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Rapid and Direct Staphylocoagulase Assay That Uses a Chromogenic Substrate for Identification of *Staphylococcus aureus*

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A rapid and direct assay of staphylocoagulase that uses chromogenic substrate Chromozym TH was developed for the routine identification of *Staphylococcus aureus*. False-positive and false-negative reactions were eliminated by measuring the activity of the staphylocoagulase-prothrombin complex directly. The method was compared with three clotting assays. The results show that the new assay is specific, quantitative, and easily automated. It offers clinical laboratories a more reliable assay of staphylocoagulase and is a valuable aid for the rapid identification of *S. aureus*.

The ability to produce staphylocoagulase is the most widely used and generally accepted criterion for identification of *Staphylococcus aureus* (3, 12). Most clinical laboratories perform a staphylocoagulase assay that is based on the ability of the enzyme to clot plasma. The clotting action of staphylocoagulase is due to a specific and stoichiometric reaction of staphylocoagulase with prothrombin, yielding a complex, i.e., staphylothrombin, which converts fibrinogen into insoluble fibrin in a manner similar to the action of physiologically formed thrombin (10, 23). Most assay methods routinely used are qualitative methods. Apart from difficulty in standardizing and quantitating these assays, false-negative and false-positive reactions may occur. False-negative reactions will take place if fibrinogen and fibrin are partially cleaved by proteases, staphylokinase-induced plasmin, or both, so that an insoluble fibrin clot cannot be formed or is dissolved (21). On the other hand, false-positive reactions are observed if prothrombin is activated by trypsin or papain-like proteases, which are present in the culture fluid of *S. aureus* (20). Recently, prothrombin activation by metalloproteases has also been described (20). The present study describes a direct assay of the activity of staphylothrombin based on limited proteolysis of a synthetic chromogenic substrate. The method is quantitative and can be automated easily. A comparison of this method with "classic" clotting assays was made by using recently isolated clinical strains of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains. *S. aureus* strains (n = 70) recently isolated from various clinical sources were kindly provided by W. J. van Leeuwen, Department of Bacteriology, National Institute for Public Health, Bilthoven, The Netherlands. The strains were characterized as *S. aureus* on the basis of phage and biotyping according to the method of Blair and Williams (4) and Kloos and Schleifer (12), respectively. For biochemical characterization, the API Staph system (API System S.A.; La Balm les Grottes, France) was used. Another 20 clinical isolates with the morphological characteristics of *Staphylococcus* spp. (yellow-white colonies with a smooth glistening surface and staining as gram-positive grapelike clusters) were also tested. These strains were nontypable with the International Phage Typing set and were not clearly identified to the species level according to the (restrictive) manufacturer's manual of the API Staph system. For controls, the following staphylocoagulase-negative reference strains were used: *Staphylococcus haemolyticus* SM102, *Staphylococcus intermedius* CF238, *Staphylococcus hyicus* CBCC1462 (obtained from W. E. Kloos, Department of Genetics, North Carolina State University, Raleigh), and *Staphylococcus epidermidis* 407 (obtained from J. Verhoef, Department of Medical Microbiology, University of Utrecht, The Netherlands).

Prothrombin - proconvertin - Stuart - Prower factor-antihemophilic factor B. A concentrate of the blood clotting factors II, VII, X, and IX was prepared according to the method of Soulier et al. (16).

Cultivation conditions. The organisms were cultivated statically in test tubes (16 by 100 mm) containing 5 ml of nutrient broth (Difco Laboratories; Detroit, Mich.) or brain heart infusion medium (Difco) at 37°C for 18 h. Bovine serum albumin (Sigma Chemical Co.; St. Louis, Mo.) was added to a final concentration of 0.2% (wt/vol) to these media to study its effect on staphylocoagulase production.

Deoxyribonuclease. Deoxyribonuclease production was screened on agar plates containing 1% deoxyribonucleic acid (Difco), as described in the manufacturer's manual.

Protease. Protease production was determined with azocasein (Sigma) according to the method of Hazen et al. (9).

Staphylocoagulase. Staphylocoagulase was produced from *S. aureus* strain 104, as described by Engels, Kamps, and van Boven (6), and purified by chromatography on a bovine prothrombin-Sepharose 4B affinity column according to the method of Igarashi, Morita, and Iwanaga (11).

