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Single-Tube Mixed Agglutination Test for the Detection of Staphylococcal Protein A

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A simple, rapid mixed agglutination test using sheep erythrocytes (SRBC) sensitized with rabbit hemolysin and intact viable staphylococci is described for the detection of bound staphylococcal protein A. Soluble protein A was heat extracted from 50 clinical isolates as well as the Cowan I and Wood 46 strains of *Staphylococcus aureus* and titered by a hemagglutination test using sensitized SRBC and dilutions of soluble protein A. Protein A could be detected in all of these supernatants including that of *S. aureus* Wood 46, a strain generally considered to be protein A negative. These organisms were later retested by the mixed agglutination test and even those staphylococcal isolates expressing very low heat-extractable soluble protein A concentrations (1:2 titers) were positive, confirming the sensitivity of the test. In a screen of clinical isolates, only 4 of 235 (1.8%) coagulase-positive isolates were negative in the mixed agglutination test. Of 25 coagulase-negative isolates, none yielded a positive reaction.

In 1958, Jensen (5) described an antigen of *Staphylococcus aureus* that precipitated a globulin fraction of all normal human serum tested. This antigen has since been designated staphylococcal protein A. Protein A is covalently linked to the outermost portion of the *S. aureus* peptidoglycan (12) and uniquely interacts with only the Fc portion of immunoglobulin G (13). Because protein A reacts in a nonimmune fashion with only the Fc portion of the immunoglobulin G molecule, the interaction has been described as "pseudoimmune" by Forsgren and Sjoquist (4).

The occurrence of protein A in staphylococcal isolates has been demonstrated by various methods. Forsgren (2) evaluated broth supernatants of 800 staphylococcal isolates for the presence of extracellular protein A, using a sensitized sheep erythrocyte assay system. He found that 98.9% of 700 coagulase-positive, deoxyribonuclease-positive isolates produced protein A and that 2.0% of 100 coagulase-negative isolates also produced both deoxyribonuclease and protein A. Kronvall et al. (9) found that 90.0% of 156 coagulase-positive staphylococci adsorbed ^{125}I-labeled myeloma immunoglobulin G, and that none of 46 coagulase-negative staphylococci did. Winbald and Ericson (15), employing a sensitized erythrocyte assay system similar to that of Forsgren (2), have shown with a rapid slide technique that 88.3% of coagulase-positive staphylococcal isolates were positive for protein A. This percentage of protein A-positive isolates could be increased to 93.3% if a tube agglutination assay was included. It seemed worthwhile, therefore, to determine if the sensitivity of sheep erythrocyte hemagglutination tests could be increased to detect organisms that produce very small quantities of protein A, and to correlate this result with coagulase production.

MATERIALS AND METHODS

Bacteria. *S. aureus* (coagulase positive) isolates were obtained from the Bacteriology Section, West Virginia University Clinical Laboratories. The Cowan I strain of *S. aureus* NTCC (8530) and the Wood 46 strain of *S. aureus* were kindly provided by Goran Kronvall, University of Lund, Lund, Sweden.

Staphylococcus epidermidis and *Micrococcus* spp. (coagulase negative) isolates were obtained from the Bacteriology Section, West Virginia University Clinical Laboratories.

Coagulase determinations were performed on all isolates using the tube test with plasma obtained from Difco Laboratories, Detroit, Mich.

Growth conditions. All bacterial isolates were grown in 5-ml volumes of Trypticase soy broth (BBL, Baltimore, Md.) for 18 h at 37°C. The bacteria were then washed three times with veronal-buffered saline (VBS), pH 7.4, and diluted to a uniform bacterial concentration, as judged by absorption at 650 nm.

Extraction of protein A. Staphylococcal protein A was extracted from *S. aureus* Cowan I and Wood 46, *S. epidermidis* and 50 coagulase-positive clinical isolates by a modification of the method of Jensen (5) as described previously by Kronvall and Williams (8). The washed bacteria were boiled for 1 h in M-15

phosphate buffer (pH 5.9). The bacteria were sedimented by centrifugation, and the supernatant fluid was adjusted to pH 3.0 with 1.0 N HCl. After 1 h at room temperature the precipitated protein A was harvested and dissolved in VBS.

