Use of Fluorescence In Situ Hybridization for Rapid Identification of Staphylococci in Blood Culture Samples Collected in a Portuguese Hospital^{†‡}

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Fluorescence in situ hybridization was used for the direct identification of staphylococci in blood cultures collected at a Portuguese hospital where staphylococci account for up to 35% of clinically relevant blood cultures. The assay was able to detect the presence/absence of staphylococci and distinguish *Staphylococcus aureus* from coagulase-negative staphylococci in 4.5 h.

Staphylococci—in particular, coagulase-negative staphylococci (CoNS)—are the pathogens most frequently isolated in blood cultures (2, 17). However, many of these CoNS are contaminants from skin flora, and their isolation from blood cultures may not correspond to infection (8). Thus, the rapid and reliable identification of staphylococci and the differentiation between *Staphylococcus aureus* and CoNS are essential to optimize diagnostic and therapeutic decisions.

Analysis of blood samples usually involves culturing in automated systems, followed by Gram staining, subculturing, and biochemical testing. Altogether, this procedure takes 24 to 48 h to provide identification after microbial growth detection. Methods that allow the direct identification of bacteria in positive blood cultures, such as fluorescence in situ hybridization (FISH), which detects nucleic acids within intact cells, are thus essential to overcome the delaying step of subculture (4, 10, 13, 14).

The purpose of the present study was to develop and evaluate the performance of a FISH protocol for the direct detection of staphylococci in positive blood cultures from a large teaching hospital in Lisbon (Portugal), where these bacteria account for up to 35% of all clinically relevant positive blood cultures.

The following oligonucleotide probes labeled at the 5' end with fluorochrome Cy3 (Thermo Hybaid, Ulm, Germany) were used for the FISH assays: EUB338 (5'-GCTGCCTCCCGTA GGAGT), targeting eubacteria (1); EUK516 (5'-ACCAGAC

TTGCCCTCC), targeting eukaryotes (1); and three additional 16S rRNA-targeted probes designed in this work, STA2 (5'-C ATATCTCTGCGCATTTC) and STA3 (5'-GCACATCAGC GTCAGT), targeting *Staphylococcus* spp., and Sau66 (5'-AA GCTTCTCGTCCGTTCG), specific for *S. aureus* (Fig. 1). Probe specificity was evaluated by testing these sequences against 16SrRNA gene sequences available in GenBank.

Although several authors have referred to the advantages of working with peptide nucleic acid probes, in our work we felt no need to use these probes, which are considerably more expensive than oligonucleotide probes.

The optimization of the FISH experimental procedure, particularly cell lysis and the evaluation of probe specificity, was carried out with a set of reference strains obtained from the Culture Collection of the Molecular Genetics Laboratory of Instituto de Tecnologia Química e Biológica (ITQB/UNL): *Staphylococcus aureus* NCTC8325, *Staphylococcus epidermidis* ATCC 14990^T, *Staphylococcus saprophyticus* ATCC 15305^T, *Staphylococcus haemolyticus* ATCC 29970^T, *Staphylococcus lugdunensis* ATCC 43809^T, *Staphylococcus hominis* ATCC 27844^T, *Staphylococcus simulans* ATCC 27848^T, *Staphylococcus warneri* ATCC 27836^T, *Staphylococcus schleiferi* subsp. *schleiferi* ATCC 43808^T, *Micrococcus caseolyticus* ATCC 13548^T, and *Escherichia coli* ATCC 35218. The yeast *Cryptococcus neoformans* PYCC3957^T, from the Portuguese Yeast Culture Collection (PYCC), was also included as a positive control for the EUK516 probe. An additional group of 39 staphylococcal clinical isolates previously identified by biochemical and molecular techniques, which included *S. aureus* (22 isolates), *S. epidermidis* and *S. haemolyticus* (7 isolates each), and *S. hominis* (3 isolates) (12, 15, 16), was also included for probe evaluation.

For the FISH assays with the reference strains, cultures were grown to exponential phase and fixed with 4% paraformaldehyde as previously described (1). One microliter of fixed cell suspension was deposited in individual wells of 12-well Teflon glass slides (Superior Marienfeld, Lauda-Koenigshofen, Germany) and dried at 37°C. Before hybridization, the staphylo-

