

Evaluation of FISH for Blood Cultures under Diagnostic Real-Life Conditions

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Background: The study assessed a spectrum of previously published in-house fluorescence in-situ hybridization (FISH) probes in a combined approach regarding their diagnostic performance with incubated blood culture materials.

Methods: Within a two-year interval, positive blood culture materials were assessed with Gram and FISH staining. Previously described and new FISH probes were combined to panels for Gram-positive cocci in grape-like clusters and in chains, as well as for Gram-negative rod-shaped bacteria. Covered pathogens comprised *Staphylococcus* spp., such as *S. aureus*, *Micrococcus* spp., *Enterococcus* spp., including *E. faecium*, *E. faecalis*, and *E. gallinarum*, *Streptococcus* spp., like *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae*, *Enterobacteriaceae*, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella* spp., *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Bacteroides* spp.

Results: A total of 955 blood culture materials were assessed with FISH. In 21 (2.2%) instances, FISH reaction led to non-interpretable results. With few exemptions, the tested FISH probes showed acceptable test characteristics even in the routine setting, with a sensitivity ranging from 28.6% (*Bacteroides* spp.) to 100% (6 probes) and a specificity of >95% in all instances.

Conclusion: If sophisticated rapid diagnostic methods like mass spectrometry from blood culture materials are not available, FISH provides an option for rapid differentiation for laboratories in resource-limited settings.

Keywords: fluorescence in-situ hybridization, blood culture, rapid diagnostics, molecular diagnostics, sepsis

Introduction

Fluorescence in-situ hybridization (FISH) is a diagnostic technique which allows the identification of pathogens on species or genus level by the binding of short fluorescence-tagged pathogen-specific DNA probes to ribosomal RNA of microorganisms. Subsequently, analysis is performed under a fluorescence microscope. Use of multi-well slides allows the application of whole panels of FISH probes with the same specimen in a parallel approach [1]. This procedure, the so-called multi-probe concept, allows the exclusion of auto-fluorescence and non-specific binding by assessing fluorescence intensity of probes, which should not lead to positive results. If positive signals are provided by different FISH probes leading to contradicting results, the overall result is “non-interpretable” but at least not “false positive” in this way.

Nearly 20 years ago, FISH was identified as an easy and rapid procedure to provide additional information in addition to Gram staining from positive blood culture materials. As early as at the beginning of the new millennium, small and in part poorly evaluated probe panels for the identification of blood culture pathogens were published [2], but insufficient standardization limited the implementation of the technique for the diagnostic routine setting [3].

Due to the experience that various previously published probes did not show acceptable performance characteristics in our hands, we started to develop new probes and to publish them individually [4–7]. Accordingly, some of the data presented here have partially been published in a pathogen-specific way. Now, the entire data-set is reevaluated in a concluding synopsis.

Due to the continuous evaluation process, the data are incoherent. This means that the probe sets were adjusted in the course of the study, if binding characteristics of individual probes were considered as inappropriate. In the here described assessment, only the results for the probes and screening algorithms, which were proven to be useful in our hands and could thus be used in the course of the whole evaluation, were described. Due to the stepwise, Gram-staining-dependent, species-specific evaluation approaches, the here described FISH assessments strongly overlap with previous reports [4–7]. However, the samples are not identical, and therefore, the results are not equal.

In the meantime, MALDI-TOF-MS (matrix assisted laser desorption ionization–time of flight–mass spectrometry) is the predominantly applied method for the early identification of pathogen species from incubated blood culture materials [8, 9]. Nevertheless, cost-efficient FISH could be an option for resource-limited settings, where expensive MALDI-TOF-MS equipment is not affordable, e.g., in non-industrialized tropical areas [1, 7]. However, this requires availability of a fluorescence microscope and of well-trained laboratory personnel, so manpower can compensate a lack of sophisticated technology.

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In the here described study, the diagnostic performance of predominantly published FISH probes for blood culture diagnostics was assessed during a two-year interval under real-life-like conditions in a diagnostic routine laboratory of a German university hospital.

Materials and Methods

Study Setting. During a two-year interval, positive blood cultures were assessed by FISH in addition to Gram staining, as soon as they were detected positive by automated blood culture. The FISH results were compared with the definite diagnostic results after cultural growth and biochemical identification via VITEK or API (bioMérieux, Marcy-l'Étoile, France). The study was performed at the Institute for Medical Microbiology of the University Hospital of Ulm.

Inclusion and Exclusion Criteria. Positive blood culture materials were included in the FISH assessment if they showed either Gram-positive cocci in grape-like clusters or Gram-positive cocci in doubles or chains or Gram-negative rod-shaped bacteria. Positive blood culture materials with other morphology of microorganisms in Gram-stain and negative blood culture materials were excluded. There was no exclusion of copy strains, and both aerobic and anaerobic cultures from the same patients were considered as distinct samples. However, materials were excluded from further assessment if they led to non-interpretable FISH results due to multiple positive FISH signals in spite of only one type of observed morphology in Gram-stain as it may occur due to autofluorescence phenomena or in the case of failed reactions of the internal control FISH. In such instances, FISH cannot be interpreted [1]. Applying these exclusion criteria, 21 samples had to be excluded as summarized in Table 1.

