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Faster Identification of Pathogens in Positive Blood Cultures by Fluorescence In Situ Hybridization in Routine Practice

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Rapid identification of microorganisms in blood cultures is required to optimize empirical treatment at an early stage. Fluorescence in situ hybridization (FISH) can reduce the time to identification of microorganisms in growth-positive blood cultures. In this study, we evaluated the performance, time to identification, and potential clinical benefits of FISH compared to those of conventional culture methods in routine practice. After Gram staining, blood culture fluids were simultaneously further identified with FISH and with conventional culture methods. Results and points in time of FISH and culture identification (provisional and final identifications) were collected and compared. For 91% of microorganisms, the genus or family name was identified, and for 79%, the species name could be attributed. The sensitivity and specificity of the individual probes exceeded 95%, except for the *Enterobacteriaceae* probe (sensitivity, 89%). Cross-hybridization was obtained with the *Klebsiella pneumoniae* probe for *Klebsiella oxytoca*. The time gains of FISH and final culture identification were more than 18 h for bacteria and 42 h for yeasts. With FISH, *Staphylococcus aureus* was differentiated from coagulase-negative staphylococci 1.4 h faster than by provisional identification ($P < 0.001$). In conclusion, FISH allows rapid and reliable identification of the majority of microorganisms in growth-positive blood cultures. The substantial time gain of identification with FISH may allow same-day adjustment of antimicrobial therapy, and FISH is especially useful if no provisional identification is obtained. With further extension of the number of probes and a reduction in turnaround time, FISH will become a very useful diagnostic tool in the diagnosis of bloodstream infections.

Bloodstream infection by bacteria or fungi is a serious clinical condition, associated with increased morbidity and mortality compared to the morbidity and mortality associated with localized infections (12). Empirical antimicrobial therapy is usually initiated in ill patients and continued for several days before final results of blood cultures are available. Decreasing the time to microbiological diagnosis of bloodstream infection is important to enable adequate pathogen-based antimicrobial therapy at an early stage and to improve outcome (6, 8, 13). For example, a rapid diagnosis of candidemia in a patient with fever in an intensive care unit would imply early antifungal therapy. Quick determination at the species level could immediately streamline this antifungal treatment to the specific *Candida* species. Also, fast differentiation of *Staphylococcus aureus* from coagulase-negative staphylococci in a blood culture could help the microbiologist in consultation with the clinician to discriminate a serious infection from a possible contamination. A rapid diagnosis could improve prognosis, reduce the length of hospitalization, and decrease the use of inadequate and broad-spectrum antibiotics, thereby reducing the potential development of resistance and possible side effects (e.g., less hepatotoxicity after streamlining of amoxicillin-clavulanic acid to flucloxacillin) (1, 3, 11).

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Various methods have been assessed to reduce the time required for identification of microorganisms in blood cultures, including fluorescence in situ hybridization (FISH), PCR algorithms, and rRNA probe matrices (10, 14, 22). The use of FISH or rRNA probes is attractive because growth-positive blood cultures contain sufficient bacteria to be detected by microscopy; therefore no amplification step (as in PCR) is required. Another advantage of FISH and probe matrices compared to PCR is that a single slide or a microtiter plate is used with an array of probes, whereas multiple individual PCRs (one per pathogen) have to be performed. This makes PCR more labor-intensive than FISH or probe matrices.

The FISH technique is based on the hybridization of probes to target rRNA followed by microscopical detection of fluorescence. Probes for FISH consist of either an oligonucleotide or a peptide nucleic acid (PNA) backbone with a fluorescent label. Evaluation of 115 growth-positive blood cultures showed that FISH with oligonucleotide probes is a sensitive method for identification of microorganisms in blood cultures (10). In this study 95% of microorganisms were identified at the family level, and 78% and 66% were identified at the genus and species level, respectively. The sensitivity and specificity of individual probes included in this study were 100%. Similar figures were found in another study, except the *Staphylococcus aureus* probe had a much lower sensitivity (67%) (9). PNA probes have been used in several studies for the identification of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans* in blood cultures. The sensitivities