Staphylocoagulase assays. (i) Chromogenic substrate method. After centrifugation of the cultures at 2,500 × *g* for 15 min, 30 μl of supernatant fluid was mixed with 120 μl of reagent mixture containing 72 mM triethanolamine buffer (pH 8.4), 144 mM NaCl, 166 μM Chromozym TH (Boehringer; Mannheim, West Germany) and 5 μl of human prothrombin–proconvertin–Stuart-Prower factor–antihemophilic factor B solution containing 50 μg of prothrombin per ml as the prothrombin source. In this assay, staphylocoagulase activates prothrombin to staphylothrombin. The thrombin-like activity of this complex cleaves the synthetic tripeptide Chromozym TH (*N*-tosyl-glycyl-L-prolyl-L-arginyl-*p*-nitroanilide.hydrochloride) by limited proteolysis, liberating the yellow *p*-nitroaniline (18, 22). The reaction was performed in microtiter plates (Greiner; Nürtingen, West Germany). After incubation for 1 and 2 h at 37°C, the absorbances were measured with a Titertek Multiskan (Flow Laboratories Ltd.; Irvine, United Kingdom) at 405 nm and were corrected automatically for blank medium reactions. Since the staphylocoagulase-negative reference strains gave absorbances lower than 0.05 after 1 h of incubation, absorbances in the range of 0 to 0.05 were recorded as negative reactions, and absorbances exceeding the value of 0.05 were recorded as positive. One unit of staphylocoagulase was defined as the amount of enzyme giving an increase in absorbance at 405 nm of 1.0 per min at 37°C.

(ii) Tube inoculation method. Twenty-five microliters of the culture fluid was inoculated into 0.5 ml of citrated human plasma and incubated at 37°C. After 1 and 4 h, the mixtures were screened for clotting. Any degree of clotting constituted a positive reaction. Flocculent or fibrous precipitates were recorded as negative (13).

(iii) Tube supernatant fluid method. After centrifugation of the cultures at 2,500 × *g* for 15 min, 0.1 ml of supernatant was mixed with 0.1 ml of citrated human plasma and incubated at 37°C. After 1 and 4 h, the mixtures were screened for clotting according to the criteria described for the tube inoculation method.

(iv) Staphylocoagulase plates. In a method comparable to those described by Van der Vijver, Kraayeveld, and Michel (19) and Parisi, Baldwin, and Sottile (15), brain heart infusion agar plates containing 10% (vol/vol) citrated human plasma were used. After inoculation and incubation at 37°C, staphylocoagulase production was indicated by opalescent haloes (diameter, 4 to 10 mm) surrounding the colonies.

RESULTS

It has been shown previously (7) that a direct determination of staphylothrombin activity can be performed by using a chromogenic substrate. In this assay method, a linear relationship was obtained not only between the incubation time and the hydrolysis of Chromozym TH with different amounts of purified staphylocoagulase (Fig. 1) but also between the concentrations of staphylocoagulase and their corresponding reaction rates (Fig. 2). Linearity of the activities was observed up to absorbances of 0.9 to 1.0. In this experiment, the amounts of purified staphy-

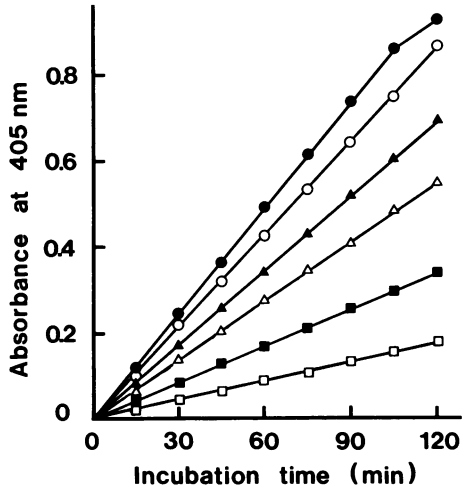

FIG. 1. *Time course of the hydrolysis of Chromozym TH with different amounts of staphylocoagulase. The reactions were performed in microtiter plates at 37°C with different amounts of purified staphylocoagulase, as described in the text. The final concentrations in the reaction mixtures were 81 ng/ml (●), 68 ng/ml (○), 54 ng/ml (▲), 41 ng/ml (△), 27 ng/ml (■), and 14 ng/ml (□).*