Crude protein A was extracted from *S. aureus* Cowan I by an 18-h lysozyme digestion according to the method of Forsgren (1). Purification was achieved by a modification of the method of Kronvall (7). Briefly, lysozyme-extracted protein A preparations were adjusted to pH 3.5 with 1.0 N HCl to precipitate some extraneous staphylococcal proteins. The supernatant was adjusted to pH 7.4 and added to 20 ml of Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.), to which human immunoglobulin G had been coupled with glutaraldehyde (14). The suspension was incubated 18 h at 4°C with constant mixing and washed three times with VBS, and the protein A was eluted from the gel with 3 M KSCN. The protein A was dialyzed extensively against VBS and stored at −20°C until use.

Tube agglutination tests. Sheep erythrocytes (SRBC) were obtained and stored at 4°C as a 50% suspension in Alsevers solution. A 1% suspension of SRBC was sensitized with rabbit anti-SRBC (BBL) at the next highest dilution of hemolysin that did not give nonspecific agglutination with the erythrocytes. The cells were then washed three times with VBS and adjusted to a 2% suspension.

For the tube mixed agglutination assay, organisms from broth cultures were washed three times with VBS and resuspended to the original volume in VBS, and 0.5 ml of this suspension was added to a separate tube along with 50 μl of the sensitized SRBC. This concentration of SRBC increased the sensitivity of the test and gave more consistent mixed agglutination patterns. The microorganism suspensions regularly had an absorbance of 0.9 to 1.0 optical density reading at 650 nm. The results of the test could be read from 2 to 5 h after addition of SRBC.

A hemagglutination test was used to assay for soluble extracted protein A using 0.5 ml of the bacterial extract instead of whole, viable bacterial cells and 50 μl of sensitized SRBC. The hemagglutination test was also performed in microtiter plates (Cooke Engineering Co. [Dynatech], Alexandria, Va.) using 50 μl of the purified protein A preparations and 50 μl of a 0.25% SRBC suspension.

RESULTS

Distribution of protein A among 50 clinical isolates. Protein A was heat extracted from a random group of 50 coagulase-positive clinical isolates and the Cowan I and Wood 46 strains of *S. aureus* as well as one *S. epidermidis* isolate. The staphylococcal extracts were diluted and tested for protein A reactivity using sensitized SRBC. The distribution of protein A reactivity is presented in Fig. 1. The extracts of coagulase-positive staphylococci exhibited ranges of protein A reactivity from a dilution of 1:2 to a dilution of 1:128, with the Wood 46 strain exhibiting an end point of 1:4. The *S. epidermidis* control extract has not been included in this distribution. Representative staphylococcal isolates that exhibited variable protein A reactivity ranging from a dilution of 1:2 to 1:128 were subcultured and used throughout the remainder of the study as isolates, producing variable amounts of protein A.

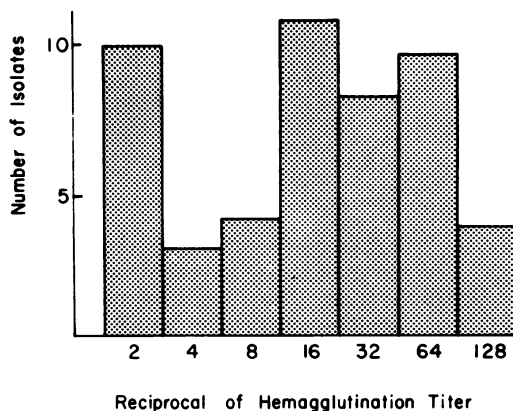

FIG. 1. *Distribution of heat-extracted protein A among 50 clinical isolates of S. aureus using sensitized SRBC. Protein A was extracted by boiling and concentrated as in Materials and Methods. The soluble extracted protein A was then titered by hemagglutination.*

