sterile wooden applicator stick and emulsified into the microtube plasma. The inoculated strips were then placed in an API incubation tray containing approximately 3 ml of tap water on the bottom of the tray, covered with the lid, and placed in an incubator at 35 to 37°C.

Specificity and sensitivity. For the purpose of this investigation, specificity was defined as the ability to accurately discriminate bacteria that do not produce coagulase from those that do. Therefore, specificity was considered to be 100% when no false-positive reactions were encountered. Sensitivity was defined as the ability to detect coagulase in all coagulase-producing strains.

Reading results. The tests were observed for gelation of the plasma each hour for 7 h and after 24 h of incubation. A reaction was considered positive when an organized clot of any size was formed. Reactions were considered to revert to negative when a formed clot liquefied during the 24-h period of observation.

Quality control. A positive control (*S. aureus* ATCC 25923) and a negative control (*Staphylococcus epidermidis* ATCC 12228) accompanied all experimental tests. An uninoculated control was also used to rule out spontaneous coagulation or coagulation caused by a contaminant.

RESULTS

Of the 339 clinical isolates of staphylococci tested, 139 were identified as *S. aureus*. All 139 strains produced coagulase (by at least two of the three coagulase tests used), fermented mannitol, and elaborated detectable levels of DNase. Of the remaining 209 clinical staphylococcal isolates, 70% consisted of *S. epidermidis*, 19% consisted of *Staphylococcus saprophyticus*, and 11% consisted of a number of other coagulase-negative *Staphylococcus* species. These latter bacteria are referred to below as coagulase-negative staphylococci.

Each system was tested for specificity with the 209 coagulase-negative strains of staphylococci and the 20 nonstaphylococcal isolates. No false-positive results were encountered with any of the systems tested; thus, each system had a specificity of 100%. The sensitivity of each system was measured by its ability to detect coagulase producers. The sensitivity of the API microtube method was 100%, as was that of the most sensitive conventional standard method tested. Conventional test 2 was unable to detect coagulase production by two strains that were positive for clot formation when tested with the API microtube method and with conventional test 1. These two false-negative reactions resulted in an overall sensitivity of 98.6% for conventional test 2.

Since the isolation of *S. hyicus* and *S. interme-*

TABLE 1. Hourly positive results obtained with the API microtube coagulase test and with standard coagulase plasma in a conventional macrotube test

System	No. of positive results[a] (cumulative %) at:							
	1 h	2 h	3 h	4 h	5 h	6 h	7 h	24 h
API microtube	73 (53%)	41 (82%)	21 (97%)	3 (99%)		1 (100%)		
Conventional test 1	62 (45%)	50 (81%)	21 (96%)	3 (98%)	2 (99%)		1 (100%)	
Conventional test 2	8 (6%)	24 (23%)	60 (66%)	20 (81%)	12 (89%)	5 (93%)	2 (94%)	6 (98.6%)

[a] Number of new positive results each hour after inoculation.

dius from human clinical material has not been documented to date, studies on these organisms were performed separately. Each of the three coagulase tests detected coagulase production by each of the two strains of these bacteria within 18 h of incubation at 35 to 37°C. Since the potential for confusing these species with *S. aureus* in clinical specimens is, at present, academic, they are not included in further analyses of the data.

The number of *S. aureus* strains showing positive tests for coagulase production during each hour of incubation was also investigated (Table 1). The data indicate that the microtube system detected 53, 82, 97, and 99% of the tested coagulase-positive staphylococci after 1, 2, 3, and 4 h, respectively, of incubation at 35 to 37°C. When standard plasma (conventional test 1) was used, 45% of the positive strains were detected after 1 h, 81% were detected after 2 h, 96% were detected after 3 h, and 98% were detected after 4 h of incubation. Only 81% of the positive strains, however, were detected with the other standard plasma (conventional test 2) after 4 h.

