Rapid Identification of *Staphylococcus aureus* in Blood Cultures by a Combination of Fluorescence In Situ Hybridization Using Peptide Nucleic Acid Probes and Flow Cytometry

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Fluorescence in situ hybridization (FISH) using peptide nucleic acid probes (PNAs) allows the identification of *Staphylococcus aureus* from human blood culture samples. We present data revealing that the combination of PNA FISH and flow cytometry is a possible approach for the noncultural identification of staphylococci in blood cultures.

Annually, 660,000 cases of sepsis occur in the United States, with a high mortality of up to 50% (9). Similar data were reported from Germany (13). Staphylococci are the most frequently isolated bacteria from blood cultures (1, 6). Usually, staphylococci are categorized as *Staphylococcus aureus* and non-*S. aureus* (coagulase-negative staphylococci [CoNS]). Although CoNS are the most prevalent bacteria (~35%) in blood cultures, the majority are contaminants from the normal skin flora (14). Therefore, the direct identification of staphylococci from blood cultures would allow the early diagnosis of an *S. aureus* septicemia, which is empirically best treated with, e.g., vancomycin (8).

Unfortunately, after gram staining, the definitive identification of staphylococci from blood cultures by traditional methods requires subculturing and biochemical analysis (e.g., detection of clumping factor and DNase, etc. [7]) and takes at least 24 h after the first indication of growth. Modern blood culture systems (e.g., the BACTEC 9240; Becton Dickinson, Heidelberg, Germany) allow the detection of pathogens in an early logarithmic growth phase when they contain high numbers of ribosomes (2). Therefore, the time point when growth is detected meets perfectly the requirements for the identification of pathogens by using fluorescence in situ hybridization (FISH). FISH using peptide nucleic acid (PNA) probes (PNA FISH) is a novel diagnostic technique proven to be a reliable method for the identification of *S. aureus* from blood cultures (3, 11, 12).

In order to establish a method which could replace the time-consuming microscopy of FISH (taking ~5 to 10 min per patient sample) and the bias associated with interobserver variability, we introduced a protocol for the detection and identification of *S. aureus* by PNA FISH and flow cytometry (data acquisition, ~10 to 20 s) using the *S. aureus* PNA FISH culture identification kit (AdvanDx, Woburn, MA). First, *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) control cells were hybridized in liquid buffer. For this purpose, BACTEC Plus Aerobic/F blood culture bottles, each inoculated with 3 ml of blood from a healthy volunteer, were spiked with 1 × 10⁶ to 5 × 10⁷ CFU and incubated in a BACTEC 9240 blood culture system. After growth detection, aliquots (2 ml) were taken and centrifuged (10 min, 425 × g). Erythrocytes were osmotically lysed (step 1) by adding 900 μl of distilled water for 2 min, and lysates were resuspended with 100 μl of 10× phosphate-buffered saline (pH 7.4), centrifuged to spin down staphylococci, treated with ethanol (80% ethanol for 5 min [step 2]), and centrifuged again. The resulting pellets had significantly reduced amounts of cell debris and contained the ethanol-fixed pathogens. PNA hybridization (step 3) was carried out for 90 min at 55°C in hybridization buffer with 30% formamide. The total volume of the hybridization reaction mixture was 100 μl and contained the fluorescently labeled PNA probes. After centrifugation, pathogens were washed under stringent conditions for 30 min at 55°C (step 4). Finally, cells were resuspended in 300 μl phosphate-buffered saline, and samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson). Logarithmic signal amplification was used, fluorescence acquisition was gated by light scatter parameters, and data were analyzed by using Summit analysis software (Dako Cytomation, Hamburg, Germany). For an internal control, each sample was analyzed microscopically.

Analysis of liquid PNA FISH-hybridized bacteria revealed a bright green fluorescence signal when *S. aureus* ATCC 25923 was present in the sample (Fig. 1). Rapid identification of *S. aureus* was achieved by flow cytometry using the *S. aureus*-specific PNA probe (green fluorescence), which did not hybridize with *S. epidermidis* ATCC 12228 (Fig. 1B), consistent with microscopic analysis (Fig. 1A).