Tube mixed agglutination of representative bacterial strains. The Cowan I and Wood 46 strains of *S. aureus* and the *S. epidermidis* isolate were incubated 18 h at 37°C and then processed as described in the Materials and Methods. The bacteria were then resuspended in VBS and adjusted spectrophotometrically to approximately equal concentrations. Twofold dilutions of these bacterial suspensions were made in VBS and sensitized SRBC were added. Table 1 shows the end point of agglutination for each of the staphylococcal strains. The Cowan I strain could be diluted 1:32, and surprisingly the Wood 46 strain could be diluted 1:8 and still show a positive mixed agglutination pattern. The *S. epidermidis* isolate did not agglutinate the sensitized SRBC. Those staphylococcal isolates used in the first experiment, which exhibited varying extracted protein A hemagglutination titers, were transferred to broth culture and tested by the mixed agglutination test. Included in this series of tests were five *S. epidermidis* isolates and two nonspeciated micrococcal isolates as well as the Cowan I and Wood 46 strains. The bacteria were washed three times and resuspended to original volume with VBS, and 0.5 ml of the suspension was then transferred to a separate hemagglutina-

TABLE 1. *Protein A reactivity of representative organisms in the mixed agglutination test*

Organism	Absorbance	Reciprocal of titer
S. aureus Cowan I	0.850	32
S. aureus Wood 46	0.900	8
S. epidermidis	0.750	0

tion test tube (13 by 100 mm). A 50-μl portion of a 2% suspension of sensitized SRBC was added to each of the tubes. The tubes were incubated 2 to 5 h at room temperature, at which time the mixed agglutination patterns were read. The results are presented in Fig. 2.

The first row of tubes (row A) consists of clinical isolates with extracted protein A hemagglutination titers of 1:2 to 1:128, respectively. All tubes received 50 μl of sensitized SRBC, and all showed a positive mixed agglutination pattern. In both rows the final tube is the erythrocyte control tube, which contains only erythrocytes and VBS. The first five tubes in row C and D contain the five *S. epidermidis* isolates; tubes six and seven contain the micrococcal isolates, followed by the Wood 46 and Cowan I strains, respectively. All tubes in row C received 50 μl of sensitized SRBC, and all tubes in row D received 50 μl of nonsensitized SRBC. In row C only the Cowan I and Wood 46 strains showed agglutination with the sensitized SRBC, and none of the other tubes showed a positive mixed agglutination pattern.

Demonstration of protein A among clinical isolates. Table 2 presents the results obtained when 235 coagulase-positive and 25 coagulase-negative clinical isolates were examined using the single-tube mixed agglutination test. Two hundred and thirty-one of the coagulase-positive isolates (98.2%) were positive for protein A. The bacteria that were negative were retested and confirmed to be coagulase positive. None of the 25 coagulase-negative isolates were protein A positive.

Quantitation of protein A. Twelve preparations of protein A prepared by lysozyme extraction and purified by affinity chromatography were tested for protein A by hemagglutination

TABLE 2. *Demonstration of protein A among 260 clinical isolates by the mixed agglutination test*

Isolate	No. of isolates	No. of protein A positive	Protein A positive (%)
Coagulase negative	25	0	0.0
Coagulase positive	235	231	98.2

