Detection of *Staphylococcus aureus* with biotinylated monoclonal antibodies directed against staphylococcal TNase complexed to avidin-peroxidase in a rapid sandwich enzyme-linked immunofiltration assay (sELIFA)

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Summary. For rapid identification of *Staphylococcus aureus*, a monoclonal antibody (MAb)-biotin-avidin-peroxidase complex, directed against the *S. aureus* thermostable nuclease (TNase), was formed and used in a rapid three-step sandwich enzyme-linked immunofiltration assay (sELIFA) and a three-step sandwich enzyme-linked immunosorbent assay (sELISA). The MAb-peroxidase complex was formed by incubating the biotinylated MAbs with a streptavidin-peroxidase conjugate and the complex was purified by gel permeation chromatography. When compared with a four-step MAb-based sELISA described previously, this complex permitted one reagent step to be omitted in a three-step sELISA, and the test time was significantly reduced. The test sensitivity was slightly reduced in the three-step ELISA (detection limit 1.0–2.0 ng of TNase/ml) when compared to the four-step sELISA (detection limit 0.5–1.0 ng of TNase/ml). The sELIFA method was based on the filtration of bacterial culture supernates through nitrocellulose membrane disks pre-spotted with a MAb directed against the *S. aureus* TNase, followed by detection with the MAb-peroxidase complex (three-step sELIFA). A detection limit of 0.5–2.0 ng of TNase/ml was achieved with the three-step sELIFA, depending on the filtrate volume of culture supernates. The total test time was 10–15 min when pre-spotted and blocked membranes were used. A total of 85 bacterial strains was tested in the sELIFA. All the 28 *S. aureus* strains showed positive results, but none of the 57 non-*S. aureus* strains did so, although some of these produced thermostable nuclease activity. When 75 blood cultures were tested directly in the sELIFA, 87% of the cultures with growth of *S. aureus* gave a positive result whereas all of the cultures with non-*S. aureus* gave negative results, a diagnostic sensitivity similar to that of the routine TNase enzyme test. Thus, the three-step sELIFA has potential for the rapid confirmation of *S. aureus* bacteraemia and, possibly, also for detecting *S. aureus* by direct testing of other clinical specimens.

Introduction

Infections caused by *Staphylococcus aureus* are usually diagnosed by cultural isolation of the bacteria which are then identified by the coagulase test or by one of several commercial reagents based on particle agglutination of *S. aureus*. These reagents for rapid identification usually have a high sensitivity and specificity for *S. aureus* cultured on artificial media, but not for direct identification of staphylococci in media used for blood cultures.¹ Blood cultures in which *S. aureus* has grown usually give a positive test result for thermostable nuclease (TNase) within 2–4 h after initiation of the test.^{2–4} However, this enzyme activity is not specific to *S. aureus*.^{5,6} On the other hand, the TNase protein which probably is produced

by all *S. aureus* strains,⁴ and the *nuc* gene encoding the enzyme,^{7,8} have *S. aureus*-specific sequences. Thus, monoclonal antibodies (MAbs) directed against three different *S. aureus* TNase epitopes showed specificity for bacteria of this species.⁹ In a MAb-based sandwich ELISA (sELISA), the lower detection limit for the TNase was 0.5 ng/ml.⁹ The TNase sELISA has interesting potential for same-day confirmation of *S. aureus* bacteraemia and possibly for direct application to other clinical specimens.

The conventional multilayer sELISA is time-consuming with an operation time of c. 4 h. In this study we aimed to reduce the assay time in the MAb-based detection of *S. aureus* TNase. Taking advantage of the high affinity of biotin for avidin,¹⁰ we prepared a four-component complex that contained biotinylated anti-TNase MAbs and a streptavidin-peroxidase conjugate to shorten the time required for the TNase sELISA

test. In addition, the four-component complex was used in a sandwich enzyme-linked immunofiltration assay (sELISA) to reduce the time required for immunological detection of the *S. aureus* TNase. In the sELISA, previously used in the diagnosis of several infectious diseases,¹¹ the rate of contact between the reagents is considerably increased, allowing a corresponding reduction in incubation period for each immunoreagent.

Materials and methods

Bacterial strains

The *S. aureus* strains Wood 46 and Cowan 1 were from Dr P. Oeding, Bergen, Norway. Several strains of *S. hyicus*, *S. intermedius* and *S. schleiferi* were supplied by Dr T. Wadström, Lund, Sweden. Reference strains were purchased from ATCC (American Type Culture Collection, Rockville, MA, USA) and DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Clinical strains were isolated in our laboratory. Staphylococci were identified biochemically by the automated API Staph System (API Systems SA, Montraleu, Vercieu, France), and by the Staphylase test (Oxoid). Non-staphylococcal clinical strains were identified by the appropriate API systems. Strains were preserved in Stuart's transport medium at -80°C .