FISH Procedure. Depending on the results of Gram-staining of positively tested blood culture materials, different panels of FISH probes were applied, as shown in Table 2. Specific probes were labelled with the red fluorescence dye Cy3 (cyanine), while the pan-eubacterial probe, which was used as the internal reaction control, was labelled with the green fluorescence dye FAM (carboxyfluorescein). Competitor probes are non-labelled probes which are added to block non-specific probe binding to phylogenetically closely related organisms. For enterococci and *Salmonella* spp., 2 distinct FISH probes and, in the latter case, also 2 distinct probe-competitor-probe-combinations were available (Table 2).

FISH was performed as described [1]. In short, slides with blood culture material were fixed with 100% methanol and

Table 1. Excluded strains due to non-interpretable results, i.e., multiple positive signals in the multi-probe approach or failed reactions in the internal control FISH

21 excluded strains	<i>Acinetobacter lwoffii</i> (n = 1)
	<i>Brevundimonas diminuta</i> (n = 1)
	<i>Comamonas acidovorans</i> (n = 1)
	<i>Enterococcus faecium</i> (n = 1)
	<i>Escherichia coli</i> (n = 1)
	<i>Flavimonas oryzihabitans</i> (n = 1)
	<i>Gemella haemolysans</i> (n = 2)
	<i>Koccuria</i> spp. (n = 1)
	<i>Micrococcus lylae</i> (n = 1)
	<i>Moraxella catarrhalis</i> (n = 2)
	<i>Moraxella osloensis</i> (n = 1)
	not further differentiated <i>Gemella</i> spp. (n = 1)
	not further differentiated Gram-negative rod-shaped bacteria (n = 2)
	not further differentiated <i>Pseudomonas</i> sp. (n = 1)
	<i>Pseudomonas aeruginosa</i> (n = 1)
	<i>Staphylococcus epidermidis</i> (n = 1)
	<i>Stomatococcus mucilaginosus</i> (n = 1)
	<i>Streptococcus mitis</i> (n = 1)

air-dried. If Gram-positive cocci were seen, permeabilization was performed for 5 min with a lysis buffer containing 1 mg/mL lysozyme and 2 µg/mL lysostaphine in 10 mM Tris HCl at 46 °C. No lysis was necessary for Gram-negative organisms. All probes were designed to work with 30% formamide in the hybridization buffer containing 0.9 M NaCl, 20 mM Tris HCl, and 0.01% sodium dodecyl sulphate (SDS). Hybridization time was 60 min, followed by washing for about 15 min with a washing buffer containing 0.102 M NaCl, 5 mM EDTA, 20 mM Tris HCl, and 0.01% SDS. Prior to assessment on a fluorescence microscope, counterstaining of the bacterial DNA was performed with 4',6-diamidin-2-phenylindol (DAPI).

In Silico Evaluation of the FISH Probes. In silico evaluation of all tested FISH probes was performed using the software probecheck (<http://131.130.66.200/cgi-bin/probecheck/content.pl?id=home>). The results are shown in Supplementary material 1.

Statistical Assessment. Descriptive assessment was performed to calculate sensitivity, specificity, positive predictive value, and negative predictive value using the software Microsoft Excel®.

Ethical Standards. Not applicable because no patient data or primary sample materials were used.

Results

Assessed Blood Culture Materials and Identification

Results by the Reference Methods. Altogether, a total of 733 pathogens from positive aerobic blood culture materials and 222 pathogens from positive anaerobic blood culture materials were included in the study during the two-year-assessment period. They comprised 33 materials with mixed bacterial cultures comprising 29 materials with double infections and 4 materials with triple infections.

