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Problems of thermonuclease detection for identifying Staphylococcus aureus in blood culture broths

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Abstract

The detection of thermonuclease by the Oxford strain and eight clinical isolates of *Staphylococcus aureus* in a variety of bacteriological broths with and without added blood was examined using a toluidine blue-DNA-agar plate method. In Isosensitest, brain-heart infusion, tryptic soy, nutrient and gas-liquid chromatography broths (all of which do not contain liquoid) thermonuclease detection was uncomplicated. In Bactec broths (containing liquoid) detectable thermonuclease activity was greatly reduced in the absence of blood. The addition of 10% blood to the Bactec broths restored the activity. Liquoid was shown to be responsible for the inhibition of thermonuclease activity, and its effect could be neutralised by the addition of blood, albumin, or haemoglobin. In specimens containing no blood, or insufficient blood to neutralise the liquoid in culture broths, more has to be added to prevent false negative reporting of *S aureus*. This can be done after growth at the time of thermonuclease testing.

Clinical consequences of delayed identification of *S aureus* in routine blood cultures may be serious. The application of the thermonuclease test to blood culture broths is both fast and specific.

The differentiation of *Staphylococcus aureus* from coagulase negative staphylococci is important in clinical microbiology laboratories. Traditionally, the separation of *S aureus* from coagulase negative staphylococci has been achieved using either the slide or tube coagulase tests; several commercially obtainable kits based on these tests are now available.[1-3] A disadvantage of both the traditional and kit based tests is that they were developed specifically for the analysis of bacterial colonies on solid media, and the application of these techniques to broth cultures is time consuming and generally unreliable.[1 4]

The production of deoxyribonuclease (DNase) by staphylococci correlates well with the production of coagulase, which is linked with pathogenicity.[3-8] Unfortunately, DNase production is not limited to staphylococci, and it has been shown that a variety of genera and even some coagulase negative staphylococci can be DNase producers.[2 8]

In 1971 Lachica *et al* showed that in addition to DNase, which is heat-labile, *S aureus* also produces thermonuclease—a heat-stable endonuclease (micrococcal nuclease EC 3.1.4.7) which is unique to the species.[8-11] Further to this a medium was developed which was suitable for the detection and approximate quantification of the enzyme and gave reliable results after four hours of incubation.[4-7 12 13] Subsequent studies showed the high sensitivity and specificity of the thermonuclease (TNase) test and the usefulness of its application to routine blood culture bacteriology.[5 6 13 14] TNase results are normally interpreted after two hours of incubation,[6 7] but in our hands positive TNase reactions from blood cultures are generally readable after as little as 20 minutes' incubation (data not shown).

Methods

The Oxford staphylococcus, a laboratory strain of *S epidermidis*, and eight randomly chosen clinically important *S aureus* isolates which had recently been grown from blood culture were used in the study. All bacterial strains were identified by catalase test, tube coagulase test, and Gram strain; further confirmation of identity was provided by API Staph biochemical test strips (API-BioMèrieux, Basingstoke, Hants).

The following liquid media were used: Isosensitest Broth (Oxoid Ltd); Bactec 6B, Bactec 7D, Bactec NR6A, Bactec NR7A (Becton Dickinson Ltd, Towson, Maryland, USA); nutrient broth (Oxoid); Mueller-Hinton broth (Oxoid); brain heart infusion broth (Lab-M Ltd, Bury, Lancs); GLC broth (Lab-M); tryptic soy broth (Difco Ltd, Detroit, Michigan, USA). Of these, only the Bactec media contain liquoid. All the dehydrated media were made according to manufacturers' instructions and autoclaved at 121°C for 15 minutes.

