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Direct Testing of Blood Cultures for Detection of the Serotype 5 and 8 Capsular Polysaccharides of *Staphylococcus aureus*

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Monoclonal antibodies (MAbs) reactive with serotype 5 and 8 capsular polysaccharides of *Staphylococcus aureus* have been used to test, by enzyme-linked immunosorbent assay (ELISA), blood culture fluids for the presence of *S. aureus*. A total of 748 blood cultures from 665 patients yielding 706 bacterial isolates belonging to more than 26 bacterial species were studied. All blood cultures containing bacterial strains belonging to species other than *S. aureus* were negative in ELISA. All 23 blood cultures containing serotype 5 *S. aureus* were positive in ELISA with the corresponding MAb. Out of 20 blood cultures containing serotype 8 *S. aureus*, 19 were positive with the corresponding MAb. All 5 blood cultures containing nontypeable *S. aureus* were negative in ELISA with both MAbs. This method provides reliable identification of serotype 5 or serotype 8 *S. aureus* by direct testing of blood culture fluids with ELISA.

Staphylococcus aureus, recently characterized as the "persistent pathogen" (23), remains a major cause of morbidity and mortality (4, 18) despite extensive use of antibiotics.

The most serious result of *S. aureus* infection is bacteremia directly related to high mortality (17, 20). *S. aureus* bacteremia is one of the most frequent causes of positive blood cultures (19, 26, 27). Rapid identification of bacteria in blood cultures is of utmost importance for diagnosis and treatment of bacteremia. Latex agglutination of particles coated with human plasma (5) and a lysostaphin sensitivity test (15) have been applied for rapid identification of *S. aureus* in blood cultures.

Capsular polysaccharides (CP) have been characterized in clinical isolates of *S. aureus* in humans (1, 7, 12, 13, 24) and animals (22). Surveys in humans have shown that two capsular serotypes, 5 and 8, account for about 70 to 80% of serological types.

Monoclonal antibodies (MAbs) reactive with serotype 5 or 8 CP have been described (12, 21). These MAbs have been used to serotype isolates by a two-step inhibition enzyme-linked immunosorbent assay (ELISA) detecting serotype 5 or 8 CP in culture extracts (22).

In this study, we investigated the specificity and the sensitivity of the direct detection of *S. aureus* CP in blood cultures for the diagnosis of *S. aureus* bacteremia.

MATERIALS AND METHODS

Type strains of *Staphylococcus* species other than *S. aureus*. *Staphylococcus arlettae* DSM 20672^T, *Staphylococcus auricularis* ATCC 33753^T, *Staphylococcus capitis* ATCC 27840^T (CIP 81.53), *Staphylococcus caprae* CCM 3573^T, *Staphylococcus carnosus* DSM 20501^T, *Staphylococcus caseolyticus* ATCC 13548^T, *Staphylococcus chromogenes* NCTC 10530^T (CIP 81.59), *Staphylococcus cohnii* ATCC 29974^T (CIP 81.54), *Staphylococcus epidermidis* ATCC 14990^T (CIP 81.55), *Staphylococcus equorum* DSM 20674^T, *Staphylococcus gallinarum* CCM 3572^T, *Staphylococcus haemolyticus* ATCC 29970^T (CIP 81.56), *Staphylococcus hominis* ATCC 27844^T (CIP 81.57), *Staphylococcus hyicus* ATCC 1124^T (CCM 2368), *Staphylococcus intermedius* ATCC 29663^T (CIP 81.60), *Staphylococcus kloosii* DSM 20676^T, *Staphylococcus lentus* ATCC 29070^T, *Staphylococcus lugdunensis* ATCC 43809^T, *Staphylococcus saccharolyticus* ATCC 14953^T (DSM 20359), *Staphylococcus saprophyticus* ATCC 15305^T (CIP 76.125), *Staphylococcus schleiferi* ATCC 43808^T, *Staphylococcus sciuri* ATCC 29062^T (CIP 81.62), *Staphylococcus simulans* ATCC 27848^T (CIP 81.64), *Staphylococcus warneri* ATCC 27836^T (CIP 81.65), and *Staphylococcus xylosus* ATCC 29971^T (CIP 81.66) were obtained from the American Type Culture Collection, Rockville, Md.; the Deutsche Sammlung von Mikroorganismen, Göttingen, Federal Republic of Germany; the Czechoslovak Collection of Microorganisms, J. E. Purkyne University, Brno, Czechoslovakia; and the Collection of the Institut Pasteur, Paris, France.