Altogether, 955 Gram-positive cocci in grape-like clusters, doubles, or chains, as well as Gram-negative, rod-shaped bacteria were identified. As stated above, 21 out of 955 (2.2%) samples had to be excluded, applying the exclusion criteria. These excluded strains comprised *Acinetobacter lwoffii* (n = 1), *Brevundimonas diminuta* (n = 1), *Comamonas acidovorans* (n = 1), *Enterococcus faecium* (n = 1), *Escherichia coli* (n = 1), *Flavimonas oryzihabitans* (n = 1), *Gemella haemolysans* (n = 2), *Koccuria* spp. (n = 1), *Micrococcus lylae* (n = 1), *Moraxella catarrhalis* (n = 2), *M. osloensis* (n = 1), not further differentiated *Gemella* spp. (n = 1), not further differentiated Gram-negative rod-shaped bacteria (n = 2), not further differentiated *Pseudomonas* spp. (n = 1), *P. aeruginosa* (n = 1), *Staphylococcus epidermidis* (n = 1), *Stomatococcus mucilaginosus* (n = 1), and *Streptococcus mitis* (n = 1). The remaining 934 pathogens that were included into further assessment comprised 534 Gram-positive cocci in grape-like clusters, i.e., *Staphylococcus aureus* (n = 84), *S. auricularis* (n = 1), *S. capitis* (n = 1), *S. caprae* (n = 1), *S. cohnii* (n = 1), *S. epidermidis* (n = 356), *S. haemolyticus* (n = 10), *S. hominis* (n = 9), *S. saccharolyticus* (n = 1), *S. simulans* (n = 3), *Micrococcus luteus* (n = 10), and not further differentiated coagulase negative staphylococci (n = 57). Further, 148 Gram-positive cocci in doubles or chains were observed, comprising *Enterococcus faecalis* (n = 38), *E. faecium* (n = 41), *E. gallinarum* (n = 3), *Lactococcus lactis* (n = 3), not further differentiated alpha-hemolytic *Streptococcus* spp. (n = 2), *S. agalactiae* (n = 5), *S. anginosus* (n = 6), *S. dysgalactiae* (n = 3), *S. dysgalactiae* subsp. *equisimilis* (n = 1), *S. mitis* (n = 18), *S. oralis* (n = 4), *S. pneumoniae* (n = 16), *S. pyogenes* (n = 3), *S. salivarius* (n = 1), and *S. sanguis* (n = 4). Further, 252 Gram-negative rod-shaped bacteria were identified as *Acinetobacter baumannii* (n = 1), *A. lwoffii* (n = 2), *Bacteroides fragilis* (n = 4), *B. thetaiotaomicron* (n = 1), *Citrobacter freundii* (n = 4), *C. koseri* (n = 2), *Enterobacter*

Table 2. Applied FISH probe panels for blood culture diagnostics

Target organism	Probe sequence	Reference
Pan-eubacterial probe combined with all panels		
All bacteria	5'-GCT-GCC-TCC-CGT-TAG-GAG-T-3'	[27]
Panel for Gram-positive cocci in grape-like clusters		
<i>Staphylococcus aureus</i>	5'-GAA-GCA-AGC-TTC-TCG-TCC-G-3'	[3]
<i>Staphylococcus</i> spp.	5'-TCC-TCC-ATA-TCT-CTG-CGC-3'	[3]
<i>Micrococcus</i> spp.	5'-GTA-TCT-CTA-CGG-CGA-TCG-3'	This study
Panel for Gram-positive cocci in doubles or chains		
<i>Enterococcus faecalis</i>	5'-GAA-AGC-GCC-TTT-CAC-TCT-TAT-GC-3'	[4]
<i>Enterococcus faecium</i>	5'-TTC-ACA-CAA-TCG-TAA-CAT-CCT-A-3'	[4]
<i>Enterococcus gallinarum</i>	5'-ATT-CAC-AAC-TGT-GTA-ACA-TCC-TAT-3'	[4]
<i>Enterococcus</i> spp.	5'-CAC-CGC-GGG-TCC-ATC-CAT-CA-3' and 5'-CAG-TTC-TCT-GCG-TCT-ACC-TC-3'	[4]
<i>Streptococcus agalactiae</i>	5'-GTA-AAC-ACC-AAA-CMT-CAG-CG-3'	[28]
<i>Streptococcus pneumoniae</i>	5'-GTG-ATG-CAA-GTG-CAC-CTT-3' in combination with the competitor probe 5'-GTG-ATG-CAA-TTG-CAC-CTT-3'	[3]
<i>Streptococcus pyogenes</i>	5'-CTA-ACA-TGC-GTT-AGT-CTC-TC-3'	This study
<i>Streptococcus</i> spp.	5'-GTT-AGC-CGT-CCC-TTT-CTG-G-3'	[29]
Panel for Gram-negative rod-shaped bacteria		
<i>Acinetobacter</i> spp.	5'-TTA-GGC-CAG-ATG-GCT-GCC-3'	[6]
<i>Bacteroides</i> spp.	5'-CAT-CCT-TCA-CGC-TAC-TTG-GCT-GG-3' in combination with the two competitor probes 5'-TCC-TTC-ACG-CGA-CTT-GGC-TGG-TT-3' and 5'-TCC-TGC-ACG-CTA-CTT-GGC-TGG-T-3'	This study
<i>Bacteroides</i> spp. / <i>Prevotella</i> spp.	5'-CAT-CCT-TCA-CGC-TAC-TTG-GCT-GG-3' in combination with the competitor probe 5'-TCC-TTC-ACG-CGA-CTT-GGC-TGG-TT-3'	This study
Enterobacteriaceae	5'-CCC-CCW-CTT-TGG-TCT-TGC-3'	[3]
<i>Escherichia coli</i>	5'-GCG-GGT-AAC-GTC-AAT-GAG-CAA-AGG-3'	This study
<i>Klebsiella pneumoniae</i>	5'-CCT-ACA-CAC-CAG-CGT-GCC-3'	[3]
<i>Pseudomonas aeruginosa</i>	5'-CCA-CTT-TCT-CCC-TCA-GGA-CG-3'	This study
<i>Salmonella</i> spp.	5'-TGC-GCT-TTT-GTG-TAC-GGG-GCT-3' in combination with the competitor probe 5'-GTG-CAT-TTT-TGT-GTA-CGG-GGC-3' and 5'-CTT-CAC-CTA-CGT-GTC-AGC-G-3' in combination with the competitor probe 5'-TCA-CCT-ACA-TAT-CAG-CGT-GC-3'	[7]
<i>Stenotrophomonas maltophilia</i>	5'-GTC-GTC-CAG-TAT-CCA-CTG-C-3'	[26]