Toluidine blue/DNA agar was prepared according to the method of Lachica *et al*.[11] The constituents were as follows: Trizma base (Sigma Chemicals, Poole, Dorset); Bacto DNA (Difco); toluidine blue (Sigma); and bacteriological agar number 1 (Oxoid). The medium was stored in 100 ml aliquots at 4°C and, when required, was melted in a water bath and poured in 20 ml amounts into standard 85 mm diameter Petri dishes. As TNase agar is sensitive to changes in media composition[15] each batch of media and each experimental

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Table 1 Detection of thermonuclease activity of isolates grown in Bactec and non-Bactec overnight broths with and without added blood

Broth type, with or without blood	*TNase reaction class*	
	Oxford strain	*Eight clinical isolates*
Non-Bactec broths − blood	4	4
Non-Bactec broths + blood	4	4
Bactec broths − blood	2	2
Bactec broths + blood	4	4

Bactec broths were NR6A, NR7A, 6B and 7D. Non-Bactec broths comprised Isosensitest, nutrient, Mueller-Hinton, brain-heart infusion, tryptic soy and gas-liquid chromatography.

plate were quality control tested with overnight Isosensitest broths of coagulase negative staphylococci and the Oxford strain.

Six colonies from a blood agar plate (37°C, aerobic) of the organism under test were suspended in 10 ml of Isosensitest broth. Three drops of this dilute suspension were used to seed the 10 ml broths, which were then incubated aerobically, unshaken overnight at 37°C. Sterile defibrinated horse blood (Advanced Protein Products, Brockmoor, West Midlands) was added to selected bottles to a final concentration of 10%.[15] Filter sterilised sodium polyanetholesulphonate (liquoid, Sigma) was added when required to give a final concentration of 0·035% as found in non-radiometric Bactec Broths NR6A and NR7A. When human haemoglobin (Sigma) was added, filter sterilised solutions in 0·9% saline were used to give a final concentration approximate to that found in lysed human blood (150 g L^{-1}). Albumin (Blood Products Laboratory) was also added to simulate the concentration found in human blood (45 g L^{-1}).

In the initial experiment all the clinical strains and the Oxford strain were grown in the full range of broths with and without added blood and thermonuclease tests were performed (table 1). Subsequently the effect of adding blood or liquoid to Isosensitest broths before and after growth of the Oxford strain was investigated (table 2) and the effect of different concentrations of haemoglobin and albumin on TNase activity noted (table 3).

Table 2 Effect on thermonuclease activity of addition of liquoid and blood to Isosensitest broths of S aureus (Oxford strain) before and after growth

Tube No	*Addition of:*				
	Liquoid before inoculation	*Liquoid after growth*	*Blood before inoculation*	*Blood after growth*	*TNase*
1	+	−	−	−	2
2	−	+	−	−	2
3	−	−	+	−	4
4	−	−	−	+	4
5	+	−	+	−	4
6	−	+	−	+	4
7	−	+	+	−	4
8	+	−	−	+	4

Table 3 Effect on thermonuclease activity of addition of haemoglobin, albumin, and blood to overnight Isosensitest broths of S aureus (Oxford strain) with added liquoid

Tube No	*Albumin*	*Haemoglobin†*	*Haemoglobin**	*Blood*	*TNase*
1	−	−	−	−	2
2	−	−	−	+	4
3	−	+	−	−	4
4	−	−	+	−	3
5	+	−	−	−	3

†150 g L^{-1}; *45 g L^{-1}.

Table 4 Classification of thermonuclease reaction zone sizes

Class	*Zone size (mm)*	*Zone intensity*
1	6–7	Faint clear rim, not pink
2	7–10	Faint pink colour
3	10–12	Strong pink colour
4	>12	Strong pink colour

The TNase test was performed by pipetting 3–5 ml of overnight broth into a small, screw topped serum phial and heating for 15 minutes in a 100°C heating block. The broth was cooled, vortex mixed, and centrifuged at 3000 × *g* for 10 minutes. Holes of 6 mm in diameter were punched in the thermonuclease agar and filled with boiled supernatant. Plates were incubated in air at 37°C for two hours before the test was interpreted.

The zone sizes (diameter of pink zone including 6 mm well diameter) and reaction intensities for the four classifications were as shown in table 4. These classifications were chosen as the observed zone sizes tended to fall naturally into the four groups.