Blood cultures. A total of 748 blood cultures collected from 665 patients were tested. Blood from patients was distributed into an aerobic blood culture bottle (BCB System Roche, F. Hoffmann-La Roche and Co. Ltd., Diagnostica, Basel, Switzerland; or Hemoline Aerobes, bioMérieux, Marcy-L'Etoile, Charbonnières-les-Bains, France) and an anaerobic blood culture bottle (prereduced Schaedler broth medium, Diagnostics Pasteur, Marnes-la-Coquette, France; or Hemoline Anaerobes, bioMérieux). Blood cultures were incubated at 35 to 37°C and examined twice a day for the first 2 days and then daily for the following 5 days. The time for detection of *S. aureus* ranged between 18 to 72 h. The average time to detect growth with coagulase-negative staphylococci was longer (3 to 5 days). All positive blood cultures were processed by classical bacteriological tests, and organisms were identified by conventional methods (16). Bottles of all positive and of a few negative cultures were then autoclaved, and 3 ml of each culture was retained and stored at −20°C until tested for the presence of CP.

Production of MAbs. Female BALB/c or C57BL/6 mice were immunized subcutaneously five times (days 1, 13, 25, 37, and 50) with 5×10^7 live cells of *S. aureus* prototype strains (strain Reynolds for serotype 5 and strain Becker for serotype 8) (13). Spleen cells of these mice were then fused

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on day 54 with X63-Ag8.653 (14) mouse myeloma cells. Hybridoma cells were tested for antibody production against serotype 5 or 8 CP by ELISA. Cells from positive wells were cloned by limiting dilutions in microtiter plates, and supernatants were screened by ELISA. MAbs were also checked for CP specificity by agglutination (12) with *S. aureus* prototype strains and by double diffusion against purified *S. aureus* CP of serotype 5 (8) or 8 (9). Immunoglobulin class and subclass determinations were performed by ELISA with reagents purchased from Miles Scientific (Naperville, Ill.). Hybridomas were expanded for antibody production by intraperitoneal injection into 2,6,10,14-tetramethylpentadecane (pristane)-primed mice. MAbs of isotype γ were purified from ascites fluid by ion-exchange chromatography. MAbs of isotype μ were purified on a S-200 Sephacryl column by a modified gel filtration technique producing an unusual exclusion volume of immunoglobulin M (3).

ELISA. Hybridoma cell supernatants were screened for antibody activity by using flat-bottom microplates (Immuno Microwell; Nunc, Roskilde, Denmark). These were coated by incubation at 37°C for 1 h with 1 μg of purified serotype 5 or 8 CP per ml in phosphate-buffered saline (PBS) (pH 7). The plates were washed five times with PBS supplemented with 0.1% Tween 20. Remaining binding sites were blocked by the addition of 0.5% gelatin (Prolabo, Paris, France) in PBS at 37°C for 1 h or overnight at 4°C. After washing with PBS-Tween, the wells received 100-μl volumes of test samples (supernatants of hybridoma cells). After incubation at 37°C for 1 h, the plates were washed with PBS-Tween. An anti-mouse peroxidase-conjugated immunoglobulin G (heavy- and light-chain specific) (Diagnostics Pasteur) diluted 1/1,000 in PBS-Tween supplemented with 0.5% gelatin was added to the wells. The plates were incubated at 37°C for 45 min. After washing with PBS-Tween, 0.4 mg of enzyme substrate (*o*-phenylenediamine dihydrochloride; Dakopatts, Copenhagen, Denmark) per ml in 0.1 M sodium citrate (pH 5.2), supplemented with 0.06% of 30% hydrogen peroxide, was added to each well. After 10 min at room temperature, the reaction was stopped by addition of 3 N HCl (50 μl per well), and the optical density was read at 492 nm.