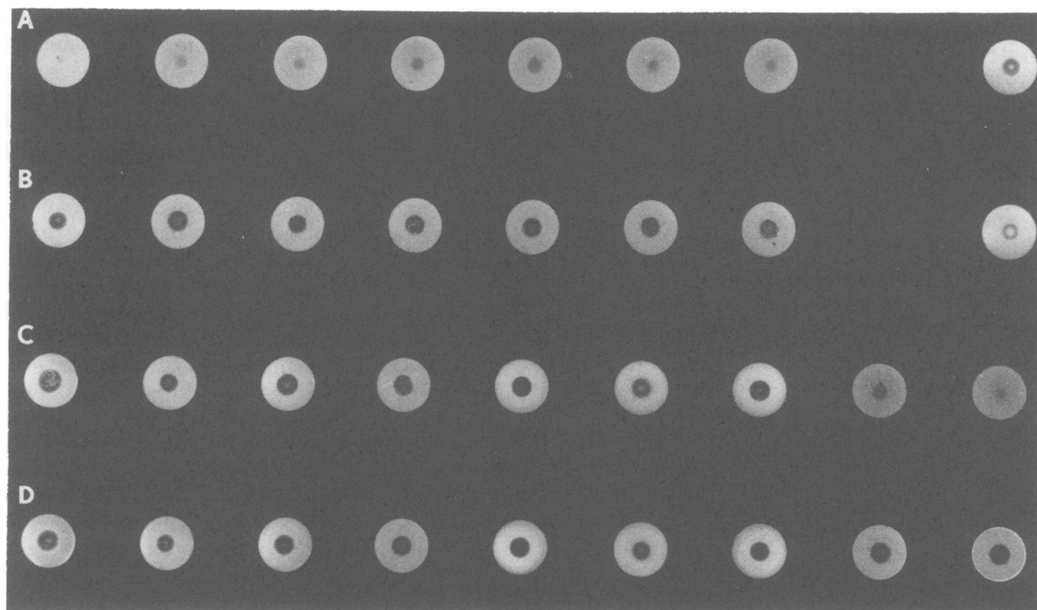

FIG. 2. *Mixed agglutination test reactions obtained with organisms of variable protein A reactivity. Row A contains S. aureus isolates with soluble protein A titers ranging from 1:2 to 1:128 (left to right) plus sensitized SRBC. Row B contains the same organisms plus unsensitized SRBC. For rows A and B the last column on the right is the erythrocyte control. Row C contains five S. epidermidis and two micrococci isolates, Wood 46 and Cowan I (left to right), plus sensitized SRBC. Row D contains the unsensitized SRBC control of row C.*

in microtiter plates. The results obtained for these preparations are presented in Table 3. The protein values were determined by the Lowry method (10) and ranged from 5 to 40 μg/ml. It can be seen that protein A reactivity varied from one preparation to another in that those preparations with the highest protein values did not necessarily have the highest protein A titers. The sensitivity of the test was indicated by the total protein at the hemagglutination end point of preparation number one, which was 0.013 ng.

DISCUSSION

The mixed agglutination test system described results from an evaluation of a variety of test situations and conditions for the rapid detection of staphylococcal protein A. Preliminary testing in this laboratory with globulin-coated particles, varying volumes and concentrations of erythrocytes and bacterial preparations, proved to be less sensitive than the optimal assay system described (i.e., 0.5 ml of the test bacterial suspension and 50 μl of a 2% suspension of hemolysin-coated erythrocytes). The test system for protein A is quite sensitive in that coagulase-positive staphylococcal isolates exhibiting extracted protein A titers of only 1:2 by hemagglutination were protein A positive by the mixed agglutination test. The hemagglutination assay in our laboratory has been shown (Table 3) to be capable of detecting protein A in samples with as little as 13 pg of protein. Secondly, a heavy bacterial suspension (optical density, 0.90) of Wood 46 could be diluted 1:8 and still be judged protein A positive in the mixed agglutination test. Heat extraction of protein A from Wood 46 also resulted in a hemagglutination titer of 1:4. The Wood 46 strain of *S. aureus* is generally considered to be protein A negative, as determined by a number of investigators (9, 11, 15), utilizing the protein A assay systems previously described. However, Forsgren and Forsum (3) have shown that the Wood 46 strain does contain protein A at a content of less than 1% of that of Cowan I. It would appear that the Wood 46 strain does contain detectable quantities of protein A, but a test system must be used that can optimally present the Fc region of immunoglobulin G for reaction with protein A.