Results

All broths showed a high turbidity after overnight incubation, indicating good bacterial growth. Initial experiments with coagulase negative staphylococci showed that negative TNase reactions (class 1) were observed in all the broths, either alone or supplemented with blood, haemoglobin, albumin or liquoid. In all three replications of each experiment the control inoculations gave a positive TNase reaction with the Oxford strain and a negative reaction with the coagulase negative staphylococci.

Thermonuclease activity was detectable in a variety of bacteriological broths containing the Oxford strain with and without added blood, but a strong positive reaction was seen in Bactec broths only in the presence of blood. The same pattern was observed with eight clinical blood culture isolates of *S aureus* (table 1).

The addition of liquoid to Isosensitest broths containing the Oxford strain either before or after overnight incubation inhibited the detection of TNase. This inhibition was neutralised by the addition of blood either before or after incubation. The addition of blood in the absence of liquoid had no effect on TNase activity (table 2). The addition of albumin, haemoglobin, or blood to Isosensitest broths containing added liquoid resulted in inhibition neutralisation that was dependent on the concentration of the compound added (table 3).

Discussion

Despite an initial reduction in the incidence of *S aureus* in the 1960s and 1970s due to the introduction of β-lactamase resistant antibiotics, the staphylococcus is currently enjoying a return to prominence as a causative agent of septicaemia.[16] Figures as high as 58% of nosocomial bacteraemias attributed to *S*

aureus have recently been reported.[16] When repeatedly isolated from "at risk" patients coagulase negative staphylococci may be significant but recent reports suggest that this is the situation in only 1–10% of cases[16] and that most coagulase negative staphylococci are introduced to blood culture broths at the time of sampling.[5]

As the morbidity and mortality associated with *S aureus* are particularly high it is essential that in blood culture broths containing Gram positive cocci the differentiation between *S aureus* and coagulase negative staphylococci is rapidly performed. None of the commercially available kits or the tube coagulase test is sufficiently sensitive or reliable to be used directly in this differentiation,[14] but the thermonuclease test is.

We have shown that in Bactec broths (6B, 7D, NR6A and NR7A) containing liquoid[17] the detectable thermonuclease activity of the Oxford strain and eight clinical strains of *S aureus* was greatly reduced. The addition of blood (1:10 in broth) neutralised this effect as did haemoglobin and albumin. When haemoglobin was added at the same concentration as albumin (45 g L^{-1}) it achieved the same degree of inhibition neutralisation. When the two compounds were added at the concentrations found in lysed human blood (45 g L^{-1} albumin, 150 g L^{-1} haemoglobin) the haemoglobin had a greater effect.

The fact that the addition of liquoid to Isosensitest broths of *S aureus* could reduce thermonuclease activity after overnight growth, and that this effect could be neutralised by addition of whole blood or blood components either before or after growth, is evidence for the process being an inhibition of enzyme activity rather than an effect on enzyme synthesis or transport. A possible explanation is that liquoid binds a proportion of the TNase enzyme thus preventing its detection. The addition of further protein in the form of blood, albumin, or haemoglobin sets up a competitive association system, resulting in a dynamic equilibrium between the individual components. Hence, if a large proportion of protein is added to the system then more of this associates with the liquoid, thus releasing more active TNase.

In normal adult blood cultures in which 3–5 ml of blood is injected into each phial there will be sufficient organic material to neutralise the liquoid and allow TNase to be detected. In paediatric blood cultures, where only small volumes of blood may be available, or in specimens such as peritoneal dialysis fluid, joint fluid or ascitic fluid, especially when clot formation has occurred, there may not be enough protein to neutralise the effect of liquoid. This may lead to difficulty interpreting the test or delayed reporting of *S aureus* isolation.

The manufacturers of the Bactec systems recommend the addition of blood to Bactec broths to facilitate the growth of liquoid sensitive and fastidious organisms. We support this recommendation and note that the addition of blood to Bactec broths containing liquoid, either before or after growth, will also prevent the occurrence of false negative thermonuclease tests. The could effectively be achieved by adding 0·5 ml of sterile defibrinated horse blood to a 5 ml aliquot of broth withdrawn for TNase testing. As the addition of blood has no adverse effects on the TNase test this should be performed on all broths containing liquoid and lacking sufficient blood for reliable TNase detection.

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