Accelerated identification of *Staphylococcus aureus* from blood cultures by a modified fluorescence *in situ* hybridization procedure

Sven Poppert,¹ Melanie Riecker,² Nele Wellinghausen,²
Hagen Frickmann³ and Andreas Essig²

Correspondence

Sven Poppert
poppert@bni-hamburg.de

¹Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

²Institute of Medical Microbiology and Hygiene, University of Ulm, Ulm, Germany

³Institute of Medical Microbiology, Virology and Hygiene, University of Rostock, Rostock, Germany

This study evaluated fluorescence *in situ* hybridization (FISH) for rapid identification of *Staphylococcus aureus* and coagulase-negative staphylococci (CoNS) directly from blood cultures. Initially, 360 blood cultures containing Gram-positive cocci were investigated by a previously described microwave-FISH procedure: 44/49 (89.8%) *S. aureus* and 298/299 (99.7%) CoNS were correctly identified. Because FISH proved useful and reliable but handling was found to be inconvenient, the method was modified by employing a recently developed slide chamber. This reduced the time required from 60 to 30 min. The simplified execution allowed integration of the method into the workflow of a routine laboratory without difficulty. The modified method proved to be highly reliable, identifying 37/37 (100%) *S. aureus* and 169/172 (98.2%) CoNS directly from blood cultures.

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INTRODUCTION

Staphylococci are the bacteria isolated most frequently from blood cultures. The fastest possible identification of *Staphylococcus aureus* and its differentiation from coagulase-negative staphylococci (CoNS) are essential for optimal treatment of infected patients (Ruimy *et al.*, 2008; Weinstein, 2003). Conventional phenotypic tests are not sufficiently reliable when applied directly to blood cultures (Chapin & Musgnug, 2003; Mehta *et al.*, 2009; Speers *et al.*, 1998). A wide variety of molecular methods are available, but most are rather complex (Mehta *et al.*, 2009; Ruimy *et al.*, 2008). A relatively easy and cost-effective molecular method for the identification of *Staphylococcus aureus* from blood cultures is fluorescence *in situ* hybridization (FISH) with either oligonucleotides of DNA (Gescher *et al.*, 2008; Jansen *et al.*, 2000; Kempf *et al.*, 2000; Peters *et al.*, 2006; Tavares *et al.*, 2008) or peptide nucleic acid (PNA) (Chapin & Musgnug, 2003; Forrest *et al.*, 2006; González *et al.*, 2004; Hartmann *et al.*, 2005; Hensley *et al.*, 2009; Ly *et al.*, 2008; Oliveira *et al.*, 2002, 2003). FISH allows species-specific staining of bacteria on a microscope slide by hybridization with fluorescently labelled oligonucleotide probes complementary to unique target sites on the rRNA gene. Compared with PNA-FISH, DNA-FISH is less standardized and less well evaluated for

the identification of *Staphylococcus aureus*. DNA probes are, however, less expensive and less prone to non-specific binding (Moter & Gobel, 2000; Oliveira *et al.*, 2002). Recently, a DNA-FISH procedure was developed that uses a microwave oven and allows a result to be obtained in 1 h, about 2 h faster than conventional PNA- or DNA-FISH (Peters *et al.*, 2006). This procedure has so far been tested with only 100 blood cultures that contained various bacteria, including in total just seven isolates of *Staphylococcus aureus* (Peters *et al.*, 2006). We therefore investigated 360 blood cultures containing Gram-positive cocci using the microwave procedure. In order to improve the ease and speed of the method further, we modified the procedure by employing a recently developed slide chamber. This modified FISH procedure was evaluated on 220 additional blood cultures in the setting of a routine diagnostic laboratory.

METHODS

Reference strains. The following reference strains were obtained from the American Type Culture Collection: *Bacillus subtilis* ATCC 6633; *Enterococcus avium* ATCC 14025; *Enterococcus casseliflavus* ATCC 12755, 25788; *Enterococcus durans* ATCC 19432; *Enterococcus faecalis* ATCC 29212, ATCC 19433; *Enterococcus faecium* ATCC 19434, ATCC 51559; *Enterococcus gallinarum* ATCC 700425; *Enterococcus hirae* ATCC 8043; *Enterococcus mundtii* ATCC 43186; *Enterococcus raffinosus* ATCC 49427; *Enterococcus saccharolyticus* ATCC 43076; *Micrococcus luteus* ATCC 7468; *Staphylococcus aureus* ATCC 25923, ATCC 29213, ATCC 43300; *Staphylococcus auricularis*

Abbreviations: CoNS, coagulase-negative staphylococci; FISH, fluorescence *in situ* hybridization; PNA, peptide nucleic acid.

ATCC 33753; *Staphylococcus epidermidis* ATCC 12228; *Staphylococcus hyicus* ATCC 11249; *Staphylococcus lugdunensis* ATCC 49576; *Staphylococcus saprophyticus* ATCC 15305; *Streptococcus agalactiae* ATCC 13813; *Streptococcus intermedius* ATCC 27335; *Streptococcus pneumoniae* ATCC 49619; *Streptococcus pyogenes* ATCC 12344.