Clot lysis was experienced with the API plasma and with one of the standard plasmas (conventional test 1) but not with the other conventional plasma (conventional test 2). All reversions occurred with rapid coagulase producers that were positive within 1 h after inoculation. Five reversions occurred with the API plasma, whereas nine reversions were observed with the standard plasma (conventional test 1). Three of the five API-plasma reversions and eight of the nine standard-plasma reversions occurred between 7 and 24 h of incubation. Also, two earlier reversions occurred with the microtube system (one at 5 h and one at 6 h), and one with the conventional macrotube test occurred after 4 h of incubation.

DISCUSSION

It is dogma in clinical microbiology to determine a microbial characteristic as accurately and as rapidly as possible. The recognition of *S. aureus*, based on the coagulase test, is a prime example of this approach. Historically, coagulase reactions were read at 4 h and on the following day (8, 11, 13, 14). Some investigators have indicated an optimal reading of 6 h followed by an overnight incubation (3). In the present investigation, 99% of all positive strains were detected at 4 h with the microtube method. Only 96.4% of the positive strains would have been detected after overnight incubation, since 5 of the 139 positive strains tested lysed the plasma clot after overnight incubation. The ideal (100% sensitivity and specificity) would have been achieved by a 4-h and a 6-h reading. Testing with plasma in the conventional tube test (conventional test 1) resulted in a 98% detection rate at 4 h, and, since nine reversions occurred, a 93.5% detection rate if only overnight readings were considered. Optimal results with the other conventional test (conventional test 2) were achieved by a 24-h reading only.

The differences in the reaction times noted with the microtube and conventional tube tests may be related to several factors, including the differences in the reaction vessels and in the bacterial inoculum/plasma ratios used in the two methods. The microtube test is performed in API microtubes which are modified, miniaturized (200-μl capacity) Ivan Hall tubes which provide an oxygen gradient that decreases from the top to the bottom of the tube (4). In addition, the conventional test exposes a single colony of bacteria to 0.5 ml of plasma, whereas the microtube test provides 1/10 of this volume for the same bacterial mass. This difference in the substrate/bacteria ratio may influence both the rate and the magnitude of the resultant coagulase reaction.

Although incubation of the coagulase test overnight or for 24 h has been recommended by several investigators (3, 8, 11, 13, 14), the following factors should be considered: plasma is usually not guaranteed to be sterile and may give false-positive or false-negative results because of contaminants; an inoculum taken from a culture on an agar plate may not be pure, with a contaminant producing false results at 24 h; and the chance of contamination is greatly increased when several technologists take samples of plasma from one reconstituted vial. These possibilities led Kloos and Smith (10) to recommend

taking only 4-h readings. For those rare strains requiring a longer incubation period (1% of the isolates in this study), the same investigators recommend that other tests be performed overnight so that results may confirm or refute the presumptive identification obtained from the coagulase results. Other investigators suggest that the thermostable nuclease test be performed on any isolate yielding doubtful results in the coagulase test (15).

In the course of this investigation, clot reversions to negative were observed with the API microtube system as well as with one of the conventional tests (conventional test 1). Reversion is caused by lysis which occurs when staphylokinase activates the formation of fibrinolysin (12). At present, staphylokinase activity is thought to be the product of several extracellular staphylococcal proteases (6). With one of the conventional macrotube plasmas (conventional test 1), one reversion occurred after 4 h of incubation, whereas eight additional reversions were observed at 24 h after inoculation. A total of five reversions occurred with the API system; one at 5 h, one at 6 h, and 3 at 24 h of incubation. No reversions occurred with the other conventional plasma (conventional test 2).

In addition to its excellent sensitivity and specificity, the API microtube method offers a convenient format for the performance of multiple coagulase tests; requires less technologist time, since it obviates the need for dispensing plasma into separate tubes; reduces the risk of contamination; and is much more economical to use than the macrotube method currently available to the clinical laboratory.

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