Culture

Bacteria were cultured on blood agar or in Brain Heart Infusion Broth (BHI; Difco) at 37°C for 16–20 h. The fluid cultures were centrifuged (12000 *g*, 10 min) and the supernate was heated (100°C , 15 min) and tested. For blood culture, the Bactec 730 system (Johnston Laboratories Inc., Towson, MD, USA) or Bactec Plus media were used. From cultures in which growth was signalled, samples were aspirated aseptically, heated at 100°C for 15 min, and centrifuged (12000 *g*, 10 min). The supernate was filtered (0.2- μm sterile membrane filter) before testing.

TNase enzyme test

The TNase enzyme test was performed as described by Berg and Maeland.² Briefly, TNase or culture supernates were heated at 100°C for 15 min, and 50 μl was applied to wells in agar plates containing DNA and *o*-toluidine blue. The plates were incubated at room temperature and observed for a pink zone around the wells after incubation for 2, 4, 6 and 24 h; the diameters of the zones were recorded.

MAbs

The anti-*S. aureus* TNase MAbs 2F11, 7F11 and 6G6 (all of the isotype IgG1) used in this study were

described previously.⁹ Each MAb was directed against distinct TNase epitope clusters. MAb IgG was isolated from ascitic fluid,¹² biotinylated¹³ with CAB-NHS ester (Gibco-BRL, Gaithersburgh, MD, USA), and stored at protein concentrations of 1 mg/ml in phosphate-buffered saline (PBS), pH 7.2, at -20°C .

Preparation of MAb-peroxidase complex

Equal volumes of biotinylated MAbs 2F11 and 6G6 (equilibrated in PBS by dialysis) were mixed and serially diluted in PBS. These dilutions were incubated in a checkerboard fashion at 4°C and 20°C with streptavidin-peroxidase conjugate (Gibco-BRL) equilibrated in PBS and serially diluted in PBS. Optimal conditions for complex formation were determined by testing against TNase in a sELISA (see *Results*). The MAb-peroxidase complex was purified on a Sephacryl S 300 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden; 3.0×43 cm). Biotinylated MAbs (400 μl , protein 1 mg/ml) were coupled to undiluted streptavidin-peroxidase (800 μl), dialysed against PBS and chromatographed with PBS as elution buffer at a flow rate of 4 ml/h; 2-ml fractions were collected.

Samples from each fraction were diluted 1 in 100 in PBS with Tween 20 0.05% v/v (PBS-T) and tested in the three-step sELISA (see below) for recording MAb-peroxidase complexes. For the estimation of free biotinylated MAbs in the fractions, samples diluted 1 in 100 in PBS-T were tested in the conventional four-step sELISA (see below). Free biotinylated MAbs were calculated by subtracting the absorbance recorded in the three-step ELISA from that recorded in the four-step ELISA.

MAb-peroxidase peak fractions were pooled (6 ml), dialysed against PBS with merthiolate 0.01%, and stored at 4°C .

Sandwich ELISA (sELISA)

The multilayer sELISA (four-steps with pre-coated plates) was performed essentially as described previously.⁹ Briefly, microtitration plates (Nunc, Roskilde, Denmark) were coated with the anti-TNase MAb 7F11 (100 μl ; 5 $\mu\text{g}/\text{ml}$ in 0.1 M carbonate buffer, pH 9.6; 20°C ; 18 h). The wells were washed with PBS-T, blocked (casein hydrolysate 1 mg/ml in PBS-T) and then incubated successively (1 h, 20°C) with the antigen-containing sample, the mixture of the biotinylated MAbs 2F11 and 6G6 (each 1 $\mu\text{g}/\text{ml}$), the streptavidin-peroxidase conjugate (1 in 1000), each with washing between the incubation steps. Bound peroxidase was detected with OPD- H_2O_2 (Organon Teknika BV, Boxtal, The Netherlands) in 0.1 M citrate-phosphate buffer (20°C , 2 min). The substrate conversion was stopped with 2 M H_2SO_4 and the colour development was measured at 492 nm.