Clinical isolates. Clinical isolates were grown from blood cultures (BACTEC 9240; Bactec PLUS aerobic/F medium; Bactec PLUS anaerobic F; and Bactec PLUS paediatric; BD at the Institute of Medical Microbiology and Hygiene, University of Ulm, Germany). The isolates were characterized phenotypically by Gram-staining; colony morphology; haemolysis on blood agar; catalase reaction; growth on agar with trehalose, mannitol and phenolphthalein diphosphate; and the presence of clumping factor (Slidex; bioMérieux). Ambiguous isolates were confirmed by RAPIDEC Staph or API Staph ID 32 (bioMérieux). When biochemical identification was impossible or discrepancies were seen with the FISH result ($n=6$), sequencing of the 16S rRNA gene was performed as described previously (Hiraishi, 1992).

FISH. For FISH, previously published probes targeting either the entire *Staphylococcus* genus (Sta 16S697: 5'-TCCTCCATATC-TCTGCGC-3') or *Staphylococcus aureus* specifically (Sau 16S69: 5'-GAAGCAAGCTTCTCGTCCG-3') were directly 5'-labelled with the red sulfoindocyanine dye Cy3 (Thermo Fisher Scientific) (Kempf *et al.*, 2000). These were used in combination with a green-fluorescing FAM-labelled eubacterial probe, EUB338, targeting almost all bacteria (Amann *et al.*, 1990). CoNS were identified as organisms that stained with the staphylococcus probe but not with the *Staphylococcus aureus* probe.

For preparation of FISH slides from subcultured bacterial colonies, suspensions of bacteria in 0.9% saline were applied to the wells of an eight-well glass slide (Menzel), air dried and fixed in methanol for 10 min. For FISH from blood cultures using the microwave FISH procedure, the blood culture medium was centrifuged at 130 *g* for 10 min to remove erythrocytes. The supernatant was centrifuged again at 1800 *g* for 5 min. The pellet was suspended in 0.9% saline and applied to an eight-well glass slide. After air drying, the slides were fixed in pure methanol for 10 min.

Permeabilization was conducted by adding 6 μ l lysis reagent, comprising lysozyme (2 mg ml⁻¹; Sigma-Aldrich) and lysostaphin (100 μ g ml⁻¹; Sigma-Aldrich) in 10 mM Tris/HCl (pH 8), to each well of the eight-well slides. The slides were incubated at 37 °C for 15 min for staphylococci or for 1 min for enterococci and

streptococci. The reaction was stopped by incubation for 3 min in methanol.

The FISH reaction was performed at a formamide concentration of 30% in a microwave oven (Sharp R208; SeaPro), as described recently (Peters *et al.*, 2006).

Modified FISH. For the modified FISH procedure, blood culture medium (0.3 ml) was not centrifuged but was diluted with 5 ml PBS. A 10 μ l sample was applied directly onto eight-well slides, air dried and fixed with methanol for 10 min. To save time, samples were prepared immediately from all blood cultures that were flagged positive. Only samples from blood cultures that later showed Gram-positive cocci in the Gram stain were investigated by FISH. Permeabilization was conducted by adding 10 μ l lysis reagent as above and incubating the slide for 5 min at 46 °C in a recently developed slide block (ThermoStat; Eppendorf). Hybridization was carried out in the same slide block at 46 °C for 10 min. The washing step was performed in 50 ml tubes in a thermoblock at 48 °C for 5 min with gentle shaking (Thermomixer; Eppendorf).

RESULTS AND DISCUSSION

Evaluation of microwave-FISH

In total, 360 blood cultures that showed Gram-positive cocci with a typical staphylococcus morphology in the Gram stain were investigated by microwave-FISH (Table 1). Only one sample per patient and only pure cultures were used for data analysis. FISH correctly identified 44/49 *Staphylococcus aureus* isolates, 298/299 CoNS and all 12 non-staphylococcal species (Table 1). For five samples with *Staphylococcus aureus*, no result was achieved directly from blood culture, because no bacteria were found on the slides and the control with the eubacterial probe was accordingly negative. One *Staphylococcus epidermidis* isolate was initially misidentified as a non-staphylococcus species because of a false-negative staining with the staphylococcus probe. All strains were correctly identified when FISH was repeated from subcultures. Three blood culture isolates had been identified as *Staphylococcus aureus* by phenotypic

Table 1. Identification of staphylococci directly from blood cultures

Method	<i>Staphylococcus aureus</i>	CoNS	<i>Micrococcus</i> spp.	<i>Rotia</i> spp.	Others*
Microwave procedure					
Isolates in total	49	299	10	1	1
Correct†	44 (89.7%)	298 (99.7%)	10 (100%)	1 (100%)	1 (100%)
Without direct FISH result	5 (10.2%)	—	—	—	—
False	—	1 (0.3%)	—	—	—
Modified procedure					
Isolates in total	37	172	4	1	6
Correct†	37 (100%)	169 (98.2%)	4 (100%)	1 (100%)	4 (66.7%)
Without direct FISH result	—	2 (1.2%)	—	—	—
False	—	1 (0.6%)	—	—	2 (33.3%)

*Comprising *Streptococcus* sp. (one strain), *Enterococcus* spp. (five strains) and *Kocuria* sp. (one strain).

