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Rapid Identification of Pathogens with the hemoFISH Test Applying a Novel Beacon-Based Fluorescence *in Situ* Hybridization (bbFISH) Technology in Positive Blood Culture Bottles

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Rapid and accurate identification of pathogens responsible for sepsis is essential for early and targeted antimicrobial therapy. Blood cultures are the current reference standard for detection of pathogens in blood, but culture-based identification methods are time consuming. We evaluated the hemoFISH assay by using the novel bbFISH technology for rapid and accurate identification of a broad range of microorganisms in positive blood cultures. A total of 103 positive blood culture bottles were investigated. In total, 106 bacterial species were detected in the blood cultures and subsequently identified with conventional methods. The Gram-staining indicated monomicrobial growth in 95.1% (98/103) and polymicrobial growth in 4.9% (5/103) blood cultures. In 65.0% (67/103) cultures Gram-positive, 32.0% (33/103) Gram-negative, and 3.0% (3/103) both Gram-positive and Gram-negative bacteria were identified. Depending on the Gram-staining results, either the hemoFISH Gram-positive or the hemoFISH Gram-negative panel was used. In case of a polymicrobial infection, both panels were applied. The hemoFISH assay showed a sensitivity and specificity of 100% (95% CIs of 96.34% to 100% and 30.48% to 100%, respectively). Of the 106 bacterial species, the hemoFISH assay correctly identified 55.7% ($n = 59$) to species level, 34.0% ($n = 36$) to genus level, and 7.5% ($n = 8$) to family level. The novel hemoFISH using bbFISH technology appears to be a valuable rapid tool for the identification of a broad range of microorganisms in positive blood cultures. *(J Mol Diagn 2013, 15: 835—839; http://dx.doi.org/10.1016/j.jmoldx.2013.07.007)*

Sepsis is a life-threatening medical condition that is defined as systemic inflammatory response syndrome in reaction to an infectious agent, including bacteria.[1] Although potent antibiotics and improved supportive care are available, mortality of septic patients remains high. The mortality rate ranges from 30% up to 50%, when associated with shock.[2] It was shown that rapid identification of the causal agent improves antimicrobial therapy and subsequently reduces the rate of mortality.[3–5]

Blood cultures represent the reference standard for the detection and identification of pathogens that cause bloodstream infections. Conventional culture-based identification systems are time consuming but can be improved by using additional test systems.[6,7]

Fluorescence *in situ* hybridization (FISH) is well-established especially in combination with peptide nucleic acid probes for identification of pathogens in positive blood cultures.[6,8–12] Recently, a novel beacon-based FISH assay (bbFISH) was introduced which applies DNA-molecular beacons as probes for pathogen identification in positive blood cultures. Molecular beacons are hairpin-shaped structures that consist of a probe sequence (loop) and 3′ and 5′ ends (stem) that carry a fluorophore and a quencher. The loop sequence is complementary to the target sequence, and, when hybridization occurs, the stem opens and a fluorescent signal is emitted and can be detected with

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fluorescence microscopy. Unbound probes remain in the closed confirmation and the signal is quenched.[13,14] The hemoFISH assay (miacom diagnostics, Inc., Düsseldorf, Germany) is an *in vitro* diagnostic/Conformité Européene-labeled test system that uses this bbFISH technology. Rapid identification of pathogens is accomplished by hybridization of the probes to 16S rRNA or 23S rRNA of the target microorganism. Depending on Gram-staining result the hemoFISH test offers a panel for Gram-positive and Gram-negative pathogens. The test is designed to detect >90% of occurring pathogens in septic patients either to family, genus, or species level.[15] The Gram-positive panel includes a screening for *Staphylococcus* spp and *Streptococcus* spp and the following species: *S. aureus*, *S. pneumonia*, *S. pyogenes*, *S. agalactiae*, *Clostridium perfringens*, *Enterococcus faecium*, and *E. faecalis*. The Gram-negative panel includes a screening for enterobacteriaceae, *Salmonella* spp, *Acinetobacter* spp, and the following species: *Escherichia coli*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia*, and *Haemophilus influenzae*.