TABLE 1. Tandem probes used for identification of microorganisms in blood cultures

Probe	FITC label[a]	Cy3 label
Gram-positive probes		
1	*Staphylococcus* genus	*Staphylococcus aureus*
2	*Streptococcus pyogenes*	*Streptococcus agalactiae*
3	*Streptococcus* genus	*Enterococcus* genus
4	*Enterococcus faecalis*	*Enterococcus faecium*
5	*Clostridium difficile*	*Streptococcus pneumoniae*
6	Eubacteria	*Enterobacteriaceae*
Gram-negative probes		
1	*Escherichia coli*/*Shigella* spp.	*Haemophilus influenzae*
2	*Stenotrophomonas maltophilia*	*Klebsiella pneumoniae*
3	*Bacteroides* spp./*Prevotella* spp.	*Pseudomonas aeruginosa*
4	Eubacteria	*Enterobacteriaceae*
Yeast probes		
1	*Candida krusei*	*Candida albicans*
2	*Candida dubliniensis*	*Candida tropicalis*
3	*Candida glabrata*	*Candida parapsilosis*
4	Eubacteria	Pan yeast

[a] FITC, fluorescein isothiocyanate.

and specificities of these probes were all more than 98% (4, 16, 17, 19, 21). Although the sensitivity and specificity of individual probes (oligonucleotide and PNA) are very good, the usefulness of FISH as a diagnostic test depends on the probes included in the assay and is related to the prevalence of microorganisms in a specific setting.

The theoretical turnaround time of the FISH procedure is 2.5 to 3 h, depending on the Gram stain characteristics of the microorganisms involved. However, the time to identification in the routine daily practice of a microbiology laboratory is not known. In our laboratory, growth-positive blood culture samples are subcultured on solid media directly after Gram staining to try to obtain a same-day "provisional identification." This identification provides a clue to the causative microorganism and, in combination with Gram stain characteristics and clinical presentation, may lead to adjustment of antimicrobial treatment. Provisional identification is always performed in parallel to test for a final identification and antibiotic susceptibility determination, both of which results are usually available the next morning. It is not known whether in this setting FISH is of additional value for routine culture-based identification for a faster diagnosis.

In the present study we evaluated the performance and the times to identification of microorganisms in growth-positive blood cultures by routine implementation of FISH with oligonucleotide probes compared to those of conventional culture methods.

MATERIALS AND METHODS

Study setup. Two hundred consecutive growth-positive blood cultures (BACTEC Aerobic/F, Anaerobic/F, and Peds/F; Becton Dickinson) were included in this study. For practical purposes, only bottles in which growth was detected by the automated culture machine between 8.30 a.m. and 2 p.m. during week days were included in the study. Only the first growth-positive blood culture from a single patient was included, except when the Gram stain characteristics of the microorganisms observed in subsequent positive blood cultures were different from the first one. Upon detection of growth by the automated blood culture machine (BACTEC 9240; Becton Dickinson), samples from the blood culture fluid were Gram stained. Three aliquots were taken: one was taken for performance of the appropriate subcultures for identification and antibiotic susceptibility testing, a second one was taken for FISH identification, and a third aliquot was stored at −20°C for sequence analysis of the microorganism in case of discrepant results between culture identification and FISH.

Conventional microbiological identification. Final (standard) identification of gram-positive and gram-negative bacteria and yeasts was performed with routine microbiological techniques, including biochemical characterization, agglutination tests, and automated identification of gram-negative microorganisms (VITEK 2; BioMérieux).

An additional subculture was performed to obtain a "provisional identification," which, in combination with the Gram stain result, aims to provide a same-day clue for the identity of the causative microorganism. For this purpose, subcultures of blood culture fluid were performed on blood agar and incubated at 37°C in a CO_2 incubator. If microbial growth was observed on the blood agar at the end of the day, the following routine microbiological tests were performed: a test for coagulase for differentiation of *Staphylococcus aureus* from coagulase-negative staphylococci when staphylococci were observed in the Gram stain, Lancefield grouping when beta-hemolysis was observed and streptococci were present in the Gram stain, and cytochrome oxidase to differentiate *Pseudomonas* species from other gram-negative rods in case of a Gram stain with gram-negative rods. Furthermore, subcultures were performed on blood agar with an optochine disk and in a bile esculin tube if the microorganisms observed in the Gram stain were suggestive of *Streptococcus pneumoniae* or *Enterococcus* species. These tests for provisional identification were considered only suggestive of certain microorganisms and were always confirmed by final identification.