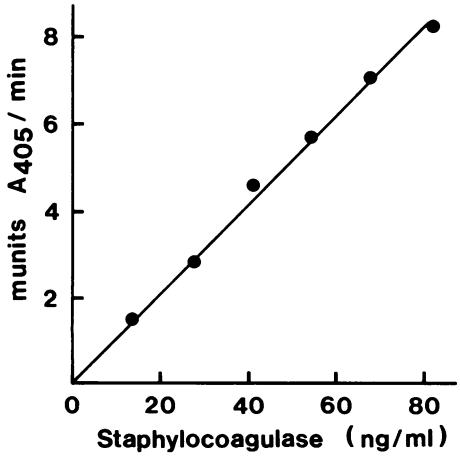

FIG. 2. *Standard curve of the linear relationship between the different staphylocoagulase concentrations and their corresponding reaction rates, calculated from the time courses shown in Fig. 1.*

locoagulase were far in excess of what was found in the supernatant fluids of the staphylococcal cultures, which gave absorbances in the range of 0 to 0.5. The influence of the cultivation medium on the production of staphylocoagulase by the *S. aureus* strains ($n = 70$), when assayed by the chromogenic substrate and the tube clotting methods, is shown in Table 1. The percentages of staphylocoagulase-positive reactions of the tested strains, subcultured in brain heart infusion medium, were 84.3, 90.0, and 84.3% with the chromogenic substrate method, the tube incubation, and the tube supernatant fluid method as the respective assay methods. After a prolonged incubation time, the percentages increased to 97.1, 98.6, and 100%, respectively. The staphylocoagulase production of one strain was variable and therefore resulted in a 98.6%—instead of a 100%—positive score for the tested organisms. The amount of staphylocoagulase produced by the strains after subculturing in nutrient broth was much lower, resulting in only 10.0, 40.0, and 35.7% positive reactions, respectively, when the assay methods mentioned above were used. A longer incubation time resulted in an increase to 40.0, 82.9, and 88.6%, respectively.

For achieving high sensitivity of the assays, the amount of staphylocoagulase produced in the 18-h cultures had to be optimized. We achieved optimization by adding bovine serum albumin to a final concentration of 0.2% (wt/vol), as recommended by Altenbern (2).

Addition of bovine serum albumin did increase the amount of staphylocoagulase produced. The effect was most obvious after subculturing in nutrient broth, using the chromogenic substrate method. An increase in positive reactions from 40 to 97.1% was observed.

Staphylocoagulase production on brain heart infusion agar plates containing plasma showed that only 51 of the 70 *S. aureus* strains (72.9%) gave positive reactions. In 11 strains, very weak or no staphylocoagulase reactions were observed. Moreover, interference by fibrinolysis took place with eight strains; thus, these strains also had to be scored as staphylocoagulase negative. Therefore, misjudging of the staphylocoagulase reaction in the latter strains cannot be ruled out. Of the staphylocoagulase-negative control strains, *S. epidermidis* and *S. haemolyticus* were determined to be staphylocoagulase negative by all the test methods. *S. hyicus* and *S. intermedius* gave a positive reaction in the clotting assays, whereas when the strains were assayed by the chromogenic substrate method, positive reactions were not observed. It was demonstrated that *S. hyicus* produced a proteolytic enzyme, which may explain the false-positive reaction in the clotting assays.

The additional 20 staphylococcal strains produced only trace amounts of staphylocoagulase in the clotting assays. However, in the chromogenic substrate assay, four strains gave a positive reaction which was not inhibited by 5 mM ethylenediaminetetraacetic acid. The additional positive deoxyribonuclease reaction indicated that these four strains could be identified as *S. aureus*.

Because false-positive reactions could have been caused by the presence of metalloproteases in the *S. aureus* strains, the chromogenic substrate assay was performed also in the presence of 5 mM ethylenediaminetetraacetic acid, as described by Wegrzynowicz et al. (20). For this assay, no influence of ethylenediaminetetraacetic acid addition on the staphylocoagulase reaction could be demonstrated.

For routine use in a clinical laboratory, it is preferable to complete the identification within 1 day. Therefore, the cultivation time of the strains in brain heart infusion medium supplemented with 0.2% bovine serum albumin was reduced from 18 to 4 h. In spite of lower staphylocoagulase activities (data not shown), 98.6% of the chromogenic substrate assay tests still gave positive reactions.