We evaluated the hemoFISH test using bbFISH technology for accurate identification of microorganisms in positive blood cultures by comparison with culture-based identification methods.

Materials and Methods

Sample Collection

Positive blood culture bottles ($n = 103$) were obtained from the Institute of Hygiene, Microbiology, and Environmental Medicine and the Department of Internal Medicine, Section of Infectious Diseases and Tropical Medicine (both from the Medical University of Graz, Graz, Austria). Samples were collected from December 2012 until March 2013. Blood cultures were obtained from patients with suspected sepsis as part of the standard hospital care and transferred to the responsible laboratories.

BACTEC Plus Aerobic/F or BACTEC Plus Anaerobic/F (Becton Dickinson Diagnostic Systems, Franklin Lakes, NJ) blood culture bottles were included in this study when identified as positive by the automated blood culture-monitoring instrument BACTEC FX (Becton Dickinson Diagnostic Systems). Only the first positive blood culture from the set of a single patient was analyzed. The samples were anonymously passed on to perform the hemoFISH assay.

Identification of Microorganisms

The clinical isolates were grown overnight on agar plates according to the bottle type and Gram-staining result. Identification was performed, depending on the suspected microorganisms either with mass spectrometry by using MALDI-TOF MS AXIMA Assurance (Shimatzu Corporation, Kyoto, Japan) and SARAMIS database (bioMérieux, Marcy l`Etoile, France) or with Vitek2 (bioMérieux). The identification was performed at the Institute of Hygiene, Microbiology, and Environmental Medicine, Medical University of Graz, which is an International Standard Organization (ISO9001,2000)-certified laboratory.

hemoFISH

The hemoFISH panel used was chosen according to the Gram-staining results. In case the Gram-staining showed a polymicrobial infection with Gram-positive and Gram-negative microorganisms, both assay panels were used. The assay was performed according to the manufacturer's recommendations. In brief, a 50 μL aliquot of the positive blood culture broth was added to 450 μL of sample buffer. After shaking, 10 μL was applied to each of the eight fields on the hybridization slide. The slide was placed on a hybridizer (a combination of a hotplate and a hybridization chamber) with a fixed temperature of 52°C and dried. Next, 10 μL of lysis buffer was added to each field with the multipipette, dried, and transferred to 95% ethanol bath for 5 minutes. Excess alcohol was allowed to evaporate. Thereafter, 10 μL of the hybridization solution was added to the fields, and the covered slide was placed in the hybridization chamber for 10 minutes. The slide was then transferred into the stop-bath for exactly 1 minute and dipped in 95% ethanol before final drying. To each field of the slide a small drop of mounting medium was added and covered with a coverslip. Examination was done with the Zeiss Axio Imager A1 microscope (Carl Zeiss AG, Oberkochen, Germany) with the use of the appropriate filters for the used fluorophores ATTO 550 (red) and FAM (green). On the first field of the slide the controls were located. The hemoFISH slide was regarded as valid, if the red channel showed fluorescent bacterial cells because of the binding of the beacons to bacterial rRNA sequences (positive control) and if the green channel was showing no fluorescent bacterial cells because no rRNA was present for binding (negative control). After verifying the controls, the remaining fields were examined, and the fields with fluorescence in the red or the green channel were indicated as positive for the respective microorganism. Slide preparation took approximately 30 minutes and microscopy took approximately 5 minutes. The overall time for testing depended on the number of specimens analyzed. Microscopy was done by one investigator, except when the result needed a second evaluation for positive or negative verification.

Results

A total number of 103 positive blood culture bottles [59.0% (61/103) BACTEC Plus Aerobic/F and 41.0% (42/103) BACTEC Plus Anaerobic/F] were tested. Culture-dependent identification with the use of the reference methods (MALDI-TOF MS or Vitek2) indicated that 95.1% (98/103)

of the blood culture bottles showed monomicrobial growth and 4.9% (5/103) showed polymicrobial growth. The Gram-staining showed Gram-positives in 65.0% (67/103), Gram-negatives in 32.0% (33/103), and both Gram-positive and Gram-negative microorganisms in 3.0% (3/103) of the cultures. According to these results, the corresponding hemoFISH panel(s) was taken for testing. Three of the samples had to be excluded because of a deficient signal in the positive control. Microorganisms identified with the reference method in these samples were *E. faecium*, *S. pyogenes*, and *Propionibacterium acnes*.