Fluorescence in situ hybridization. At the time that subcultures were performed, 5 ml of blood culture fluid was drawn for FISH. These samples were centrifuged at 1,200 rpm (~450 g) for 1 min. The supernatant fraction was inoculated on a glass slide with eight square fields; depending on the Gram stain, either six fields were inoculated for gram-positive bacteria or four fields were inoculated for gram-negative microorganisms or yeasts. The commercial SeaFAST sepsis kit (SeaPro Theranostics International B.V., Lelystad, The Netherlands) was used for FISH according to the manufacturer's protocol. Briefly, after fixation and an extra permeabilization step with lysosyme/lysostaphine in the case of a gram-positive microorganism, cells were permeabilized with three consecutive baths of 50%, 80%, and 96% ethanol each for 3 min. Oligonucleotide probes (Table 1) were applied and slides were incubated in a hybridization chamber in a water bath at 48°C for 90 min. After hybridization, unbound probe was washed off. The slides were dried and read by fluorescence microscopy (Olympus BX40; magnification, ×100) with a fluorescein isothiocyanate (absorption wavelength, 494 nm; emission wavelength, 518 nm) and Cy3 (absorption wavelength, 552 nm; emission wavelength, 570 nm) filter. The oligonucleotide probes are complementary to portions of the 16S or 23S rRNA of the specific bacteria. Bacteria were identified as coagulase-negative staphylococci if fluorescent bacteria were seen with the *Staphylococcus* genus probe and no fluorescence was observed with the *Staphylococcus aureus* probe. A mixed infec-

tion was identified if fluorescence of the *Staphylococcus aureus* probe was observed for only a proportion of bacteria with fluorescence of the *Staphylococcus* genus probe on the same field. Probes did not differentiate between *Escherichia coli* and *Shigella* spp. or between *Bacteroides* spp. and *Prevotella* spp. because of the very high homology of the rRNA sequences of these organisms.

Sequence analysis. Sequence analysis was performed on DNA of microorganisms isolated from blood culture fluids with discrepant results from culture and FISH. DNA was isolated from the stored aliquot of blood culture fluid with QIAGEN columns (QIAamp mini kit; QIAGEN), followed by PCR with 16S universal primers (466 base pairs) (5). Sequence determination was done with the BigDye Terminator cycle sequencing kit (Applied Biosystems) on the ABI Prism 3100 genetic analyzer (Applied Biosystems) by following the manufacturer's instructions.

Statistical analysis. Data for outcome and points in time were collected for Gram staining and conventional culture identification (provisional and final) by a routine laboratory technician, and results and points in time of FISH were collected by the technician who performed the FISH technique. Data were analyzed by descriptive statistics, and comparison of times to identification was performed with SPSS 10.0.

RESULTS

Two hundred blood cultures that were signaled as growth positive by the blood culture machine were included in this study. In 11 cases, no microorganisms were detected by Gram stain or culture or identified by FISH. From the remaining 189 blood cultures, a total of 200 microorganisms were isolated; two different microbial species were present in 11 blood cultures. FISH with eubacterial and pan-yeast control probes was positive in 100% of samples with bacterial ($n = 194$) and fungal (6) growth, respectively, whereas no fluorescence was observed when such growth was absent. FISH identification of the genera of gram-positive microorganisms or a family of gram-negative microorganisms was obtained for over 90% (91%; 95% confidence interval [CI], 89 to 93%) of isolates (Table 2). Genus or species name could be attributed to nearly 84% (95% CI, 81 to 87%) and 79% (95% CI, 76 to 82%) of all isolates, respectively. With FISH the presence of two pathogens was recognized in all 11 cases of mixed infection, including one blood culture with *Staphylococcus aureus* and coagulase-negative staphylococci.