In the three-step multilayer test, purified MAb-peroxidase complex was diluted 1 in 50 in PBS-T and applied after incubation with the antigen-con-

Fig. 1. Purification of a MAb-peroxidase complex by Sepharose S 300 gel permeation chromatography. Bovine serum albumin (1 mg/ml) was added as marker protein. The protein profile for the eluted fractions is shown by the curve (+); the sELISA values for the MAb-peroxidase complex (■) and free biotinylated MAbs (□) are shown by the bars.

taining sample, followed by washing and the substrate reaction.

Sandwich enzyme-linked immunofiltration assay (sELIFA)

An Octavac eight-place vacuum manifold filtration unit (Costar, Cambridge, MA, USA) was used with water suction and a filtration flow rate of 3 ml/min. Circular nitrocellulose membrane disks (Costar; 0.45 µm pore size, diameter 7 mm) were dotted with 3 µl of the MAb 7F11 (200 µg/ml in PBS) and air-dried. Remaining binding sites were blocked for 30 min with defatted dried milk (10 mg/ml in PBS-T) and the membranes were washed by filtering 1.5 ml of PBS-T. Antigen-containing solutions, biotinylated MAbs, streptavidin-peroxidase conjugates, or the complexes of these components, were filtered in a volume of 0.4 ml and washed with 1.5 ml of PBS-T between each reaction step. Colour development was accomplished with DAB-H₂O₂ (Sigma) 100 µl/disk for 3–5 min and washing the disks in water. The results were recorded by visually observing for immuno-stained spots, corresponding to the grabber MAb dot (1–2 mm in diameter).

Other methods

Protein concentrations were measured by the method of Bradford.¹⁴

Results

Preparation of MAb-peroxidase complex

Serial dilutions of a mixture of the biotinylated anti-

TNase MAbs 2F11 and 6G6 were mixed with serial dilutions of the peroxidase-streptavidin conjugate in a chequerboard fashion and incubated at 4° and 20°C for various periods. The mixtures were tested in a three-step sELISA with MAb 7F11 coated to the wells and TNase as antigen, to measure MAb-mediated binding of the peroxidase. Optimal signals were achieved when the MAb and peroxidase were present in a ratio of 1:2 (1 ml of biotinylated MAbs at 1 mg/ml mixed with 2 ml of undiluted streptavidin-peroxidase) and both were equilibrated in PBS and then allowed to react at 4°C for 30 min, then at 20°C for 60 min. Incubation at 4°C, followed by 20°C, was chosen as a standard procedure. The macromolecular complex, which contained the biotinylated MAbs bound to the streptavidin-peroxidase conjugate, was purified by gel permeation chromatography on a Sephacryl S 300 column (fig. 1). Fractions 13–15, which contained the complex, were mixed, dialysed against PBS and used for further experiments at a dilution of 1 in 50 (1 in 300 when compared to the volume before purification). Free biotinylated MAbs were eluted in fractions 16–20 (fig. 1); free streptavidin-conjugated peroxidase was not detected.

Fig. 2 shows the signals obtained for the conventional four-step sELISA and the complex-based three-step sELISA with the purified complex. The four-step procedure resulted in stronger signals, notably with TNase concentrations > 4 ng/ml. In repeated experiments, the lower limit of detection for TNase was 0.5–1.0 ng/ml with the four-step procedure and 1.0–2.0 ng/ml with the three-step procedure.

sELIFA for TNase detection

The sELIFA technique was based on the binding of the TNase filtered through nitrocellulose membranes

Fig. 2. Two-fold dilutions of commercial TNase tested by the four-step conventional sELISA (□) and by the three-step MAb-peroxidase complex-based sELISA (*-*).

to anti-TNase MAbs pre-spotted on the membranes, followed by detection of the antigen bound to this solid phase with the MAb-peroxidase complex. By filtering the immunoreagents through the solid phase an increase in contact between them was achieved, permitting considerable reduction in reaction time for each immunoreagent.

Nitrocellulose disks in an eight-well strip system

Table I. Bacterial isolates (reference or clinical strains) tested in the TNase enzymatic method and in the sELIFA

Species	Number tested	Number that gave positive results in	
		TNase test*	sELIFA†
<i>S. aureus</i>	28	28	28
<i>S. epidermidis</i>	12	0	0
<i>S. saprophyticus</i>	1	0	0
<i>S. hominis</i>	3	0	0
<i>S. haemolyticus</i>	3	0	0
<i>S. cohnii</i>	1	0	0
<i>S. intermedius</i>	6	5	0
<i>S. hyicus</i>	5	5	0
<i>S. capitis</i>	1	0	0
<i>S. schleiferi</i>	6	5	0
<i>S. sciuri</i>	1	0	0
<i>S. auricularis</i>	1	0	0
<i>S. simulans</i>	1	0	0
<i>S. lugdunensis</i>	1	0	0
<i>Streptococcus pyogenes</i>	1	0	0
<i>Streptococcus pneumoniae</i>	2	0	0
<i>Peptostreptococcus</i> spp.	1	0	0
<i>Neisseria meningitidis</i>	1	0	0
<i>Escherichia coli</i>	6	0	0
<i>Proteus mirabilis</i>	2	0	0
<i>Enterobacter cloacae</i>	2	0	0