From the 106 bacterial species identified with the reference methods, the hemoFISH correctly identified 55.7% ($n = 59$) to species level, 34.0% ($n = 36$) to genus level, 7.5% ($n = 8$) to family level, and 2.8% ($n = 3$) were unidentifiable (Table 1). The three unidentified species were regarded as true negative because they are not included in the hemoFISH panel, which leads finally to a sensitivity and specificity of 100% (95% CI of 96.34%−100% and 30.48%−100%, respectively) (Table 2).

In the monomicrobial cultures 63 Gram-positive species were detected. *S. aureus* ($n = 21$), *S. pneumonia* ($n = 4$), *E. faecalis* ($n = 3$), *E. faecium* ($n = 2$), and *S. agalactiae* ($n = 1$) were correctly identified to species level. The coagulase-negative staphylococci ($n = 27$) were correctly identified to genus level as *Staphylococcus* spp and *S. mitis/oralis* ($n = 2$), *S. gallolyticus* ($n = 1$), and *S. pluranimalium* ($n = 1$) to *Streptococcus* spp, respectively. From the 36 Gram-negative species detected in monomicrobial samples, *E. coli* ($n = 16$), *P. aeruginosa* ($n = 5$), *K. pneumonia* ($n = 2$), and *P. mirabilis* ($n = 1$) were correctly identified with the hemoFISH Gram-negative panel to species level, whereas the five isolates of *Enterobacter cloacae* were correctly identified as enterobacteriaceae to family level. *P. alcaligenes*, *Sphingomonas koreensis*, and *Bacteroides fragilis* only gave a positive signal in the positive control, indicating that bacteria were present. Precise identification was not possible, because these targets are not represented on the Gram-negative hemoFISH panel (Table 1).

From the five polymicrobial samples, one showed Gram-positive cocci only and one Gram-negative rods only but were found to be polymicrobial samples after culturing. In the Gram-positive sample *S. epidermidis* and *S. hominis* were identified and detected with the Gram-positive hemoFISH panel as *Staphylococcus* spp. In the Gram-negative sample *E. cloacae* and *K. pneumoniae* were identified and detected with the Gram-negative hemoFISH panel as enterobacteriaceae and *K. pneumoniae*. In both cases the hemoFISH assay was not able to show the presence of a polymicrobial infection, because the identification to species level was not achieved for both microorganisms. The polymicrobial samples showing Gram-positive and Gram-negative microorganisms were correctly identified according to the microorganisms present on the hemoFISH panels to species, genus, or family level (Table 1).

From the 106 slides tested, in 11 cases the readout was initially challenging because of slight autofluorescence from certain probes. These slides were reevaluated and double-checked by a second investigator. In all cases, the result obtained by the two investigators was in agreement with the culture results. The specific fluorescence was much brighter than the observed autofluorescence.

C. perfringens included in the Gram-positive hemoFISH panel did not occur in this study. The *S. pyogenes*−positive sample could not be identified with hemoFISH because of invalid controls. No results were received for the bbFISH probes in the hemoFISH Gram-negative penal for *Acinetobacter* spp, *Salmonella* spp, *H. influenzae*, *S. marcescens*, and *P. vulgaris* because all blood cultures were negative for these species.

