Simultaneous Detection and Differentiation of *Staphylococcus* Species in Blood Cultures Using Fluorescence in situ Hybridization

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Key Words

Fluorescence in situ hybridization • Bacteremia • *Staphylococcus*

Abstract

Objective: To develop a new protocol for the simultaneous detection and differentiation of *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) in blood cultures using fluorescence in situ hybridization (FISH), without cultivation and biotyping. **Materials and Methods:** Oligonucleotide probes were used to target the variable regions of the 16S rRNA of *S. aureus* and CoNS, the probes were labeled with fluorochrome Cy3 (red signal) and fluorescein isothiocyanate (green signal), respectively. It is not possible to design one probe that will hybridize to all CoNS species. Therefore, in this study two differentially labeled probes (STA and SAU) were mixed and used to detect and differentiate *S. aureus* from CoNS rapidly in a single smear. Samples of 189 positive blood cultures with Gram-positive cocci in clusters and 11 Gram-positive cocci in chains or pairs were included. **Results:** The FISH assay showed 91.8% sensitivity and 100% specificity for *S. aureus* and 100% sensitivity and 93.3% specificity for CoNS when hybridized with STA and SAU probes. Meanwhile, the assay showed 91.8% sensitivity and 100% specificity for *S. aureus* and 100% sensitivity and 90% specificity for CoNS, when hybridized with the SAU probe only.

Conclusion: Our data showed that the FISH technique was suitable for the simultaneous detection and differentiation of *Staphylococcus* sp. in blood cultures.

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Introduction

Bacteremia and sepsis are amongst the most serious health problems with an incidence that is increasing worldwide, resulting in a tripling of the number of sepsis-related deaths [1]. *Staphylococcus aureus* is the most important pathogen detected in blood culture. Bacteremia caused by *Staphylococcus* sp. often indicates a serious medical condition. Coagulase-negative staphylococci (CoNS) usually cause device-associated infections resulting from contamination. Rapid differentiation of *S. aureus* from CoNS in blood culture using fluorescence in situ hybridization (FISH) has had an important impact on vancomycin usage, length of patient hospitalization and hospital costs [2]. Rapid identification of bloodstream infections and delivery of information to treating clinicians facilitate optimization of antimicrobial therapy. Differentiation of *S. aureus* from CoNS in blood culture takes at least 24–48 h, using subculturing and biochemical analysis techniques. To decrease the time taken from the initial positive blood culture to identification of the

pathogen causing bacteremia, a simple, sensitive, specific method for rapid identification of *S. aureus* and CoNS from blood culture needs to be established. Hence we investigated the development of a new protocol for the simultaneous detection and differentiation of *S. aureus* and CoNS in blood cultures using FISH.

Materials and Methods

Reference Strains and Clinical Isolates

Eighteen reference strains representing related and clinically relevant bacterial and yeast species were obtained from the American Type Culture Collection. Two hundred blood cultures included in this study were collected from The First People's Hospital, Jingmen. Only samples detected by BacT/ALERT 3D120 (bioMérieux) between 8.00 a.m. and 11.30 p.m. on weekdays were included in this study. Upon detection of growth by the automated blood culture machine, samples from blood culture fluid were Gram-stained. Three aliquots were taken and used for: Gram staining, subculture on agar plates and FISH analysis.

Preparation of Smears

For each smear, a 15- μ l aliquot was pipetted onto a glass slide and streaked out gently. The slide was then fixed using the flame of a Bunsen burner twice. Subsequently, the smears were disinfected by immersion into 96% (vol/vol) ethanol for 10 min and air-dried.

Oligoprobes

A probe was designed for the detection of *Staphylococcus* sp. (STA probe) and another for the detection of *S. aureus* (SAU probe) [3]. The sequence of the probes was as follows: STA probe: 5'-TCC TCC ATA TCT CTG CGC-3', with the 5' end linked to fluorescein isothiocyanate; and SAU probe: 5'-GAA GCA AGC TTC TCG TCC G-3', with the 5' end linked to cyanine dye3. Probes Eub338 and Non-Eub were used simultaneously [4], as positive and negative controls, respectively. Probes were synthesized by EuroGentec BV (Maastricht, The Netherlands).

The probe specificity was evaluated by the FISH method, using reference strains representing the *Staphylococcus* genus and clinically relevant bacterial and yeast species. The diagnostic performance of the FISH method was evaluated with 200 positive blood culture samples, and the results were compared to the results obtained by conventional culture methods. The cultures comprised 189 *Staphylococcus* sp. cultures and 11 non-*Staphylococcus* cultures. Of the 189 staphylococci cultures, 49 were *S. aureus* [21 methicillin-resistant *S. aureus* (MRSA) strains, 28 methicillin-sensitive *S. aureus* (MSSA) strains], 140 were CoNS, with five different species.