aerogenes (*Klebsiella aerogenes*) ($n = 8$), *E. amnigenus* ($n = 1$), *E. cloacae* ($n = 12$), *E. hormaechei* ($n = 1$), *Escherichia coli* ($n = 126$), *Klebsiella oxytoca* ($n = 7$), *K. pneumoniae* ($n = 27$), *Morganella morganii* ($n = 1$), not further differentiated *Acinetobacter* spp. ($n = 6$), not further differentiated *Bacteroides* spp. ($n = 2$), not further differentiated *Citrobacter* spp. ($n = 1$), not further differentiated *Pantoea* spp. ($n = 1$), *P. agglomerans* ($n = 1$), *Pseudomonas aeruginosa* ($n = 30$), *Salmonella* ser Bredeney ($n = 1$), *S. ser* Enteritidis ($n = 3$), *S. ser* Typhi ($n = 2$), *Serratia liquefaciens* ($n = 1$), *S. marcescens* ($n = 4$), and *Stenotrophomonas maltophilia* ($n = 3$) (Table 3).

In-silico Evaluation and Diagnostic Performance of the Assessed FISH Probes. Details of the in-silico evaluation of the assessed FISH probes are presented in the supplementary material (Supplementary material 1) and summarized in Table 4. As indicated in Table 4, potential cross-binding was considered as likely in the case of mismatches up to 2 bases. In-silico matching with the target organisms was 26.9–100% for the 0-mismatch range, 0–100% for the 1-base-mismatch range and 0–99.9% for the 2-bases-mismatch range (Table 4). Of note, even probes that showed good binding characteristics in the later in-vitro evaluation like the probe for *S. aureus* (Table 5) were associated with only moderate matching with deposited sequences in the in-silico evaluation (Table 4).

The in-vitro performance characteristics of the assessed FISH probes are detailed in Table 5. While the specificity of all described FISH approaches was better than 95%, sensitivity ranged from 28.6% for *Bacteroides* spp. to 100% for *Micrococcus* spp., *E. gallinarum*, *S. pyogenes*, *Acinetobacter* spp., *Klebsiella pneumoniae*, and *Salmonella* spp., respectively. Low percentage values, however, were influenced by disproportional high effects of single missed bindings in the case of species which were very rarely identified, like *S. agalactiae* (Table 5).

The details on lacking or incorrect binding of FISH probes are provided in Table 6. In detail, the *S. aureus* probe failed to bind to 3 *S. aureus* isolates. Of note, the *Staphylococcus* spp. probe was once erroneously used with Gram-positive cocci in

chains and showed cross-binding with a *S. mitis* isolate. This erroneous use is not shown in Table 5. The probe altogether failed in the cases of 2 *S. aureus*, 3 *S. epidermidis*, and 4 not further identified *Staphylococcus* spp. The *E. faecalis* probe missed 2 *E. faecalis* isolates, and the *E. faecium* probe missed 8 *E. faecium* isolates. In contrast, the *E. gallinarum* probe showed cross-binding with 2 *E. faecium* isolates. The *Enterococcus* spp. probes failed to identify 3 *Enterococcus* spp. strains, i.e., 1 *E. faecalis* and 2 *E. faecium*. The *S. agalactiae* probe missed 1 *S. agalactiae* isolate, and the *S. pneumoniae* probe missed 2 *S. pneumoniae* isolates. The *S. pyogenes* probe showed cross-binding with 1 *S. anginosus* strain. The *Streptococcus* sp. probe missed 1 *S. mitis* but identified all 3 *Lactococcus lactis* isolates. Therefore, it has to be considered as a *Streptococcus* spp./*Lactococcus* spp. probe. The *Bacteroides* spp. probe missed 5 out of 7 *Bacteroides* spp., i.e., 3 *Bacteroides fragilis*, 1 *Bacteroides thetaiotaomicron*, and 1 not further identified *Bacteroides* sp. strain if used with both competitor probes against *Prevotella* spp. and *Porphyromonas* spp. If it was used as a *Bacteroides* spp./*Prevotella* spp. probe in conjunction with only the competitor probe against *Porphyromonas* spp., 3 out of 7 *Bacteroides* spp., i.e., 1 *Bacteroides fragilis* and 2 not further identified *Bacteroides* spp. were missed. The *Enterobacteriaceae* probe failed in 4 instances with 4 *E. coli*. The *Escherichia coli* probe missed 5 *E. coli* and showed cross-binding with 1 *Klebsiella oxytoca* isolate. The *Klebsiella pneumoniae* probe showed cross-binding with 1 *E. amnigenus* strain, 2 *Klebsiella oxytoca* strains, and 1 *Serratia marcescens*. The *P. aeruginosa* probe missed 3 *P. aeruginosa* isolates, and the *S. maltophilia* probe missed 1 out of 3 *S. maltophilia* in blood culture.