In 5/162 cases (3%), identification with the species-specific probes included in the assay was not optimal: in two isolates of *Staphylococcus aureus*, no FISH identification was obtained, and in one case, *Staphylococcus aureus* was misidentified as coagulase-negative *Staphylococcus*. No fluorescence was observed with the species-specific probe for two isolates of *Escherichia coli*. Thus, the sensitivity of the available species-specific probes was 97% (95% CI, 96 to 98%) (Table 3). The specificity of the species-specific probes was 95% (95% CI, 91 to 98%); one isolate was identified by FISH as *Haemophilus influenzae*, while by conventional techniques, identification of the isolate was not compatible with *Haemophilus influenzae* but was given the description nonfermenting gram-negative rods. Sequence analysis of the isolate revealed *Roseomonas* species. Finally, fluorescence was observed with the *Klebsiella pneumoniae* probe for one isolate of *Klebsiella oxytoca*. The fluorescence in this sample was weaker than expected from our experience with the *Klebsiella pneumoniae* probe but sufficiently evident relative to background fluorescence to be considered a positive result. Sequence analysis of this strain confirmed the conventional identification as *Klebsiella oxytoca*. In two isolates, no fluorescence was observed for *Staphylococcus aureus*, as mentioned before. Thus, the sensitivity of the genus-specific probes was 98% (95% CI, 95 to 99.5%) and the specificity 100%. The sensitivity and specificity of the *Enterobacteriaceae* probe were 89% (95% CI, 84 to 94%) and 100%, respectively, because no fluorescence was obtained in four cases of *Proteus mirabilis* and one case of *Morganella morganii*.

TABLE 2. Identification with FISH of gram-positive and gram-negative microorganisms in growth-positive blood cultures

Microorganism	No. of samples positive by culture	No. of samples positive by FISH[c]	
		Genus or family	Genus or species
Gram-positive microorganisms			
Coagulase-negative staphylococci	94	94	94
Staphylococcus aureus	20	18	17
Streptococcus pneumoniae	2	2	2
Streptococcus pyogenes	1	1	1
Other streptococci[a]	9	9	
Enterococcus faecalis	2	2	2
Enterococcus faecium	2	2	2
Other enterococci	1	1	
Candida albicans	5	5	5
Candida tropicalis	1	1	1
Other[b]	6		
Total	143	135	124
Gram-negative microorganisms			
Escherichia coli	23	23	21
Klebsiella pneumoniae	6	6	6
Enterobacter cloacae	6	6	
Proteus mirabilis	4		
Nontyphoid salmonellae	3	3	
Other *Enterobacteriaceae*[d]	3	2	
Pseudomonas aeruginosa	6		6
Other[e]	6		
Total	57	46	33

[a] These include *Streptococcus mitis* ($n = 3$), *Streptococcus oralis* ($n = 2$), hemolytic *Streptococcus* group C ($n = 2$), hemolytic *Streptococcus* group G, and *Streptococcus sanguis*.

[b] This group consisted of diphtheroid rods ($n = 2$), *Propionibacterium acnes* ($n = 2$), *Corynebacterium* species, and *Bacillus cereus*.

[c] Values for gram-negative microorganisms include identification of the family with the *Enterobacteriaceae* probe and identifications of the genus or species. For gram-positive microorganisms, identifications are made only for genus and species.

[d] Other *Enterobacteriaceae* include *Serratia marcescens*, *Klebsiella oxytoca*, and *Morganella morganii*.

[e] These include *Acinetobacter anitratus* ($n = 2$), nonfermenting gram-negative rods ($n = 2$), *Fusobacterium nucleatum*, and *Aeromonas caviae*.

