Rapid Identification of Staphylococcus aureus by Using Fluorescent Staphylocoagulase Assays

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Two rapid (1-h) assays for the detection of Staphylococcus aureus staphylocoagulase were developed by using the fluorogenic thrombin substrates N-t-boc-Val-Pro-Arg-7-amido-4-methylcoumarin (VPA) and N-t-boc-β-benzyl-Asp-Pro-Arg-7-amido-4-methylcoumarin (BB). The assays were compared to the tube coagulase test and latex agglutination (LA) (Sanofi Diagnostics Pasteur, Guildford, Surrey, United Kingdom) by using 406 clinical isolates of staphylococci, and they produced positive and negative predictive values of 99.2 and 99.1% for LA, 98.9 and 92.7% for VPA, and 98.9 and 99.1% for BB. Fluorescent assays used colonies from solid media, thereby eliminating the need for broth cultures, and were performed in microtiter trays, thus making them suitable for large-scale screening.

Staphylococcus aureus is one of the most frequently encountered pathogens in clinical samples (7, 10), while methicillin-resistant S. aureus (MRSA) is an important nosocomial pathogen (9, 15). A rapid, reliable test to identify these organisms is of considerable relevance (10, 15). The detection of staphylocoagulase by the tube coagulase test (1, 4, 16) is the "gold standard" for identification of S. aureus from human sources (3, 7, 13, 16); however, variations in the quality of the plasma (3, 12, 16) and problems in the interpretation of the test (1, 3, 4, 11–13, 16) can lead to misidentification (1, 12).

Tube coagulase tests are incubated for 24 h (1, 3, 12, 13, 16) to ensure detection of weak coagulase producers, thus increasing the risk of false-negative results due to proteolysis (4, 11) or false-positive results due to the activation of prothrombin by proteases (4, 11) or metalloproteases (4). Latex agglutination (LA) tests which detect S. aureus clumping factor, protein A, and capsular polysaccharides (7, 10) may give false-positive results with other organisms (11a) and staphylococci (10) and may have low sensitivity with MRSA (7, 10).

Chromogenic and fluorogenic staphylocoagulase assays have been developed and used to study purified staphylocoagulase (5, 6) and broth supernatants (3, 4, 11). This report details two new fluorescent assays for staphylocoagulase using colonies on solid media and compares these to the classical tube coagulase test and an improved LA test using clinical isolates of staphylococci.

Bacterial strains. A total of 406 clinical isolates of staphylococci were tested. Isolates were grown and maintained on Columbia horse blood agar. All isolates were identified as catalase-positive, gram-positive cocci and by the tube coagulase test.

Tube coagulase test. Strains were incubated for 18 h in 10 ml of brain heart infusion (BHI) broth (Lab M, Bury, United Kingdom) at 37°C. A 200-μl volume of this broth was added to 500 μl of Bacto Coagulase Plasma (Difco Laboratories, East Molesey, United Kingdom) in a sterile plastic universal container and mixed. Tests were incubated at 37°C and examined at 4 and 24 h. Coagulation was graded as 1+ to 4+ (12).

S. aureus (NCTC 6571) was included as a positive control and Staphylococcus epidermidis (NCTC 11047) was included as a negative control with each run.

LA. The Pastorex Staph Plus test (Sanofi Diagnostics Pasteur, Guildford, United Kingdom), which detects clumping factor, staphylococcal protein A, and capsular polysaccharides was used according to the manufacturer’s instructions.

Fluorescent staphylocoagulase assays. Isolates were subcultured onto Columbia blood agar overnight before testing. Tests were performed in round-bottomed microtiter trays. A 25-μl volume of BHI broth containing 0.2% bovine albumin/fraction V (Sigma Ltd., Poole, United Kingdom) and 25 μl of prothrombin (factor V) diluted at 1 mg/ml in distilled water (Sigma) were pipetted into each well. Four distinct colonies or a 2-mm streak through confluent growth of the organism tested was emulsified in the BHI-prothrombin solution to a turbidity greater than a no. 10 MacFarland standard.

The plates were incubated at 37°C for 40 min before 100 μl of N-t-boc-Val-Pro-Arg-7-amido-4-methylcoumarin (VPA) (Sigma) (0.5 mg/ml in 0.05 M Tris, pH 8.0) was added, the solution was mixed, and the plates were incubated for a further 20 min. The degree of fluorescence was determined by using a Labtech Biolite F1 fluorescent microtiter plate reader (Labtech Ltd., Litchfield, United Kingdom) with filters set at 365 and 440 nm and sensitivity at 28. Readings above 8,000 were taken as positive. Positive, negative, and reagent controls were included with each batch, with S. aureus NCTC 6571 as the positive control and S. epidermidis NCTC 11047 as the negative control. The same technique was repeated with N-t-boc-β-benzyl-Asp-Pro-Arg-7-amido-4-methylcoumarin (BB) (Sigma) (0.5 mg/ml in 0.05 M Tris, pH 8.0).