Discussion

The application of FISH for rapid blood culture diagnostics is not a new innovation. In parallel to MALDI-TOF-MS for rapid blood culture diagnostics, FISH was evaluated in various commercial and non-commercial approaches for this purpose.

Table 3. Distribution of species in samples which were included in the assessment

Groups	Species	Numbers of species	
534 Gram-positive cocci in grape-like clusters	<i>Staphylococcus aureus</i>	84	
	<i>Staphylococcus auricularis</i>	1	
	<i>Staphylococcus capitis</i>	1	
	<i>Staphylococcus caprae</i>	1	
	<i>Staphylococcus cohnii</i>	1	
	<i>Staphylococcus epidermidis</i>	356	
	<i>Staphylococcus haemolyticus</i>	10	
	<i>Staphylococcus hominis</i>	9	
	<i>Staphylococcus saccharolyticus</i>	1	
	<i>Staphylococcus simulans</i>	3	
	<i>Micrococcus luteus</i>	10	
	not further differentiated coagulase negative staphylococci	57	
	148 Gram-positive cocci in doubles or chains	<i>Enterococcus faecalis</i>	38
		<i>Enterococcus faecium</i>	41
		<i>Enterococcus gallinarum</i>	3
		<i>Lactococcus lactis</i>	3
		not further differentiated alpha-hemolytic <i>Streptococcus</i> spp.	2
		<i>Streptococcus agalactiae</i>	5
		<i>Streptococcus anginosus</i>	6
<i>Streptococcus dysgalactiae</i>		3	
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i>		1	
<i>Streptococcus mitis</i>		18	
<i>Streptococcus oralis</i>		4	
<i>Streptococcus pneumoniae</i>		16	
<i>Streptococcus pyogenes</i>		3	
<i>Streptococcus salivarius</i>		1	
<i>Streptococcus sanguis</i>		4	
252 Gram-negative rod-shaped bacteria		<i>Acinetobacter baumannii</i>	1
		<i>Acinetobacter lwoffii</i>	2
		<i>Bacteroides fragilis</i>	4
	<i>Bacteroides thetaiotaomicron</i>	1	
	<i>Enterobacter aerogenes</i>	8	
	<i>Enterobacter amnigenus</i>	1	
	<i>Enterobacter cloacae</i>	12	
	<i>Citrobacter freundii</i>	4	
	<i>Enterobacter hormaechei</i>	1	
	<i>Escherichia coli</i>	126	
	<i>Citrobacter koseri</i>	2	
	<i>Klebsiella pneumoniae</i>	27	
	<i>Klebsiella oxytoca</i>	7	
	<i>Morganella morganii</i>	1	
	not further differentiated <i>Acinetobacter</i> spp.	6	
	not further differentiated <i>Bacteroides</i> spp.	2	
	not further differentiated <i>Citrobacter</i> spp.	1	
	not further differentiated <i>Pantoea</i> spp.	1	
	<i>Pantoea agglomerans</i>	1	
	<i>Pseudomonas aeruginosa</i>	30	
	<i>Salmonella</i> ser Bredeney	1	
	<i>Salmonella</i> ser Enteritidis	3	
	<i>Salmonella</i> ser Typhi	2	
	<i>Serratia liquefaciens</i>	1	
	<i>Serratia marcescens</i>	4	
	<i>Stenotrophomonas maltophilia</i>	3	

Commercial applications comprise the use of patent-protected peptide-nucleic-acid (PNA)-FISH probes as developed and distributed by AdvanDx, Inc. (Vedbæk, Denmark) for the identification of pathogens in incubated blood culture materials. Respective PNA FISH approaches for the rapid diagnosis of blood culture pathogens comprised agents like *Acinetobacter* spp., *C. albicans*, *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. aureus* [10–14].

Molecular beacon DNA probes for FISH-based blood culture diagnostics in a commercial hemo-FISH kit (miacom diagnostics, Düsseldorf, Germany) for both Gram-positive and Gram-negative pathogens have been introduced as well [15],

next to various in-house approaches based on DNA FISH probes [5–7, 16–20].