The presence of CP in blood cultures was detected by a two-step inhibition ELISA. In step 1, MAbs bound to CP present in test samples. In step 2, bound antibodies were not able to bind to the CP-coated microtiter plates. Plate 1 was blocked with 0.5% gelatin in PBS at 37°C for 1 h. After washing with PBS-Tween, the wells received 100-μl volumes of test samples (undiluted supernatants of autoclaved blood culture) and 100 μl of MAbs diluted in PBS-Tween, supplemented with 0.5% gelatin, at a concentration giving an optical density at 492 nm of 0.2 to 0.5 and determined by preliminary titration. After incubation at 37°C for 1 h and then overnight at 4°C, 100-μl samples per well were transferred to plate 2, which had been previously coated with purified CP and blocked with gelatin. This plate was incubated at 37°C for 1 h and, after washing with PBS-Tween, conjugate was added to the wells and the plate was incubated at 37°C for 45 min. After washing with PBS-Tween, enzyme substrate was added, and after 10 min at room temperature, the reaction was stopped and the optical density was read at 492 nm. For each ELISA run, negative controls (wells not receiving test samples but PBS-Tween supplemented with 0.5% gelatin) and titration of purified CP were performed to determine assay sensitivity.

The CP content in blood cultures was determined by the same ELISA technique. A standard titration curve of each CP was plotted for the absorbance of serially diluted samples (0.03 ng/ml to 32 ng/ml) of purified CP. The contents of CP in the supernatants of autoclaved blood cultures assayed at different dilutions were determined from the standard titration curve and expressed in nanograms per milliliter.

TABLE 1. Microorganisms isolated in blood cultures[a]

Microorganism	No. of isolates
Escherichia coli	93
Salmonella species	8
Klebsiella pneumoniae	27
Enterobacter species	11
Serratia species	1
Proteus species	8
Morganella morganii	3
Haemophilus influenzae	1
Pseudomonas species	29
Xanthomonas species	1
Moraxella species	1
Acinetobacter species	11
Flavobacterium meningosepticum	1
Bacteroides species	22
Staphylococcus aureus	48
Coagulase-negative staphylococci	265
Micrococcus species	19
Streptococcus pneumoniae	26
Group B streptococci	11
Group C streptococci	2
Group G streptococci	2
Viridans group streptococci	25
Enterococcus species	20
Bacillus species	12
Clostridium species	4
Corynebacterium species	55

[a] Only one isolate of one bacterial species or group from each patient is included.

RESULTS

Microorganisms isolated in blood cultures. A total of 706 bacterial strains were isolated, including only one isolate of one bacterial species or group from each patient. More than 26 bacterial species were studied, and numbers of isolates of each bacterial species or group are shown in Table 1.

Capsular serotyping of *S. aureus* isolates. Only one isolate from each patient was included. Among 48 *S. aureus* strains isolated from blood cultures, 23 contained serotype 5 CP, 20 contained serotype 8 CP, and 5 were nontypeable with MAbs specific for serotype 5 or 8 CP.

Detection of serotype 5 and serotype 8 CP in blood cultures. All blood cultures from which bacterial strains belonging to species other than *S. aureus* were isolated were negative in ELISA. Organisms belonging to *Staphylococcus* species other than *S. aureus* were, for the most part, *S. epidermidis*. Since the focus of this work is the accurate identification of *S. aureus*, type strains of other recognized *Staphylococcus* species, including two recently described species (10), were tested in ELISA. All these strains were negative in ELISA.

Only one blood culture from each patient was included. All 23 blood cultures containing *S. aureus* serotype 5 were positive in ELISA with serotype 5 CP-specific MAbs. Of 20 blood cultures containing *S. aureus* serotype 8, 19 were positive in ELISA with serotype 8 CP-specific MAbs. All five blood cultures containing nontypeable *S. aureus* were negative in ELISA with serotype 5 or 8 CP-specific MAbs.