The mixed agglutination test system indicated that 98.2% of coagulase-positive isolates were also positive for protein A. This result is in good agreement with the work of Forsgren (2), who found that 98.9% of 700 coagulase-positive staphylococcal isolates were also positive for heat-extracted, soluble protein A. This close correlation to the results of Forsgren is better than that previously described by Kronvall et al. (9) or Winblad and Ericson K(15). In addition to increased sensitivity, the mixed tube agglutination test is specific. *S. epidermidis* and micrococcal isolates do not give positive test results. Finally, the mixed agglutination test is relatively rapid in that test results can be read as early as 2 h after preparation.

The test system described is sensitive and specific and shows strong correlation with the extensive work of Forsgren (2). The assay system is easily prepared and therefore can be applied to both clinical as well as research screening for staphylococcal protein A. The correlation between coagulase production and protein A expression as judged by this test system allows investigators another parameter by which to screen *S. aureus* isolates.

TABLE 3. *Quantitation of purified protein A samples by HA[a] titer*

Protein A preparation	Protein (μg/ml)	Reciprocal of HA titer	Total protein[b] at HA end point (ng)
1	33	131,072	0.013
2	40	8,192	0.240
3	30	8,192	0.183
4	25	8,192	0.154
5	21	8,192	0.128
6	14	2,048	0.342
7	11	2,048	0.263
8	18	1,024	0.889
9	14	512	1.367
10	5	512	0.488
11	8	256	1.563
12	7	64	5.469

[a] HA, Hemagglutination.

[b] Determined by dividing protein concentration of protein A preparation by HA titer and then multiplying by 0.05 ml.

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LITERATURE CITED

1. **Forsgren, A.** 1969. Protein A from *Staphylococcus aureus*. VIII. Production of protein A by bacterial and L forms of *Staphylococcus aureus*. Acta Pathol. Microbiol. Scand. 75:481-490.
2. **Forsgren, A.** 1970. Significance of protein A production by staphylococci. Infect. Immun. 2:672-673.
3. **Forsgren, A., and U. Forsum.** 1970. Role of protein A in non-specific immunofluorescence of *Staphylococcus aureus*. Infect. Immun. 2:387-391.
4. **Forsgren, A., and J. Sjoquist.** 1966. Protein A from *S. aureus*. I. Pseudoimmune reaction with human gamma globulin. J. Immunol. 97:822-827.
5. **Jensen, K.** 1958. A normally occurring staphylococcus antibody in human serum. Acta Pathol. Microbiol. Scand. 44:421-428.

6. **Kabat, E. A., and M. M. Mayer.** 1967. Experimental immunochemistry, 1st ed. Charles C Thomas, Springfield, Ill.
7. **Kronvall, G.** 1973. Purification of staphylococcal protein A using immunosorbents. Scand. J. Immunol. 2:31-36.
8. **Kronvall, G., and R. C. Williams, Jr.** 1969. Differences in antiprotein A activity among IgG subgroups. J. Immunol. 103:828-833.
9. **Kronvall, G., J. H. Dossett, P. G. Quie, and R. C. Williams, Jr.** 1971. Occurrence of protein A in staphylococcal strains: quantitative aspects and correlation to antigens and bacteriophage types. Infect. Immun. 3:10-15.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
11. **Reiss, J.** 1973. The immunofluorescence absorption test (IFAT) in the estimation of staphylococcal protein A. Contrib. Microbiol. Immunol. 1:98-101.
12. **Sjoquist, J., J. Movitz, I. B. Johansson, and H. Hjelm.** 1972. Localization of protein A in the bacteria. Eur. J. Biochem. 30:190-194.
13. **Sjoquist, J., and G. Stalenheim.** 1969. Protein A from *S. aureus*. IX. Complement fixing activity of protein A-IgG complexes. J. Immunol. 103:467-473.
14. **Ternynck, T., and S. Avrameas.** 1972. Polyacrylamide-protein immunoadsorbents prepared with glutaraldehyde. FEBS Lett. 23:24-28.
15. **Winbald, S., and C. Ericson.** 1973. Sensitized sheep red cells as reactants for *S. aureus* protein A. Acta Pathol. Microbiol. Scand. Sect. B 81:150-156.