Technical methods

A solid medium for visual demonstration of coagulase production by *Staphylococcus aureus*

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The tube test for free coagulase is the most reliable method for the distinction between *Staph. aureus* and *Staph. albus* in the clinical laboratory, since the whole plasma-containing solid media (Penfold, 1944; Lack, 1957; Esber and Faulconer, 1959) show false-positive reactions with coagulase-negative staphylococci (Williams and Harper, 1946; Klemperer and Haughton, 1957; Lotter and Horstman, 1967). These non-specific reactions appear practically eliminated in the medium of Klemperer and Haughton (1957) because of its low plasma content (3%). In practice this medium has the inconvenience that any new batch of plates must be prepared with fresh active plasma. As the clotting activity of coagulase is based on its reaction with a coagulase-reacting factor in plasma, which is identical with prothrombin (Tager, 1956; Soulier and Prou, Wartelle, 1967), a prothrombin preparation could also be used. The object of the present paper is to describe a solid medium for the detection of coagulase production using a stable human prothrombin preparation.

Material and Methods

The tube coagulase test was performed with Difco plasma according to the prescriptions of the manufacturer, the slide test according to Cadness-Graves, Williams, Harper, and Miles (1943). Staphylococcal strains were identified according to the criteria of Baird-Parker (1963), the Enterobacteriaceae and group A haemolytic streptococci according to the methods described by Bailey and Scott (1970).

Methods of Prothrombin Preparation

Five hundred ml frozen plasma (-20°C) was the starting material. The plasma pooled from two

Received for publication 30 September 1971.

donors was obtained from the blood bank. For adsorption of the prothrombin a suspension of 20% (w/v) aluminiumhydroxide (Moist gel, BDH) and 20% (w/v) ethanolyzed cellulose (Grycksbo, Pappersbrück, Sweden) was prepared (Bruning, 1970). The eluting fluid consisted of a 0.25 M disodium hydrogen phosphate solution supplemented with 1% (v/v) of a 0.3 M disodium-EDTA (Analar, BDH) solution preventing spontaneous thrombin formation by calcium ions. After thawing for 30 minutes at 37°C , the prothrombin was adsorbed by adding 25 ml aluminiumhydroxide suspension to 500 ml plasma in a plastic centrifuge tube and manual mixing for five minutes at room temperature. Prolonged adsorption must be avoided because of loss of prothrombin activity (Bruning, 1970). After centrifugation at 700 *g* for 20 minutes at 4°C , the supernatant fluid containing the γ -globulins was decanted. Elution of prothrombin occurred by adding 50 ml phosphate-EDTA solution to the precipitate. After magnetic stirring for 20 minutes at room temperature, a second centrifugation was performed at 16000 *g* for 20 minutes at 4°C . The supernatant fluid, about 50 ml, was decanted. The pH was then adjusted to 7.5 by slowly adding 1 volume 0.5 M sodium-dihydrogen phosphate solution with a pH of 4.2 to 20 volumes of the eluate under constant manual mixing. The eluate was sterilized by successive filtration through a 1.2 and 0.45 μm Millipore membrane filter. Finally the eluate was checked for prothrombin activity according to the method described by Loeliger and Koller (1952) and lyophilized and stored at 4°C in ampoules of 1 ml.

Preparation of the Coagulase Medium

A 3% bovine fibrinogen solution was prepared as follows: 1800 mg bovine fibrinogen (Poviet, Amsterdam, containing 66.1% clottable protein) was added to 60 ml saline and solubilized by magnetic stirring for one to two hours. After filtering through a Whatman no. 1 filter, the solution was sterilized through a Seitz filter (EKS no. 6). The lyophilized prothrombin preparation was reconstituted in 1 ml saline and subsequently diluted to 30 ml in saline. For preparing the standard medium 50 ml freshly prepared fibrinogen solution and 30 ml diluted prothrombin preparation were added to 1 litre

	Number Tested	Number of Positive Reactions			
		Difco Tube Coagulase Test	Plate Coagulase Test	Slide Coagulase Test with Difco Plasma	Slide Coagulase Test with Human Plasma
<i>Staph. aureus</i> from nasal carriers	100	100	100	89 ¹	93 ¹
<i>Staph. aureus</i> from clinical material	200	200	200	—	—
<i>Staph. albus</i> from clinical material	100	0	0	0 ²	1 ²

Table The results of the plate test for coagulase production as compared with the tube and slide test in *Staph. aureus* and *Staph. albus*.