By serial dilution of bacterial suspensions and FISH analysis, the detection limit of FISH was determined to be 10^3 microorganisms/ml.

Conventional Microbiological Identification

Positive blood cultures demonstrating Gram-positive cocci in clusters by Gram stain were subcultured onto 5% sheep blood agar plates (bioMérieux) and incubated overnight at 35°C with 5–10%

CO₂. Possible staphylococci isolates were subject to catalase and tube coagulase tests (bioMérieux), the API Staph Strip test (bioMérieux).

Establishment of FISH Assay

The assay was performed as previously described by Kempf et al. [3] and Jansen et al. [5] with some modifications. Two modifications were made to the original FISH protocols; lysis conditions were changed for complete lysis of *S. aureus* and CoNS and the hybridization temperature and formamide concentration were modified for decreasing the hybridization time, to ensure the high stringency of hybridization. Briefly, Gram-positive staphylococci were permeabilized by incubating the fixed slide with lysozyme (1 mg/ml for 10 min at 30°C) followed by lysostaphin (Sigma) (60 U/ml for 10 min at 30°C), each dissolved in 10 mM Tris (pH 8.0). The smears were then washed and 100 μ l of oligonucleotide probe (10 ng/ml) was added onto slides and hybridized at 50°C for 90 min (20 mM Tris-HCl, 0.9 M NaCl, 0.1% SDS, 10% formamide, pH 7.2). After hybridization, the slides were washed for 10 min at 50°C in washing buffer (20 mM Tris-HCl, 0.9 M NaCl, pH 7.2) and mounted with VectaShield (Vector Laboratories, Burlingame, Calif., USA). Immediately after preparation, the slides were evaluated using an epifluorescence microscope (Olympus BX40). Aliquots of all samples were tested in parallel with the positive control probe, Eub338, and the negative control probe, Non-Eub.

Optimization of Hybridization

Most CoNS species were not sensitive to lysostaphin with the exception of a few strains. To increase the penetration ability of CoNS into the bacteria cell, a high concentration of lysostaphin (60 U/ml) is preferable. Hybridization buffer with 10% formamide can strengthen the stringency of FISH and yield more specific results.

Data Analysis

For each probe, the sensitivity (defined as the proportion of blood samples that contained the target organism and gave a positive result by FISH analysis) and the specificity (defined as the proportion of blood samples that did not contain the target organism and gave a negative result by FISH analysis) were calculated. Microorganisms obtained in parallel using conventional blood culture techniques were defined as the gold standard.

Results

Evaluation of Probe Specificity

The SAT probe correctly detected all 12 *Staphylococcus* reference strains, showed no cross-reactivity with other species, including closely related *Streptococcus* sp. and *Enterococcus* sp. The SAU probe hybridized specifically to four MSSA reference strains and two MRSA reference strains, and showed no cross-reactivity with other CoNS species. The two probes were therefore highly specific and hybridized to their respective target species/genus only and not to related bacterial species/genus (table 1).

Table 1. Specificity evaluation of STA and SAU probes with the reference strains

Strains	Code	STA	SAU
<i>Staphylococcus aureus</i>	ATCC33862	+	+
<i>Staphylococcus aureus</i>	ATCC29213	+	+
<i>Staphylococcus aureus</i>	ATCC29737	+	+
<i>Staphylococcus aureus</i>	ATCC25923	+	+
<i>Staphylococcus aureus</i>	ATCC33591	+	+
<i>Staphylococcus aureus</i>	ATCC33593	+	+
<i>Staphylococcus epidermidis</i>	ATCC12228	+	–
<i>Staphylococcus epidermidis</i>	ATCC14990	+	–
<i>Staphylococcus cohnii</i>	ATCC35662	+	–
<i>Staphylococcus haemolyticus</i>	ATCC29970	+	–
<i>Staphylococcus sciuri</i>	ATCC29060	+	–
<i>Staphylococcus schleiferi</i>	ATCC43808	+	–
<i>Streptococcus pneumoniae</i>	ATCC49619	–	–
<i>Streptococcus agalactiae</i>	ATCC27956	–	–
<i>Enterococcus faecalis</i>	ATCC29212	–	–
<i>Enterococcus faecium</i>	ATCC27270	–	–
<i>Candida albicans</i>	ATCC90028	–	–
<i>Micrococcus luteus</i>	ATCC49732	–	–