TABLE 1. *Comparison of different assays of staphylocoagulase produced by 70 strains of S. aureus in relation to various cultivation media*

Cultivation medium	% Positive staphylocoagulase reactions at indicated assay incubation time					
	Chromogenic substrate method		Tube inoculation method		Tube supernatant method	
	1 h	2 h	1 h	4 h	1 h	4 h
Brain heart infusion	84.3	97.1	90.0	98.6	84.3	100
Brain heart infusion + 0.2% bovine serum albumin	98.6	98.6	97.1	100	98.6	98.6
Nutrient broth	10.0	40.0	40.0	82.9	35.7	88.6
Nutrient broth + 0.2% bovine serum albumin	74.3	97.1	54.3	87.1	52.9	97.1

DISCUSSION

Staphylocoagulase production has been established as one of the definitive characteristics of *S. aureus* (3). However, rapid identification of *S. aureus* has not always been possible because most assays may take 24 h to produce a positive result. In general, two tests can be performed: a tube test and a slide test. Although slide tests are performed in various laboratories, it must be emphasized that this assay involves the clumping factor and not staphylocoagulase activity (1).

The classic clotting methods were very reliable, as expected (Table 1). However, when the criteria of Sperber and Tatini (17) were strictly followed, a considerably lower number of positive reactions were recorded in the clotting assays because, for about 15% of the strains, no firm opaque clots were formed after 4 h, owing to fibrinolysis and related reactions. For this reason, longer assay times should be avoided also (13). Therefore, misjudging of the reaction in the clotting assays can occur easily if one has no experience in this field.

The staphylocoagulase plate assay, which is in principle comparable to the method described by Van der Vijver et al. (19), and the quantitative radial slide assay method described by Kohl and Johnson (14) did not yield very reliable results. The relatively low numbers of positive staphylocoagulase reactions observed (72.9%) were probably due to fibrinolysin or related enzymes produced by the *S. aureus* strains. Kohl and Johnson might not have observed this interfering enzyme reaction because, in contrast to the strains used in this study, their test strains were from food rather than clinical sources.

Although false-negative and false-positive reactions in the clotting assays can be prevented by using a mixture of different protease inhibitors (20, 21), an assay based on a direct and specific measurement of staphylothrombin activity is preferred. From this study, it can be concluded that the chromogenic substrate assay is more reliable than the clotting assays. Even with the 20 weak staphylocoagulase-producing staphylococcal strains, staphylocoagulase could be demonstrated positively in four strains. The assay is specific, since *S. intermedius* and *S. hyicus* will not give a positive result. Staphylocoagulase production by *S. intermedius* and *S. hyicus*, as reported by Hájek (8) and Devriese (5), needs further investigation.

Cultivation of the *S. aureus* strains in brain heart infusion medium supplemented with 0.2% bovine serum albumin and an assay time of 1 h resulted in a 98.6- to 100%-positive score (Table 1). For routine assays, a cultivation time of 4 h enables a staphylocoagulase assay within 1 day. To eliminate possible false-positive reactions, we recommend adding 5 mM ethylenediaminetetraacetic acid to the assay mixture. Prothrombin-proconvertin-Stuart-Prower factor-antihemophilic factor B can be obtained from almost every blood transfusion center at reasonable prices, especially in the case of outdated samples. Analyzing the data quantitatively, we could not observe a correlation between the amount of staphylocoagulase produced and phage type or origin of the *S. aureus* strains. Also, there was no correlation between origin and phage type.

Nevertheless, this test enables a quantitative assay of staphylocoagulase and provides the means for setting up an epidemiological survey with large numbers of *S. aureus* strains to investigate a possible relationship between the amount of staphylocoagulase produced and the virulence of *S. aureus* on the one hand and the role of the enzyme in the pathogenesis of *S. aureus* on the other. In addition, the availability of a simple quantitative method for the assay of staphylocoagulase makes it possible to monitor staphylocoagulase activities during enzyme purification. Since the assay can be performed in microtiter plates, high numbers of samples can be tested automatically.

In conclusion, the major advantages of this test are not only the short assay time but also the possibility of screening large numbers of strains or staphylocoagulase samples in a quantitative and specific way.

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