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Strain	Access number	STA2 Probe	STA3 Probe	Sau66 Probe
		CTTTACGCGTCTCTATAC	TGACTGCGACTACAG	GCTTGCTGCTCTT-CGAA
<i>S. aureus</i>	(D83357)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGGACGAGAA-GCTTSC-TTCTCT
<i>S. epidermidis</i>	(D83363)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGACGAGAA-GCTTSC-TTCTCT
<i>S. saprophyticus</i>	(D83371)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGATAGAA-GCTTSC-TTCTTT
<i>S. haemolyticus</i>	(D83367)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGACGAGAA-GCTTSC-TTCTTT
<i>S. lugdunensis</i>	(AB009941)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGATAGAA-GCTTSC-TTCTTT
<i>S. hominis</i>	(X66101)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGACGAGAA-GCTTSC-TTCTTT
<i>S. schleiferi</i>	(883568)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGGACGAGAA-GCTTSC-TTCTTT
<i>S. simulans</i>	(D83373)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGACGAGAA-GCTTSC-TTCTCT
<i>S. warneri</i>	(L37603)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGATGTGCGAAAGC	AGTCGAGCGAACGATAGAA-GCTTSC-TTCTTT
<i>M. caseolyticus</i>	(D83359)	TAGCGGTGAAATGCGCAGAGATATGGAGGAA	GTCTGTACTGACGCTGAGGTGCGAAAGC	AGTCGAGCGAACGGACGAGAGTCTTSCACTCTCT
<i>E. coli</i>	(X80724)	TAGCGGTGAAATGCGTAGAGATATGGAGGAA	GACGAAGCTGACGCTCAGGTGCGAAAGC	AGTCGAGCGTATACAGGACGAGCTTSC-TGCTTT

FIG. 1. Alignments of STA2, STA3, and Sau66 probes with the 16S rRNA gene complementary target sequences of *Staphylococcus* spp., *M. caseolyticus*, and *E. coli*. These sequences correspond, respectively, to nucleotides 686 to 716, 742 to 770, and 59 to 93 of the *E. coli* 16S rRNA (3). Mismatches are indicated by the highlighted cells.

coccal cells were permeabilized by one of the following protocols: (i) treatment with an enzyme mix of 750 µg/ml lysostaphin (Sigma Chemical Company, St. Louis, MO) and 5 mg/ml lysozyme (Sigma) for 1 h at 37°C (11); (ii) incubation with 1 mg/ml lysozyme for 10 min at 30°C, followed by 1 mg/ml lysostaphin for 5 min at 30°C (10); or (iii) incubation with 1 mg/ml lysozyme for 15 min at 37°C, followed by incubation with 10 µg/ml lysostaphin for 5 min at the same temperature (adapted from reference 9).

For each hybridization reaction, 10 µl of hybridization solution (20% formamide, 0.9 M NaCl, 20 mM Tris-HCl [pH 8.0], 0.01% sodium dodecyl sulfate) containing 30 ng of each probe was added to each well and the slides were incubated for 3 h at 46°C. Both the permeabilization and hybridization procedures were performed in a saturated humid chamber. Thereafter, the slides were incubated in 50 ml of prewarmed wash solution (0.215 M NaCl, 20 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.01% sodium dodecyl sulfate) for 15 min at 46°C and air dried in the dark. The results were observed using an Olympus BX50 microscope (Olympus Optical Co., Hamburg, Germany) equipped with a filter for Cy3.

Of the three permeabilization protocols tested, the one described by Kempf and colleagues (10) provided the best compromise between cell integrity and hybridization signal intensity. Although STA2 would theoretically hybridize in a region with higher accessibility to the 16S rRNA molecule (6), FISH assays using STA2 and STA3 probes with the reference strains demonstrated that the two probes hybridized equally well, with STA3 showing higher specificity toward the *M. caseolyticus* negative control, as would be expected from the in silico analysis (Fig. 1). Therefore, STA3 was used in the remaining assays.

The Sau66 probe hybridized only with *S. aureus* strains, showing no unspecific hybridization with the other staphylococcal species tested, except for a weak hybridization with the *S. schleiferi* subsp. *schleiferi* reference strain, as expected by the in silico analysis (Fig. 1) and also reported with other *S. aureus* probes (7). Because of the very low incidence of *S. schleiferi* subsp. *schleiferi* as a causative agent of sepsis, this cross-reactivity was not considered diagnostically significant.

The specificity of the hybridization signals obtained with these probes was further evaluated by hybridizing the same reference strains with the 18S rRNA-targeted universal eu-

karyote probe EUK516. As expected, only the *C. neoformans* cells emitted hybridization signals with this probe, indicating that no unspecific binding occurs between the probes used and the staphylococcal cellular matrix. Further testing of probes STA3 and Sau66 with the 39 staphylococcal isolates of clinical origin confirmed the correct hybridization of these probes to their target species.

The optimized FISH protocol using probes STA3 and Sau66 was then applied to the direct identification of staphylococci in 92 blood cultures obtained from the bacteriology laboratory of a 1,300-bed teaching hospital (Lisbon, Portugal), with medical, surgical, pediatrics, intensive care, and emergency medicine units, during June to October 2005. These corresponded to blood cultures from patients with suspected general bloodstream infection, which were inoculated in aerobic (BacT/Alert FA, bioMérieux, Marcy l'Etoile, France), anaerobic (BacT/Alert FN), or pediatric (BacT/Alert PF) blood culture bottles. This collection included 77 blood cultures identified in the clinical laboratory as containing staphylococci, 14 cultures containing other bacteria, and 1 culture with the yeast *Candida parapsilosis*. A sterile blood culture was also tested to evaluate the interference of culture medium components on the hybridization results.