For the presented study, in-silico evaluation of the probes was repeated with the updated database. The discrepancy, as exemplarily described for *S. aureus* in the Results section, between the observed in-vitro performance of the probes and the relatively poor in-silico matching is known from previous assessments. Even the current databases are not perfect, and older entries with wrong identifications are still included. Accordingly, in-vitro evaluations cannot be avoided.

This study assessed various published and few unpublished DNA FISH probes in a real-life-like observation at a German university hospital for their suitability for diagnostic FISH from blood culture materials. As detailed in the results, sensitivity and specificity in this real-life-like approach were slightly lower than suggested by previous evaluations under study conditions [16–20] including our own previous species-specific assessments [4–7]. Under the study conditions, cultured bacteria were directly tested by FISH in all contradictory cases, and in some cases, FISH was right in the end. In the here described assessment, such test repetition was not included, and the results were presented as obtained under routine-like conditions. Although phenotypical differentiation was used as reference standard for this assessment, it cannot be excluded that FISH results were nevertheless correct in some discrepant cases, as phenotypical methods can infrequently lead to incorrect results as well [21].

Anyway, very poor performance characteristics with less than 80–90% matching were only observed, like in the case of sensitivity of the *S. maltophilia* probe, if only very few samples were part of the assessment. Individual false reactions had a lot of impact under these circumstances, an undeniable limitation of the presented single-center assessment. As impressively shown for the example of the *Bacteroides* spp. probe, increases in specificity due to the adding of several competitor probes [1] can lead to reduction of sensitivity to unacceptably low values. Further, there have been various changes in the taxonomy of anaerobic bacteria in the recent years, and the applied biochemical identification procedures were shown to be associated with limited reliability as well [22].

The assessment of the usefulness of FISH probes requires the consideration of various factors. For example, FISH-based differentiation of staphylococci was confirmed to show good performance characteristics under routine-like conditions. Only a small probe panel was necessary for the discrimination of Gram-positive cocci in grape-like clusters, and FISH allowed a clearly visible microscopic distinction between coagulase-negative staphylococci and the clinically much more relevant species, *Staphylococcus aureus*. Successful commercial applications have been described [23].

For microorganisms with other morphology in Gram stain, like yeasts, which were not in the scope of this assessment but detailed elsewhere [24–25], Gram-positive cocci in doubles or chains, or even Gram-negative rod-shaped bacteria, considerably larger probe panels are required for differentiation. Especially for Gram-negative bacteria, FISH-based differentiation without resistance data is also less likely to allow clear-cut therapeutic decisions just based on the species identity. For anaerobic bacteria, FISH-based identification using the described approach currently makes little sense due to poor performance and debatable therapeutic consequences. Anyway, identification of anaerobic bacteria is difficult with conventional phenotypic methods as well [22], potentially resulting in many incorrect entries in the databases. Next to this, nomenclature of these species is still in progress. If this

Table 4. In-silico matching of the assessed probes with target sequences in the 0-mismatch range (0 MM), the 1-base-mismatch range (1 MM), and the 2-bases-mismatch range (2 MM) (–, not matches indicated)