†The FISH assay allows identification to the species level only for *Staphylococcus aureus*. All other bacteria were classified either as CoNS or as non-staphylococci.

methods but were identified as CoNS by FISH. Sequencing revealed these strains as *Staphylococcus hominis* (two strains) and *Staphylococcus haemolyticus* (one strain). One *Kocuria* species had been misidentified as CoNS by phenotypic methods, but was correctly recognized as a non-staphylococcus species by FISH.

DNA-FISH using the microwave procedure thus proved to be highly reliable. However, application of the method in a routine laboratory was hampered by the need for sophisticated preparation steps and rather complicated handling of the microwave oven, which required pre-heating and complex calibration.

Development and evaluation of a modified FISH procedure

A simplified and even faster FISH procedure was therefore developed. The effects of the modifications were tested using subcultures of *Staphylococcus aureus* (ATCC 29213) and spiked blood cultures (data not shown). The different incubation times were reduced until the results visibly deteriorated, but for the final protocol, timing was increased to provide a safety margin. The centrifugation steps were omitted in order to save time and to improve the recovery of *Staphylococcus aureus*, on the assumption that *Staphylococcus aureus* tends to form clusters that may be lost with the erythrocytes during spinning. The staphylococcus cell walls were permeabilized for 5 min at 46 °C in a recently developed slide chamber instead of for 15 min at 37 °C in an incubator. Using the same slide chamber for the hybridization allowed shortening of this step from 18 to 10 min, because heat transmission in the slide block is even faster than in the microwave. The washing step was conducted in 50 ml tubes by gentle shaking in a Thermomixer, which allowed shortening of the washing step from 14 to 5 min. The total required time was thereby reduced from approximately 60 min to only 30 min, and the whole procedure was considerably more convenient.

The modified FISH procedure was evaluated using 27 reference strains as listed in Methods and six clinical isolates of *E. faecium*, *Streptococcus bovis*, *Streptococcus gallolyticus*, *Streptococcus gordonii*, *Streptococcus mitis* and *Streptococcus oralis*. FISH produced correct results with 32/33 tested bacteria. Unfortunately, the *Bacillus* stained clearly positive with the general *Staphylococcus* probe and weakly positive with the *Staphylococcus aureus* probe. These cross-reactions also occurred when FISH was performed in the classic way in an incubator. Fortunately, these cross-reactions do not impair the value of FISH for the identification of staphylococci in blood cultures, because bacilli, being Gram-positive rods, cannot be confused with staphylococci in a Gram stain.

In the next step, the applicability and reliability of the modified FISH procedure in a routine setting was tested using additional blood cultures showing Gram-positive

cocci in the Gram stain. First, 55 blood cultures comprising 48 CoNS and seven *Staphylococcus aureus* were investigated using both FISH procedures. In this set, both procedures achieved correct results in all cases except that both procedures missed one CoNS. In these cases, no result was achieved because no bacteria were detected on the slide with the failed procedure, and in both cases a correct result was obtained with the other procedure.

After this successful pre-evaluation, the modified method was integrated into the workflow of the diagnostic laboratory and executed by routine personnel without difficulties. In total, 165 additional blood cultures were investigated, meaning that, overall, 220 blood cultures were examined with the modified method. Of these, all 37 *Staphylococcus aureus* and 169/172 CoNS were correctly diagnosed (Table 1). Two enterococci were misidentified as CoNS and as *Staphylococcus aureus*. This was probably caused by technical mistakes by less-experienced personnel, as correct results were achieved when FISH was repeated from pure culture, and the probes did not show any cross-reaction with the reference strains of enterococci. In addition, the staphylococcus probe (Sta 16S697) had been tested, in the scope of a previous study, with 60 enterococci and streptococci without showing any cross-reaction (Wellinghausen *et al.*, 2007, unpublished data). One *Staphylococcus capitis* isolate was misidentified as *Staphylococcus aureus*. In this single case, the *Staphylococcus aureus* probe showed some faint cross-reaction when FISH was repeated from pure culture. In contrast to the microwave-FISH procedure, all samples with *Staphylococcus aureus* were clearly assessable and correctly identified by the modified procedure.

The modified FISH procedure thus showed high sensitivity and specificity. The short time to result represents a significant advantage in comparison with conventional DNA and PNA-FISH procedures, as well with PCR, as it makes simultaneous reporting of the findings of Gram staining and FISH possible. This allows quick initiation of optimal antibiotic therapy, leading to improved patient outcome and cost savings (Barenfanger *et al.*, 1999; Forrest *et al.*, 2006; Ly *et al.*, 2008; Ruimy *et al.*, 2008).

In conclusion, FISH proved to be highly reliable for rapid identification of staphylococci from blood cultures. The modifications to the method considerably facilitated and accelerated the application in a routine diagnostic setting.

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