Table 2. Results of FISH analysis of 200 blood culture samples

Conventional identification	Specimens	FISH identification			Non- <i>S. aureus</i>
		EUB338	STA	SAU	
<i>Staphylococcus aureus</i> (MSSA)	28	28	28	26	2
<i>Staphylococcus aureus</i> (MRSA)	21	21	21	19	2
<i>Staphylococcus epidermidis</i>	110	110	110	0	110
<i>Staphylococcus hominis</i>	16	16	16	0	16
<i>Staphylococcus haemolyticus</i>	9	9	9	0	9
<i>Staphylococcus simulans</i>	3	3	3	0	3
<i>Staphylococcus capitis</i>	2	2	2	0	2
<i>Enterococcus faecalis</i>	4	4	0	0	0
<i>Streptococcus pyogenes</i>	2	2	0	0	0
<i>Streptococcus agalactiae</i>	1	1	0	0	0
<i>Streptococcus sanguinis</i>	1	1	0	0	0
<i>Streptococcus pneumoniae</i>	1	1	0	0	0
<i>Micrococcus</i> sp.	2	2	0	0	0
Total	200	200	189	45	144

Diagnostic Sensitivity and Specificity

The results summarized in table 2 showed 91.8% sensitivity and 100% specificity for *S. aureus* and 100% sensitivity and 93.3% specificity for CoNS, when hybridized with the STA and SAU probes, whereas the assay showed

91.8% sensitivity and 100% specificity for *S. aureus* and 100% sensitivity and 90% specificity for CoNS, when hybridized with the SAU probe only. Two MRSA and two MSSA strains were not detected by the SAU probe.

Discussion

The rapid identification of *S. aureus* and CoNS has direct clinical significance in the prompt initiation of antibiotic treatment or adjustment of a current antibiotic regimen. Conventional identification takes 24–48 h after a positive BacT/ALERT result. This delay has led to a practice of widespread broad-spectrum antimicrobial therapy for patients. However, 85% of CoNS-positive results in some clinical settings represent contamination; therefore, fast differentiation of *S. aureus* from CoNS would help the microbiologist, in consultation with the clinician, to discriminate a serious infection from a possible contamination [6]. This study developed a FISH assay that enabled the differentiation between *S. aureus* and CoNS on a single smear, and that could be easily completed within 2 h.

By testing reference strains and checking the BLASTN database, the two probes were shown to have good theoretical specificity. By examination of 200 clinical samples, high detection sensitivity and specificity were achieved by FISH compared with conventional methods of identification. Two MRSA and MSSA strains were not detected using the SAU probe, which may be attributable to the rigid cell wall of *S. aureus*. It was observed that a small proportion of *S. aureus* strains emitted only moderate fluorescence signals (compared to the intensive fluorescence signals usually emitted) probably due to the strain-specific characteristics of the cell wall composition and variable sensitivity to the enzyme [5]. As a result, a high concentration of lysostaphin was applied to ensure complete lysis of *S. aureus* strains, enabling the SAU probe to easily penetrate the cell in all strains.

After treatment with the lytic enzyme, the adherence between bacteria cells and the slide was effected, and the washing procedure must be performed gently to avoid cell loss. The procedure for implementing the FISH assay is as follows: once a positive BacT/ALERT result was obtained, the smear and Gram stain should be prepared, then the appropriate probe is selected depending on the Gram stain characteristics of the microorganisms involved. The results of this study suggest that FISH can segregate *S. aureus* from CoNS successfully in positive blood cultures.

A probe specific for all CoNS could not be designed on the basis of conserved 16S rRNA sequences, so this study selected genus-specific staphylococci and species-specific *S. aureus* probes to screen CoNS. Species that react with the STA probe rather than the SAU probe were defined as CoNS. The diagnostic specificity was strengthened by using two probes (93.3%) versus a single SAU probe (90%). Without the STA probe to detect *Staphylococcus* sp., *Micrococcus* sp. could be falsely identified as CoNS. Compared with FISH, analysis involving polymerase chain reaction (PCR) can provide high sensitivity; however, there are several problems associated with this technique, including a high percentage of false positives. Even if DNA is detected, it is unclear whether it actually represents live invading microorganisms or simply dead pre-sorbed microorganisms, or microorganisms engulfed in, and killed by, polymorphonuclear leukocytes [3]. The use of FISH is attractive because growth-positive blood cultures contain sufficient bacteria to be detected by microscopy, and therefore no amplification step (as in PCR) is required [6]. A new technique, peptide nucleic acid probe

FISH, is now available [7, 8], but most of the peptide nucleic acid probes are much more costly than DNA probes and this limits its application [9]. In summary, FISH has the potential to become a very useful tool in the diagnosis of bloodstream infections.

FISH is a rapid and reliable technique for distinguishing *S. aureus* from CoNS from positive blood cultures within 2 h. Such a decrease in the diagnostic turnaround time would have significant benefits for the rapid diagnosis and treatment of patients with suspected bacteremia. Future studies to improve the sensitivity of the assay for *S. aureus* detection and the specificity of CoNS detection should be performed.

Conclusion

Our data showed that the FISH technique was suitable for the simultaneous detection and differentiation of *Staphylococcus* sp. in blood cultures.

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