Serial blood cultures from two patients were studied. Of seven blood cultures from patient 1, three contained sero-

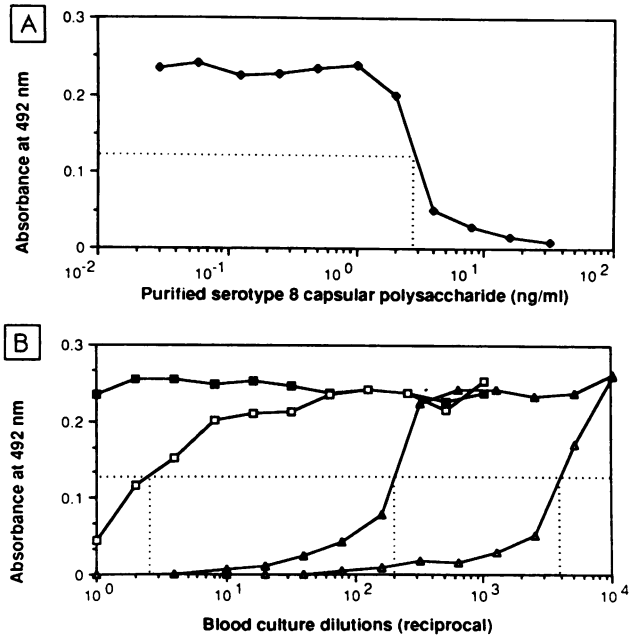

FIG. 1. Quantification of serotype 8 CP by inhibition ELISA. Purified CP (♦) and samples from negative (■), low-positive (□), high-positive (▲), and very high-positive (△) blood culture fluids were serially diluted to form titration curves. · · · · · , Determination of the amounts yielding 50% inhibition.

type 5 CP detectable in ELISA, and *S. aureus* serotype 5 was isolated from these three blood cultures. From the other cultures, which were negative in ELISA, no *S. aureus* was isolated, but coagulase-negative staphylococci (three cultures) and enterococci (one culture) were isolated. Of 23 blood cultures tested from patient 2, nine blood cultures, from which *S. aureus* serotype 5 was isolated, contained serotype 5 CP detectable in ELISA. All other blood cultures (n=14) with no detectable bacterial growth were negative in ELISA.

Concentration of CP in blood cultures. Figure 1 shows an example of quantitation of the serotype 8 CP in inhibition ELISA. The amounts of purified serotype 5 or 8 CP which gave an inhibition of 50% (2.9 ng/ml in the example shown in Fig. 1A) varied from 1 to 5 ng/ml. Concentrations of serotype 5 CP in 22 blood cultures and of serotype 8 CP in 19 blood cultures are shown in Fig. 2. These concentrations varied from 2 to 2,000 ng/ml for serotype 5 CP and from 3 to 12,000 ng/ml for serotype 8 CP.

DISCUSSION

Both coagulase-positive and coagulase-negative staphylococci have been shown to be very commonly isolated from blood cultures (2, 11, 25, 26), and these observations are confirmed in our study (313 staphylococcal isolates among a total of 706 isolates). However, the significance of *S. aureus* isolates in blood cultures is different from that of coagulase-negative staphylococcal isolates. *S. aureus* is usually of clinical significance. The widespread use of antibiotics and the use of invasive procedures in traumatized or immunocompromised patients have abetted the emergence of novel pathogens such as coagulase-negative staphylococci (18). Although potentially associated with catheters or prosthetic

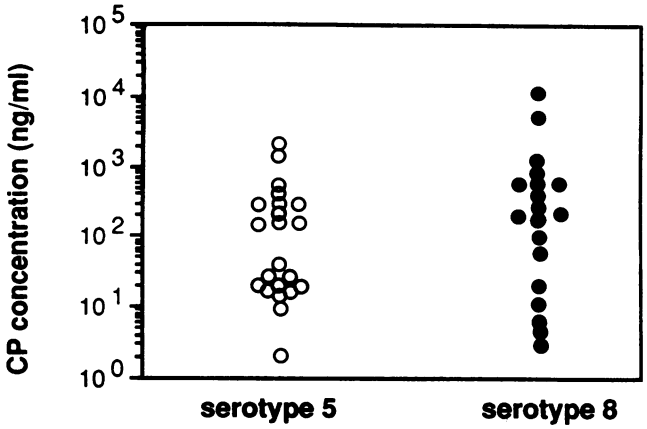

FIG. 2. Distribution of concentrations of serotype 5 CP (○) and serotype 8 CP (●) in positive blood culture fluids.

devices, coagulase-negative staphylococci are still often found to be contaminants. Rapid procedures capable of distinguishing *S. aureus* from coagulase-negative staphylococci directly from blood culture fluids would be clinically useful.