Next, we investigated whether staphylococci previously isolated from blood cultures of septicemic patients can be detected and identified by PNA FISH using flow cytometry. Again, blood culture bottles were inoculated, each with 3 ml blood, and spiked with staphylococcal blood culture isolates from the microbiological routine laboratory which were previ-
ously identified by standard laboratory methods. In total, 38 blood culture bottles were spiked with staphylococci isolated randomly and in chronological order in January and February 2005. Thirty-three of these staphylococcal isolates were unique, and five of them were isolated twice on different days. Immediately after growth detection in BACTEC 9240, PNA FISH using flow cytometry was performed (representative examples are given in Fig. 1C and D). For control reasons, the liquid-hybridized bacteria were additionally analyzed microscopically (data not shown). Data revealed no discrepancies between S. aureus-identification by standard laboratory methods, by microscopic, and by flow cytometric PNA FISH analysis. Thus, we obtained a sensitivity and specificity of 100%, as all S. aureus isolates (n = 8) were identified correctly by the use of PNA FISH and flow cytometry. The S. aureus-specific PNA probe did not hybridize to any of the CoNS isolates (n = 30; included S. epidermidis [n = 29] and S. capitis [n = 1]), which was reflected by a missing green fluorescence signal in flow cytometry. These data were confirmed objectively by comparing the means of the green fluorescence intensities from histogram analysis (mean intensity of green fluorescence from S. aureus isolates [n = 8], 966.6 ± 450.0; mean intensity for CoNS isolates [n = 30], 675 ± 450).
Isolates \( [n = 30] \), 11.8 ± 6.9. Calculated from these data, the mean of fluorescence intensities obtained with PNA FISH from \( S. \text{aureus} \) isolates was 17- to 32-fold higher than that of those obtained from CoNS. Therefore, \( S. \text{aureus} \) can be clearly distinguished from CoNS by PNA FISH using flow cytometry in less than 4 h.

Furthermore, \( S. \text{schlieferti} \), previously reported to weakly cross-react with the \( S. \text{aureus} \)-specific PNA probe in microscopic analysis (3), could be clearly distinguished from \( S. \text{aureus} \) by PNA FISH using flow cytometry (mean green fluorescence intensities were as follows: for \( S. \text{aureus} \) ATCC 25923, 225.9; for \( S. \text{epidermidis} \) ATCC 12228, 16.3; for \( S. \text{schlieferti} \) subsp. \( S. \text{schlieferti} \) ATCC 43808, 79.3; for \( S. \text{schlieferti} \) subsp. \( S. \text{coagulans} \) ATCC 49545, 19.1).

In this study, we have shown that the combination of PNA FISH and flow cytometry is a highly reliable noncultural method for the rapid detection and identification of \( S. \text{aureus} \) directly from blood cultures. Results obtained with PNA FISH using flow cytometry did not reveal any discrepancies in sensitivity or specificity compared to standard laboratory methods. However, it has to be mentioned that the widespread use of FISH and flow cytometry in clinical microbiology might be limited by the fact that expensive flow cytometers are usually not included in the standard equipment of routine laboratories. So-called “bench-top” flow cytometers, which nearly match fluorescence microscopes in both price and size, might represent a reasonable, cheap, and space-saving alternative to conventional flow cytometers.

We have previously demonstrated that FISH with rRNA-targeted DNA probes represents a useful method for fast noncultural identification of pathogens grown in blood cultures (5, 6). This method was hampered, however, by the facts that (i) FISH of staphylococci needs enzymatic pretreatment; (ii) even after this pretreatment, a significant number of the pathogens were not accessible for DNA FISH probes due to insufficient membrane permeabilization (6); and (iii) centrifugation of such permeabilized staphylococci for performing DNA FISH and flow cytometry led to the destruction of the bacteria (4). In contrast, PNA FISH does not need such permeabilization pretreatment, as PNA probes contain an uncharged neutral backbone, enabling PNA probes to penetrate the hydrophobic cell wall easily (10). Therefore, PNA FISH expands the capacity of DNA FISH and flow cytometry on gram-positive cocci, which were not included in our earlier studies (4).

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