¹One autoagglutination excluded. ²Five autoagglutinations excluded.

autoclaved molten brain heart infusion agar (Difco) at 45°C, mixed, and poured immediately. (A temperature above 45°C transforms the fibrinogen in fibrin resulting in difficulties in reading of the plate due to the action of staphylokinase.)

Results

The prothrombin activity of six different prothrombin preparations varied from 300 to 350% as compared with our standard of normal human plasma. To determine the optimal prothrombin content in the medium, different plates with undiluted and 1/5, 1/10, 1/30, 1/60, 1/90, and 1/150 diluted prothrombin preparation were spot inoculated (5 mm or less) with eight strong and seven weak coagulase-producing strains. The narrow zones of opacity of the weak reactions became negative or difficult to read with the prothrombin dilution 1/90. The optimum incubation time of the plates is 18 hours at 37°C. After 18 hours of incubation fibrinolysis or proteolysis can give disturbing effects. In view of the above result a medium containing a prothrombin preparation in a dilution of 1/30 was adopted as the standard.

On this medium 300 strains of *Staph. aureus* and 100 strains of *Staph. albus* were tested and the results were compared with the tube and slide coagulase test. It can be seen in the Table that complete agreement exists between the results obtained with the coagulase plate test and the tube test. *Staph. albus* gave no false positive reactions. The slide test with Difco plasma was in 10% false negative, with fresh human plasma in 4%. No false positive reactions were obtained with five strains of the common genera of Enterobacteriaceae and haemolytic streptococci.

When retested after one year, the prothrombin activity of the lyophilized prothrombin preparation used in the experiments described above remained unchanged. This was also the case when this preparation was used in the solid medium and tested with the series of seven weak coagulase-producing staphylococcal strains, indicated before. If kept at 4°C, the quality of the plates remained unchanged for one month.

Comment

From the results it appears that a coagulase plate with bovine fibrinogen and prothrombin as a source of CRF is well suited for routine bacteriology and constitutes a convenient method for the identification of *Staph. aureus*.

From a practical point of view the preparation of the medium is simple. From 500 ml plasma 50 ml prothrombin preparation can be made, lyophilized and stored in 1 ml ampoules. After reconstitution and 1/30 dilution of the content of such an ampoule 1 l medium or 70 plates can be poured. The yield of one plasma fractionation is sufficient for the preparation of 3000 plates.

It appears that the use of prothrombin instead of whole plasma has solved the problem of inactivity of some plasmas as the preparation is devoid of the γ -globulins excluding activity of anticoagulase (Lominski and Roberts, 1946; Tager and Hales, 1948).

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Lysis of red cells from haematological disorders

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Erythrocytes from patients with acute and chronic leukaemias or myeloproliferative disorders treated with N-acetyl-L-cysteine are more sensitive than normal erythrocytes to lysis in acidified serum and in the 'sucrose' lysis test. Furthermore, erythrocytes from acute leukaemias and myeloproliferative disorders exhibit a lower acetylcholinesterase activity than normal red cells. Erythrocytes from aplastic anaemia and thalassaemic syndromes behave normally. Several workers have shown that normal erythrocytes, following incubation with a variety of substances, behave like erythrocytes of paroxysmal nocturnal haemoglobinuria (PNH). N-acetyl-L-cysteine (N-Ac) is one of these substances (Dé Sandre, Vettore, Corrocher, Cortesi, and Perona, 1968).

Materials and Methods

Erythrocytes were obtained before any treatment from patients suffering from various haematological disorders; erythrocytes from 20 normal subjects were used as controls. One volume of venous blood was mixed with 3 volumes of Alsever solution. After centrifugation the erythrocytes were separated and treated with a solution of 0.613 M N-Ac (BDH) as described by De Sandre *et al* (1968).

The haemolysis test in acidified serum (pH 6.2) was performed as described by Dacie and Lewis (1968). A pool of fresh human sera from healthy group AB and Rh(D)-positive donors was used. The 'sucrose' lysis test was performed as described by Jenkins, Hartmann, and Kerns (1967). The AchE activity of the erythrocytes was measured as described by Michel (1949).

Results

Table I shows that whilst erythrocytes from patients with aplastic anaemia and thalassaemic syndromes treated with N-Ac behave like normal erythrocytes, erythrocytes from patients with acute leukaemia, chronic leukaemias (both myeloid and lymphatic),

Received for publication 30 November 1971.