The existence of CP in clinical *S. aureus* isolates and the predominance of capsular serotypes 5 and 8 in the United States (1) and Europe (7, 12, 24) offer the possibility of identification of *S. aureus* isolates which possess these CP by immunological methods directly in blood cultures.

We used in this study MAbs reactive with serotype 5 or 8 CP in a two-step inhibition ELISA which was previously applied to the serotyping of human (7) or animal (22) isolates of *S. aureus*. The specificity of this method was excellent since, among 748 blood cultures examined, all organisms other than *S. aureus* yielded negative results in ELISA. The specificity for *S. aureus* of serotype 5 or 8 CP-specific MAbs has been previously established in bacterial agglutination assays by using several *S. aureus* strains, three strains of *S. epidermidis*, and strains belonging to four species of gram-negative bacilli (21). Our study extended this observation to 658 clinical isolates belonging to 25 species or groups other than *S. aureus* and to the type strains of 25 *Staphylococcus* species other than *S. aureus*.

The sensitivity of this method was good since all 23 *S. aureus* serotype 5 isolates and 19 of 20 *S. aureus* serotype 8 isolates were directly detected in blood culture fluid by ELISA. The false-negative result obtained with a serotype 8 isolate could be due to a technical error since coagulase-negative staphylococci were isolated from other blood cultures of the same patient. This result could also be due to the fact that the amount of serotype 8 CP present in this blood culture was below the detection limit of the ELISA. This hypothesis is consistent with the very high range of the variation of concentrations of CP observed in the positive blood cultures (from 2 to 12,000 ng/ml) and with the sensitivity of this method (1 ng/ml).

Nontypeable *S. aureus* isolates were not detected by ELISA, and this result confirms the specificity of our MAbs for serotype 5 or 8 CP. One part of these nontypeable strains may elaborate CP belonging to other capsular serotypes (13) and, in this case, further preparation of MAbs reactive with these CP will improve the percentage of *S. aureus* isolates identified by ELISA in blood cultures. The detection of the other part of these nontypeable *S. aureus* strains, which may not elaborate CP, might be possible by using MAbs reactive

with other antigenic structures specific for *S. aureus* such as teichoic acid, protein A, or coagulase. Further studies are, however, necessary to examine these possibilities. Nevertheless, the observation that a high proportion (43 of 48) of *S. aureus* blood isolates were either of serotype 5 or of serotype 8 confirms previous results (7) and shows that an ELISA with only two MAbs (serotype 5 or serotype 8 specific) is able to detect up to 90% of *S. aureus* bacteremia isolates.

The study of serial blood cultures from two patients shows the reproducibility of the direct detection of CP in blood cultures by ELISA, since encapsulated *S. aureus* was always isolated from blood cultures positive in ELISA. Moreover, all blood cultures from the same patients with no detectable bacterial growth were negative in ELISA.

The purpose of this study was to evaluate the reliability of the direct detection by ELISA of *S. aureus* CP in blood culture fluids. Our study of the specificity, sensitivity, and reproducibility of this method shows its reliability.

Technical improvements are, however, necessary before this method can be used for clinical applications. These improvements should be directed towards the extraction procedure of the CP from bacterial cells and towards the procedure of the ELISA. The autoclaving of bacterial cells is efficient in releasing *S. aureus* CP (8, 9) and safe and convenient in processing a great number of samples. It is, however, less convenient if applied to only a few clinical samples. Other methods using chemical products or enzymes, such as lysostaphin, which has been shown to be efficient in releasing CP from *S. aureus* cells (9), could be applied in clinical routine tests.

In this study, we have used a two-step procedure for the ELISA which is time-consuming and adapted to the simultaneous processing of a great number of samples. The simple and rapid ELISA procedures used in the kits for detection of group A streptococci directly from throat swabs (6) could certainly be adapted to the detection of *S. aureus* CP in blood culture fluids. Such a kit could be very useful for the direct identification and serotyping of *S. aureus* in blood cultures. This possibility, together with the observation that there is a correlation between capsular serotype of *S. aureus* and resistance to oxacillin (7), may help the clinician to better evaluate and treat *S. aureus* infections.

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