Probe ^a	Target organism	0 MM	1 MM	2 MM
ACS 16S 729	<i>Acinetobacter</i> spp.	61.7% 3114/5046	57.6% 276/479	9.7% 45/463
Entero all (variant 1: “A” at the wobble position)	<i>Enterobacteriales</i>	58.1% 1906/3282	47.0% 7110/15123	51.0% 521/1022
Entero all (variant 2: “T” at the wobble position)	<i>Enterobacteriales</i>	46.5% 6526/14023	55.7% 2220/3987	54.0% 594/1101
ENF 16S 191	<i>Enterococcus faecalis</i>	47.5% 322/678	18.8% 3/16	0% 0/1
ENU 23S 140 Beimfohr	<i>Enterococcus faecium</i>	97.3% 36/37	–	25.0% 1/4
EGA 16S 141	<i>Enterococcus gallinarum</i>	66.7% 2/3	–	–
ENC 16S 221	<i>Enterococcus</i> spp.	52.4% 1403/2677	11.7% 27/230	0.3% 4/1260
ENC 176 23S kurz	<i>Enterococcus</i> spp.	86.2% 125/145	0% 0/59	0% 0/999
ESC 16S 468	<i>Escherichia coli</i>	28.3% 1983/7009	29.5% 165/559	7.9% 3/38
Klpn23S	<i>Klebsiella pneumoniae</i>	82.1% 55/67	0% 0/7	0% 0/2403
Psae Ulm 16S 182	<i>Pseudomonas aeruginosa</i>	17.0% 747/4406	0.7% 8/1088	0.1% 6/4926
Sal 23S 331	<i>Salmonella</i> spp.	95.1% 273/287	75.0% 3/4	0% 0/2236
Sal Yer 23S 1705 Komp	none, prevention of cross-reaction with organisms other than <i>Salmonella</i> spp.	100% 2198/2198	87.5% 239/273	29.9% 120/401
SalYer 23S 1705	<i>Salmonella</i> spp.	94.9% 281/296	12.5% 34/271	0% 0/2246
Komp Sal 23S 331	none, prevention of cross-reaction with organisms other than <i>Salmonella</i> spp.	100% 2181/2181	94.8% 165/174	99.0% 308/311
Stalle 16S	<i>Staphylococcus</i> spp.	38.7% 2154/5571	0.9% 51/5484	0.02% 17/99651
Stau16S Kempf	<i>Staphylococcus aureus</i>	68.1% 451/662	25.0% 14/56	0% 0/1052
Stemal Hogardt	<i>Stenotrophomonas maltophilila</i>	26.9% 77/286	14.3% 315/2207	6.7% 81/1211
Saga (variant 1: “A” at the wobble position)	<i>Streptococcus agalactiae</i>	94.1% 32/34	65.0% 134/206	–
Saga (variant 2: “C” at the wobble position)	<i>Streptococcus agalactiae</i>	65.2% 135/207	93.9% 31/33	–
Spneu	<i>Streptococcus pneumoniae</i>	97.4% 683/701	1.6% 20/1220	0.3% 7/2050
Spn Komp	none, prevention of cross-reaction with bacteria other than <i>Streptococcus pneumoniae</i>	98.6% 1197/1214	74.8% 2051/2741	99.9% 2141/2144
Strep Franks = Str 16S 492 Jansen	<i>Streptococcus</i> spp.	43.6% 3725/8553	3.1% 39/1239	0.03% 10/39762
Bact 16 S 389	<i>Bacteroides</i> spp.	2.2% 463/21395	0.3% 27/9376	0.2% 17/9436
Bact Komp	none, prevention of cross-reaction with <i>Porphyromonas</i> spp.	99.3% 3110/3132	98.4% 29461/29941	99.9% 16677/16691
Bact Komp 2	none, prevention of cross-reaction with <i>Prevotella</i> spp.	100% 10857/10858	97.9% 21873/22351	99.5% 4896/4921
Mic	<i>Micrococcus</i> spp.	78.4% 355/453	34.0% 34/100	4.5% 24/528
Spy1	<i>Streptococcus pyogenes</i>	72.2% 143/198	100% 1/1	0% 0/2

^aProbe names as stated in the supplementary material 1.

process is finished and better databases are available, designing better FISH probes for anaerobic bacteria may be possible in the future.

While the newly introduced *Bacterioides* spp. probe showed limited reliability, considerably better results were observed for the new probes for *Micrococcus* spp., for *S. pyogenes*, and, to a lesser extent, for the probe targeting *P. aeruginosa*. Accordingly, these new probes represent useful additions to diagnostic FISH probe panels. In our hands, the newly introduced *S. pyogenes* probe was more reliable than the previously published one [26] (data not shown), and it could be useful to evaluate it with other sample materials as well.

For several published FISH probes and probe-competitor-probe combinations, acceptable performance characteristics could be shown. Accordingly, FISH can provide rapid

preliminary pathogen identification from positive blood culture materials in resource-limited settings, where sophisticated and expensive approaches like MALDI-TOF-MS are not available for financial reasons. If published DNA-probes without patent protection are used, the price of material costs for a FISH reaction is less than one dollar if basic laboratory equipment like an incubator and a fluorescence microscope is available. The application of the multi-probe concept as described above reduces the risk of false positive results, making this algorithm based on Gram-morphology highly useful for FISH-applications. As usual for molecular methods, however, in-house FISH probe panels have to be thoroughly evaluated for diagnostic purposes and cannot just be blindly taken from previous publications to be applied in the diagnostic routine setting.