* Positive test defined by a pink zone with diameter > 1 cm after incubation for 24 h.²

† Positive test defined by visual detection of an immunostained spot on the nitrocellulose membrane disks.

Fig. 3. Four-fold dilutions of commercial TNase (400 µl) tested in sELIFA: A, by a four-step procedure; B, by a three-step complex-based procedure. Positive (+) or negative (-) test result is shown to the left of each well. The control (0) is a test without TNase.

were spotted with the anti-TNase MAb 7F11 and blocked. TNase, wash solutions, and purified MAb-peroxidase complex were filtered through the membrane disks at a flow rate of 3 ml/min. The test was performed in c. 10 min when prepared disks (pre-spotted and blocked) were available. Fig. 3 shows a lower TNase detection limit of 2.0 ng/ml when the

Table II. Identification of *S. aureus* directly from blood cultures by the TNase enzymatic test, the TNase sELISA, and the TNase sELIFA

Blood culture isolate	Number tested	Number (%) that gave a positive result in		
		TNase test*	sELISA†	sELIFA*
<i>S. aureus</i>	40	30 (75)	37 (92)	35 (87)
<i>S. epidermidis</i>	14	0	0	0
<i>S. haemolyticus</i>	2	0	0	0
<i>S. schleiferi</i>	1	1	0	0
<i>S. hominis</i>	2	0	0	0
<i>Str. pyogenes</i>	1	0	0	0
<i>Str. pneumoniae</i>	3	0	0	0
<i>Peptostreptococcus</i> spp.	1	0	0	0
<i>N. meningitidis</i>	1	0	0	0
<i>E. coli</i>	6	0	0	0
<i>P. mirabilis</i>	2	0	0	0
<i>Ent. cloacae</i>	2	0	0	0
No bacteria	5	0	0	0

* Positive tests are defined as described in table I.

† Positive test defined as mean OD₄₉₂ > mean of OD₄₉₂ for negative controls plus 2.5 SD.

purified MAb-based complexes were used. The lower detection limit was 0.5 ng/ml when the four-step procedure was performed. The sensitivity was increased to 1.0–0.5 ng/ml if 2 ml of TNase was filtered instead of 0.4 ml in the three-step approach.

TNases ELIFA for identification of *S. aureus*

Reference strains and clinical isolates of staphylococci were cultured in BHI broth, and 400 µl of the heated culture supernates were tested for TNase in the three-step procedure, and 25 µl tested by the TNase enzymatic method. A total of 85 strains was tested in the sELIFA, with a test time of c. 10 min for each run. All of the strains previously identified as *S. aureus* by other methods gave positive results in both the sELIFA and the TNase enzymatic test (table I). All the non-*S. aureus* strains gave negative sELIFA results although some of these strains produced TNase activity.

Testing of blood cultures with sELIFA

The possibility of using the MAb-based sELIFA for rapid identification of clinical *S. aureus* isolates was examined. A total of 75 blood-culture bottles from 75 different patients was tested after signalling of bacterial growth, and five bottles without growth were tested. The positive cultures yielded *S. aureus* (40), non-*S. aureus* staphylococci (19) or other bacteria (16) as determined by standard laboratory tests (table II). The sELIFA permitted identification of the isolate in 87% of the *S. aureus*-positive cultures within 15 min after pre-treatment of the samples (heating, centrifugation and filtration). The three-step sELISA and the TNase enzymatic test required 2 and 24 h, respectively, for recording the final results (table II). All cultures from which *S. aureus* was grown and the TNase test result

was positive gave positive results in both the sELISA and sELIFA, but the sensitivity of the immunological tests was better than that of the TNase test. One culture with a non-*S. aureus* strain (*S. schleiferi*), which gave a positive result in the TNase test, gave negative results in the sELISA and sELIFA (table II). Some cultures from which a few *S. aureus* colonies were grown gave negative results in all three tests. We have found that with different *S. aureus* isolates, 10⁵–10⁶ cfu/ml of blood culture are required for detectable levels of the TNase in the antibody-based tests. By adding purified TNase to blood culture media with or without whole blood, incubating for several days, and testing by the four-step sELISA, whole blood components were shown to have both an immediate and a time-dependent negative effect on the sELISA signals.