There was a need to adapt the previously optimized FISH protocol for the analysis of blood cultures. In particular, due to the high density of the culture medium, which complicated cell homogeneous treatment and proper fixation to the slide, the 12-well slides were replaced by normal microscope slides, into which 10 to 20 µl of blood culture was streaked and air dried. There was no need to use specific reagents such as poly-L-lysine to increase the adherence of specimens to the slides. The cells were fixed with 1:1 ethanol-phosphate-buffered saline because of the simplicity of this protocol and because long storage of the samples before their analysis was not needed. The remaining experimental protocol followed the procedures previously optimized.

In general, the staphylococcal cells showed good hybridization signals, which allowed their rapid detection in the blood cultures, even in the samples with low cell concentrations (data not shown). We did not observe interference of the charcoal with the probes or autofluorescence from the medium components, with a few exceptions, which did not influence the interpretation of the FISH signals.

TABLE 1. Results of the direct identification of *Staphylococcus* spp. by FISH in blood cultures previously identified by conventional laboratory techniques

Conventional identification ^a	No. of specimens	FISH identification with probe ^b :			No. of discrepancies
		EUB338	STA3	Sau66	
Gram-positive bacteria					
<i>S. epidermidis</i>	25	25	25	0	0
<i>S. aureus</i>	22	22	22	22	0
<i>S. hominis</i>	16	16	16	0	0
<i>S. haemolyticus</i>	9	9	9	1	1
<i>S. simulans</i>	3	3	3	0	0
<i>Staphylococcus capitis</i>	2	2	2	0	0
<i>E. faecalis</i>	4	4	1	0	1
<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
Gram-negative bacteria					
<i>Pseudomonas aeruginosa</i>	2	2	0	0	0
<i>Acinetobacter baumannii</i>	1	1	0	0	0
Yeast					
<i>C. parapsilosis</i>	1	0	0	0	0
Total	92	91	78	23	2

^a Identification provided by the hospital laboratory using conventional techniques.

^b FISH identification with probes targeting eubacteria (EUB338), *Staphylococcus* spp. (STA3), and *S. aureus* (Sau66).

The FISH results correlated well with the identifications obtained at the hospital laboratory using conventional techniques, and only two discrepancies were found (Table 1). The STA3 probe hybridized correctly with the 77 *Staphylococcus* species isolates tested and with only 1 of the 15 nonstaphylococcal samples tested. This culture, previously identified as *Enterococcus faecalis*, contained a mixture with CoNS cells that were readily identified by FISH. The 22 positive blood cultures containing *S. aureus*, which included both methicillin-susceptible and methicillin-resistant *S. aureus* isolates, hybridized correctly with the Sau66 probe, whereas the remaining 70 non-*S. aureus* blood cultures showed no hybridization with this probe, except for a culture previously identified as containing *S. haemolyticus*, which turned out to be a polymicrobial culture. Thus, the rapid detection of polymicrobial or mixed cultures is another advantage of FISH assays (7).

The Sau66 probe revealed high levels of sensitivity (100%) and specificity (98.6%), with positive and negative predictive values of 95.6% and 100%, respectively, for the identification of *S. aureus*. These results are similar to those reported by others (4, 7, 10, 13, 14).

Other groups have recently reported positive outcomes for the introduction of FISH-based assays in the routines of their clinical laboratories (5, 14). The study presented here is a pioneer in Portugal, where for the first time FISH was applied to the analysis of blood cultures in parallel to the routine analysis. The FISH protocol optimized in this study provided results for the presence/absence of staphylococci and the differentiation between *S. aureus* and CoNS within 4.5 h, which constitutes a time savings of 24 to 48 h over the conventional laboratory identification.

Due to the critical role played by the laboratory in the

management of bloodstream infections in large hospitals such as the one in this study, where *S. aureus* and CoNS accounted for 18.9% and 16.1%, respectively, of all bacteria isolated from blood cultures during the year of sample analysis, rapid access to this information is of utmost importance. This method may constitute a valuable tool complementary to conventional laboratory analysis by providing a same-day result that can be used to streamline antimicrobial therapy, thus preventing excessive vancomycin usage, improving patient prognoses, and reducing the length of hospitalization (5).

Our experience showed that FISH is a rapid, reliable, and appropriate method for the daily work routine. Except for the epifluorescent microscope, the protocol does not involve special equipment. Also, with the exception of labeled probes, which are used in very low concentrations, the method does not involve expensive reagents. Additionally, the methodology does not require technical expertise, as most of the procedures are routine in the workflow of the microbiologic clinical laboratory. The total hands-on time is short (less than 1 h), thus not involving the need of extra personnel.

Based on the promising results obtained, we believe that in the future, FISH will become a valuable diagnostic tool for the direct identification of microorganisms in blood cultures that is complementary to conventional laboratory techniques.

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