Of the 406 clinical isolates tested, 297 were positive by the tube coagulase test and were identified as S. aureus. Of these, 295 (99.3%) were LA positive while 294 (98.9%) were positive by the two fluorescent assays (Table 1). Two strains identified as S. aureus by the API STAPH system (Biomerieux, Basingstoke, United Kingdom) and by the tube coagulase test were reproducibly LA negative. Forty MRSA strains were positive with all three assays.

Two S. aureus strains gave negative results with both VPA and BB fluorescent assays. One S. aureus strain was VPA negative and BB positive, and one strain was VPA positive and BB negative (Table 1). Of the 109 coagulase-negative strains, one
was repeatedly LA positive and was identified by the API STAPH system as *S. epidermidis*. Eight strains (7%) were VPA positive, while one strain (0.9%) (different from the previous eight) was BB positive (Table 2).

The positive and negative predictive values of the Pastorex LA test, the VPA fluorescent assay, and the BB fluorescent assay were 99.3 and 99.1%, 98.9 and 92.7%, and 98.9 and 99.1%, respectively.

Tube coagulase tests are widely used to detect staphylocoagulase production for identification of *S. aureus* (1, 3, 4, 7, 16). Problems have been encountered with this test due to variations in the type of plasma used, incubation time, and degree of clotting achieved, and definitive results may be unavailable until up to 24 h after initial isolation (3, 12, 13, 16).

Direct detection of the staphylocoagulase-prothrombin complex could offer a rapid, reliable assay for identification of *S. aureus* (4), and specific and sensitive chromogenic assays for staphylocoagulase have been described (3, 4). The more sensitive fluorescent substrate VPA has been used to study staphylocoagulase kinetics and proved to be rapid, accurate, and simple to use (5, 8). Previous chromogenic and fluorogenic assays have been based on detection of staphylocoagulase in broth culture fluids or in semipurified form. These assays are not suitable for use in routine laboratories, where most organisms are grown on solid media. Staphylocoagulase is present and exposed on the cell surface (2) and is released into culture fluids through proteolysis (5). False-positive reactions observed with culture fluid staphylocoagulase assays may be due to direct activation of prothrombin by trypsin or papain-like proteases (4). Proteolytic release of staphylocoagulase may result in degradation of its COOH terminus, producing heterogeneity (5, 6), and may explain discrepancies observed between tube coagulase and direct staphylocoagulase tests using culture fluids. The use of colonies grown on solid media as described in this study may remove these problems while dispensing with the need to grow strains in broth culture first. A linear relationship exists between reaction rates and staphylocoagulase production (4), with optimal production depending on supply of growth factors from a rich medium (7). This study shows that overnight growth of *S. aureus* on Columbia blood agar plates produces sufficient staphylocoagulase for detection in 1 h. Fluorescence values ranged from 9,100 to 76,000, with the majority between 30,000 and 60,000. The positive control, negative control, and reagent blanks gave average readings of 30,000, 3,000, and 2,000, respectively.

Extending assay incubation time beyond 1 h slightly increased fluorescence values with *S. aureus* strains but significantly increased the number of false-positive reactions observed (data not shown). Between 0% (8) and 20% (5) of coagulase-negative staphylococci (CNS) can produce positive reactions with chromogenic assays. Pseudocoagulase activity in CNS has been described (3, 14) and may contribute to this false-positive reaction rate.

In this study, 7.3 and 0.9% of the CNS were positive by the VPA and BB assays, respectively (Table 2). Protease inhibitors such as hirudin and aprotinin can prevent false-positive reactions in clotting assays (4) but failed to prevent false-positive reactions with fluorogenic substrates in this study (data not shown), suggesting that these may be due to mechanisms other than proteolytic activation of prothrombin.

Fluorescent staphylocoagulase assays appear to be simple, rapid, and specific tests for identification of *S. aureus* colonies grown on solid media. The BB assay is as sensitive as the VPA assay but is more specific. Our results suggest that VPA or BB assays are valid alternatives to LA or the tube coagulase test for the identification of *S. aureus* or could provide rapid and reliable confirmation of LA tests in the laboratory. Fluorogenic assays are well suited to large-scale screening and can provide quantitative values with standardized inocula.

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### REFERENCES