Discussion

TNase activity is a characteristic feature of *S. aureus*, although it is not specific to *S. aureus*.^{5,6} However, data previously reported have indicated that the *nuc* gene encoding the *S. aureus* TNase and the enzyme protein have *S. aureus*-specific sequences.^{8,9} Thus, provisional data have indicated that both amplification of the *nuc* gene by the polymerase chain reaction and MAb-based detection of the TNase have potential both for the identification of *S. aureus* isolates and for the rapid confirmation of *S. aureus* infection by direct testing of clinical specimens.^{9,15}

The MAb-based four-step sELISA, previously used to detect TNase,⁹ has an operation time of c. 4 h. In the present study we aimed to reduce the operation time by (a) preparation of a four-component MAb-biotin-streptavidin-peroxidase complex for use in a three-step multilayer sELISA, (b) applying the sELIFA which can reduce operation time by the

increased rate of complexing between the immuno-reagents¹¹ and (c) a combination of the two approaches.

The high affinity of avidin for biotin¹⁰ explains our findings that a four-component complex was formed and remained stable after purification by gel filtration. Optimal conditions for complex formation required a ratio of 1:2 of the biotinylated MABs and peroxidase-streptavidin conjugate. Improved complex formation was observed when incubation was started at 4°C and then changed to 20°C; there is no obvious explanation for this. Free MABs were separated from the four-component complex by gel filtration (fig. 1), and the signal-to-noise ratio was increased when these purified complexes were used, compared with the use of unpurified complexes. This favours the use of the purified four-component complex. In the complex-based sELISA, the lower detection limit was somewhat higher, but the assay time was reduced when compared to the conventional multistep sELISA (fig. 2).

These results have confirmed the findings of IJsselmuiden *et al.*¹¹ and Shields *et al.*¹⁶ that ELIFA markedly reduced the assay time without affecting the sensitivity considerably, when compared with other multilayer enzyme-linked immunoassays, e.g., sELISA. Thus, the *S. aureus* TNase was detected with an assay time of 10 min when nitrocellulose membrane disks pre-coated with the grabber MAB and with the remaining binding sites blocked were used. During optimisation of the sELIFA we did not add the chosen blocking reagent (dried milk 1%) to immunoagents, to avoid clogging of the membranes or viscosity-induced hindrance of the filtration flow.¹⁷ A high concentration of Tween 20 (> 0.05%) was also omitted to avoid detachment of reagents bound to the solid phase,^{11,18} and adequate signal-to-background discrimination was achieved by using PBS with Tween 20 0.05% (fig. 3). The lower detection limit for both the

complex-based three-step and conventional four-step sELIFAs was 2 ng/ml, if the filtration volume was 0.4 ml, but was lowered to 0.5 ng/ml if filtered volumes were increased to 2 ml. It is likely that the sensitivity may be further increased by reducing the flow rate.

All 28 *S. aureus* strains were identified correctly by the sELIFA when culture supernates of these bacteria were tested. However, the most important application of the sELIFA may be the direct identification of *S. aureus* in blood cultures, since a prompt and reliable detection of *S. aureus* is essential in bacteraemia, which may be life-threatening. In the present study, the sensitivities of the TNase enzyme test, sELISA and sELIFA were 75%, 92%, and 87%, respectively, for the direct detection of *S. aureus* in blood cultures. When evaluated for rapid identification of *S. aureus* directly from blood cultures, the sensitivity of six different agglutination reagents which are commercially available ranged from 38.6 to 77.3%.¹ The sELIFA has the advantage of rapid identification of *S. aureus* after heating and centrifugation of samples of the blood cultures, the assay time is similar to that of agglutination tests, and the clinician can be informed of the results within minutes of the initiation of testing. Blood cultures from which *S. aureus* was isolated, but which gave negative results in our tests, contained comparatively few bacteria. Preliminary experiments have also indicated that components of whole blood reduce the signals for TNase both in the enzymatic test and in the antibody-based tests, and modifications of the methods to eliminate these inhibiting factors will, therefore, be important in increasing the sensitivity of the test. In addition, the tests should have potential for the direct detection of *S. aureus* in other clinical specimens, such as material from abscesses or various body fluids, notably the sELIFA because of its short assay time. This application of the test has not yet been investigated.

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