TABLE 3. Sensitivities and specificities of probes for identification of microorganisms in blood cultures[a]

Probes	Sensitivity	Specificity
Species specific	157/162 (97)	36/38 (95)
Genus specific	129/131 (98)	69/69 (100)
Enterobacteriaceae	40/45 (89)	155/155 (100)
Eubacterial control	194/194 (100)	6/6 (100)
Pan-yeast control	6/6 (100)	194/194 (100)

[a] Values are numbers of positive samples/numbers of samples tested (percentages).

TABLE 4. Times to identification of FISH compared to those of culture identification[a]

Microorganism	FISH TTI (h)	Provisional identification		Final identification	
		TTI (h)	Time gain (h)[b]	TTI (h)	Time gain (h)
Staphylococci	4.1	5.5	1.4 ± 1.2	22.9	18.8 ± 1.6
Streptococci	4.5	5.8	1.3 ± 1.1	23.1	18.6 ± 1.3
Enterococci	4.7	7.1	2.4 ± 1.3	23.1	18.4 ± 1.7
Enterobacteriaceae	3.8			23.1	19.3 ± 1.5
Pseudomonas aeruginosa	3.6	5.7	2.1 ± 0.9	23.7	20.1 ± 1.1
Yeasts	4.9			47.0	42.1 ± 1.4

[a] TTI, mean time to identification.
[b] Difference in times to identification in hours (means ± standard deviations) between FISH and provisional culture.

The turnaround time of FISH in routine practice was 3 h 45 min (range, 2.3 to 5.7 h) for gram-negative and 4 h 12 min (range, 2.8 to 5.6 h) for gram-positive microorganisms. Turnaround time data from final and provisional identifications were recorded for 178 (89%) samples. Provisional identification was made with 167 samples (94%); 11 blood cultures were growth positive after 12 a.m., and no growth was observed in subcultures the same day. Provisional identification was obtained for 107 blood cultures with staphylococci ($n = 81$), streptococci ($n = 16$), enterococci ($n = 4$), and *Pseudomonas aeruginosa* ($n = 6$). In the other 60 cases, provisional culture identification was not possible, because the specific tests for these microorganisms, such as for *Escherichia coli*, were not included.

The time gain by FISH compared to final conventional identification of microorganisms in blood cultures was quite substantial—more than 18 h (Table 4). For yeasts, the time gain to final identification was more than 42 h ($P < 0.001$). By FISH, *Staphylococcus aureus* could be differentiated from coagulase-negative staphylococci significantly faster than a provisional identification could be obtained ($P < 0.001$). FISH provided identification more than 2 h faster than provisional culture identification of *Pseudomonas aeruginosa*.

DISCUSSION

Blood cultures have been performed for over a century, but little progress has been made in conventional culture-based methods to reduce the time to diagnosis (18). Our evaluation of the performance of FISH for the identification of microorganisms in blood cultures and the time gain to direct identification showed that FISH is a valuable addition to the identification techniques available to the clinical microbiologist. In our study, for more than 90% of samples, we identified the genus name of gram-positive microorganisms or family name of gram-negative microorganisms, and for 79%, the species name was attributed to the microorganism. The number of microorganisms identified by the assay could be further increased by inclusion of probes for viridans streptococci, *Enterobacter cloacae*, and *Proteus mirabilis*. If these probes had been included, species identification of an additional 16 microorganisms (8%) would have been achieved. On the other hand, probes for *Stenotrophomonas maltophilia* and *Clostridium difficile*, which are relatively uncommon in blood cultures, are included in the assay. The spectrum of isolates encountered in a laboratory of microbiology is of relevance to the probes that should be included in the FISH assay. Factors of influence are a hospital in-patient population, the presence of a hemato-oncology ward, reference functions for general practitioners, and the number of beds in the intensive care unit. As more probes become available, a setting-specific design of the FISH assay will be possible.