Table 5. Performance characteristics of the assessed FISH probes

Target organism	Sensitivity (%; absolute numbers)	Specificity (%; absolute numbers)	Positive predictive value (%; absolute numbers)	Negative predictive value (%; absolute numbers)
<i>S. aureus</i>	96.4, 81/84	100.0, 450/450	100.0, 81/81	99.3, 450/453
<i>Staphylococcus</i> spp.	98.3, 515/524	100.0, 10/10	100.0, 515/515	52.6, 10/19
<i>Micrococcus</i> spp.	100.0, 10/10	100.0, 524/524	100.0, 10/10	100.0, 524/524
<i>E. faecalis</i>	94.7, 36/38	100.0, 110/110	100.0, 36/36	98.2, 110/112
<i>E. faecium</i>	80.5, 33/41	100.0, 107/107	100.0, 33/33	93.0, 107/115
<i>E. gallinarum</i>	100.0, 3/3	98.6, 143/145	60.0, 3/5	100.0, 143/143
<i>Enterococcus</i> spp.	96.3, 79/82	100.0, 66/66	100.0, 79/79	95.7, 66/69
<i>S. agalactiae</i>	80.0, 4/5	100.0, 143/143	100.0, 4/4	99.3, 143/144
<i>S. pneumoniae</i>	87.5, 14/16	100.0, 132/132	100.0, 14/14	98.5, 132/134
<i>S. pyogenes</i>	100.0, 3/3	99.3, 144/145	75.0, 3/4	100.0, 144/144
<i>Streptococcus</i> spp.	98.4, 62/63	96.5, 82/85	95.4, 62/65	98.8, 82/83
<i>Acinetobacter</i> spp.	100.0, 9/9	100.0, 243/243	100.0, 9/9	100.0, 243/243
<i>Bacteroides</i> spp.	28.6, 2/7	100.0, 245/245	100.0, 2/2	98.0, 245/250
<i>Bacteroides</i> spp./ <i>Prevotella</i> spp.	57.1, 4/7	100.0, 245/245	100.0, 4/4	98.8, 245/248
Enterobacteriaceae	98.0, 199/203	100.0, 49/49	100.0, 199/199	92.5, 49/53
<i>E. coli</i>	96.0, 121/126	99.2, 125/126	99.2, 121/122	96.2, 125/130
<i>Klebsiella pneumoniae</i>	100.0, 27/27	98.2, 221/225	87.1, 27/31	100.0, 221/221
<i>Pseudomonas</i> <i>aeruginosa</i>	90.0, 27/30	100.0, 222/222	100.0, 27/27	98.7, 222/225
<i>Salmonella</i> spp.	100.0, 6/6	100.0, 246/246	100.0, 6/6	100.0, 246/246
<i>Stenotrophomonas</i> <i>maltophilia</i>	66.7, 2/3	100.0, 249/249	100.0, 2/2	99.6, 249/250

Table 6. Details of lacking or incorrect binding of specific FISH probes

Probe	Failed to identify	Cross bindings
<i>S. aureus</i>	3/84 <i>S. aureus</i>	
<i>Staphylococcus</i> spp.	2/84 <i>S. aureus</i> 3/356 <i>S. epidermidis</i> 4/57 not further differentiated coagulase negative staphylococci	1/18 <i>S. mitis</i> ^a
<i>E. faecalis</i>	2/38 <i>E. faecalis</i>	
<i>E. faecium</i>	8/41 <i>E. faecium</i>	
<i>E. gallinarum</i>		2/41 <i>E. faecium</i>
<i>Enterococcus</i> spp.	1/38 <i>E. faecalis</i> 2/41 <i>E. faecium</i>	
<i>S. agalactiae</i>	1/5 <i>S. agalactiae</i>	
<i>S. pneumoniae</i>	2/16 <i>S. pneumoniae</i>	
<i>S. pyogenes</i>		1/6 <i>S. anginosus</i>
<i>Streptococcus</i> spp.	1/18 <i>S. mitis</i>	3/3 <i>Lactococcus lactis</i>
<i>Bacteroides</i> sp. probe in conjunction with the competitor probes against <i>Prevotella</i> spp. and <i>Porphyromonas</i> spp.	3/4 <i>Bacteroides fragilis</i> 1/1 <i>Bacteroides thetaiotaomicron</i> 1/2 <i>Bacteroides</i> spp.	
<i>Bacteroides</i> sp./ <i>Prevotella</i> sp. probe in conjunction with only the competitor probe against <i>Porphyromonas</i> spp.	2/2 <i>Bacteroides</i> spp. 1/4 <i>Bacteroides fragilis</i>	
Enterobacteriaceae	4/126 <i>E. coli</i>	
<i>Escherichia coli</i>	5/126 <i>E. coli</i>	1/7 <i>Klebsiella oxytoca</i>
<i>Klebsiella pneumoniae</i>		1/1 <i>E. amnigenes</i> 2/7 <i>Klebsiella oxytoca</i> 1/4 <i>Serratia marcescens</i>
<i>P. aeruginosa</i>	3/30 <i>P. aeruginosa</i>	
<i>S. maltophilia</i>	1/3 <i>S. maltophilia</i>	

^aNot mentioned in Table 5, because the probe was erroneously used with streptococci.

Conclusions

In summary, the described FISH panel is a suitable approach for rapid preliminary identification of pathogens from blood culture materials, which could be useful for resource-limited settings. Individual misidentifications may occur. The demonstrated performance under diagnostic routine-like conditions confirms the suitability of the approach for the routine setting, apart from study conditions. However, FISH probe panels have to be thoroughly adapted to individual diagnostic needs. To our experience, FISH for the rapid identification of *S. aureus* was particularly useful.

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Authors' Contributions

AR performed the assessments and was mainly responsible for the publication. SP planned and conducted the experiments. MR also conducted the experiments. HF supervised and supported AR's assessments. All authors jointly wrote and corrected the manuscript.

Conflict of Interest

Nothing to report.

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