The sensitivity and specificity of individual probes are good. In a few samples, the presence of large amounts of protein and blood cells hampered the interpretation of slides. The background fluorescence in these slides was too strong to be able to distinguish fluorescent bacteria from proteins and blood cells. Therefore, no clinically relevant conclusions could be drawn with FISH for these samples. This was the case for 4 out of 200 slides. We did not find a common denominator for these samples, such as abnormally high levels of C-reactive protein, extremely high leukocyte count, or administration of parenteral nutrition. In one instance, *Staphylococcus aureus* was incorrectly identified as a coagulase-negative staphylococcus. This blood culture was obtained from a child with osteomyelitis who recovered well with flucloxacillin and who had also *Staphylococcus aureus* cultured from two other blood culture bottles. This is a major error, since misidentification of the species of staphylococci may greatly influence the decision to start optimal antimicrobial treatment. Probe specificity was not optimal for the *Klebsiella pneumoniae* probe, which is due to cross-hybridization because of high 23S rRNA homology between *Klebsiella pneumoniae* and *Klebsiella oxytoca*. In the other case of a false-positive FISH result, the possibility of a mixed infection should be considered. This blood culture was from a 1-month-old child with respiratory failure who had *Haemophilus influenzae* and *Moraxella catarrhalis* cultured from his sputum. Fluorescent bacteria were observed with the *Haemophilus influenzae* probe in FISH, but *Roseomonas* species were identified by sequence analysis of the microorganisms cultured. *Roseomonas* species are a known cause of bacteremia, although uncommon (2, 20). *Haemophilus influenzae* and *Roseomonas* species are not closely related, and the *Haemophilus influenzae* probe is not complementary to the *Roseomonas* species 16S rRNA. Therefore, the presence of a mixed infection rather than cross-hybridization of the probe may be the most likely explanation of the FISH result in this blood culture. The *Enterobacteriaceae* probe failed to hybridize in four cases of *Proteus mirabilis* and one case of *Morganella morganii*. This probe is not complementary to the 16S rRNA of both species, as previously described for *Proteus* species (10). This should be considered when thinking of changes in antimicrobial treat-

ment based on the presence or absence of fluorescence with the *Enterobacteriaceae* probe.

FISH provides a same-day identification of the majority of microorganisms in blood cultures, and the turnaround time is considerably faster than microbiological culture. This can allow a more rapid streamlining of antimicrobial therapy, which potentially reduces mortality and length of hospitalization (1, 3, 11). An important example is the time gain to microbiological diagnosis by FISH in cases of blood cultures with yeasts. Although the number of cases with candidemia was small in this study, the time gain is very valuable in this serious condition of the frequently immunocompromised host, because FISH identification allows same-day adjustment of antifungal treatment. However, in our setting, the clinical value of identification provided by FISH seems limited in those blood cultures for which a provisional identification can be obtained. For example, *Staphylococcus aureus* bacteremia is distinguished from bacteremia caused by coagulase-negative staphylococci only 1 h faster with FISH than by provisional identification. The influence on antimicrobial management of FISH seems therefore not substantially different from the impact of the somewhat slower provisional identification in these cases. However, a provisional identification can frequently not be obtained, because blood cultures are growth positive in the afternoon or microorganisms for which no provisional identification is possible, such as *Enterobacteriaceae*, are involved. In these cases, the results of same-day identification with FISH are very useful.

FISH identification requires more than 4 hours after Gram staining before results are available. Most therapy interventions with regard to bloodstream infections are made at the time of phlebotomy and on the basis of notification of Gram stain characteristics rather than on the basis of microbiological identification or susceptibility determination (7, 15). It is questionable whether clinicians will routinely wait 4 hours until FISH results are available before a start or change of antibiotics. A further reduction in the time to diagnosis of microorganisms in blood cultures by FISH therefore seems required to extend the potential influence on clinical management of FISH identification.

In conclusion, FISH is a rapid and reliable technique for the identification of the vast majority of microorganisms in growth-positive blood cultures and provides faster identification than conventional culture. The applicability of FISH in routine practice is dependent on the probes included in the assay, the epidemiology of microorganisms isolated from blood cultures in an individual setting, and the local routine of provisional identification. A decrease in the turnaround time to less than 1 h would extend the potential use of FISH considerably. With the reduction in the turnaround time of the FISH procedure and extension of the panel of probes, FISH will become a very useful diagnostic tool in the diagnosis of bloodstream infections.

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None of the authors have financial interests to declare.

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