Discussion

Our study presents the first evaluation of the new bbFISH technology (hemoFISH) for rapid identification of microorganism(s) in positive blood cultures. Several studies describe the importance of rapid bacterial identification for beneficial management of septic patients.[3−5] The Gram-staining result of a positive blood culture may have an effect for antimicrobial treatment adaption, as shown by Hautala et al[16]; however, the precise identification of the microorganism causing the infection can improve the treatment decision.[12,16]

The hemoFISH Gram-positive panel correctly differentiated *S. aureus* from coagulase-negative staphylococci, which represent the most frequently isolated microorganisms in this study (Table 1). Rapid identification of *S. aureus*, as the most important Gram-positive pathogen in septic patients, and accurate differentiation from coagulase-negative staphylococci mainly associated with contamination from normal skin flora, gives impetus to avoid unnecessary antibiotic therapy.[15,17] On the basis of these facts several FISH methods are available for identification of staphylococci[18,19]; in addition, the precise identification of streptococci and enterococci is of importance. In our study *S. pneumonia* and *E. faecalis*, together with *E. faecium*, were the second-most frequently detected genera. Identification to species level of these genera is of interest because penicillins are useful drugs against *S. pneumonia* or *E. faecalis* but fail as treatment for *E. faecium*. This makes the differentiation of these species in one method very useful.[8,20]

When Gram-negative rods are detected with Gram-staining, a broad spectrum of species is detectable with the hemoFISH Gram-negative panel. As confirmed in this study, *E. coli* represents the most frequent Gram-negative microorganism in blood cultures (Table 1).[15] However, it has to be considered that an rRNA-specific probe for *E. coli* always detects *Shigella* spp because of their sequence homology.[8] *E. cloacae* together with *P. aeruginosa* were the second-most common Gram-negative microorganism detected in this study. *E. cloacae* could only be identified to family level

Table 1 Correctly Identified Microorganisms in Positive Blood Cultures with the Use of hemoFISH

Identification with reference methods*	Level of identification with hemoFISH				
Microorganisms	No.	No. family	No. genus	No. species	No. with no ID
Monomicrobial samples Gram-positive (n = 63)					
Staphylococcus aureus	21			21	
S. epidermidis	18		18		
S. haemolyticus	5		5		
S. hominis	3		3		
S. simulans	1		1		
S. warneri	1		1		
Streptococcus pneumoniae	4			4	
Enterococcus faecalis	3			3	
E. faecium	2			2	
S. mitis/oralis	2		2		
S. agalactiae	1			1	
S. gallolyticus	1		1		
S. pluranimalium	1		1		
Gram-negative (n = 32)					
Escherichia coli	16			16	
Enterobacter cloacae	5	5			
Pseudomonas aeruginosa	5			5	
Klebsiella pneumoniae	2			2	
Proteus mirabilis	1			1	
P. alcaligenes	1				1
Sphingomonas koreensis	1				1
Bacteroides fragilis	1				1
Polymicrobial samples Gram-positive and Gram-negative (n = 11)					
S. epidermidis	1		1		
S. hominis	1		1		
E. cloacae	1	1			
K. pneumoniae	1			1	
P. aeruginosa	1			1	
E. faecium	1			1	
Stenotrophomonas maltophilia	1			1	
S. hominis	1		1		
Citrobacter freundii	1	1			
K. ornithinolytica	1	1			
S. anginosus	1		1		
Total, no. (%)	106	8 (7.5)†	36 (34.0)†	59 (55.7)†	3 (2.8)†

*Reference methods were MALDI-TOF MS and Vitek2.
†Percentage of the total.
ID, identification.

because rRNA-specific probes that target *E. cloacae* are not available because of sequence homologies with other bacteria from the enterobacteriaceae family. Because other important enterobacteriaceae can be identified to species level, one may find identification only to the family level helpful. The correct identification of *E. coli*, *K. pneumoniae*, *P. mirabilis*, enterobacteriaceae, and *P. aeruginosa*, as achieved in the Gram-negative panel, can serve as primary driver of antimicrobial selection, especially if growth failure may occur.[21]

The three microorganisms that could not be identified with hemoFISH were *P. alcaligenes*, *S. koreensis*, and

Table 2 Comparison of the hemoFISH to Culture-Based Microbe Identification in Positive Blood Cultures

	Culture-based identification				
	No. of specimens (n = 103)	Sensitivity (%) [95%CI]	Specificity (%) [95%CI]	PPV (%) [95%CI]	NPV (%) [95%CI]
hemoFISH		100 [96.34–100]	100 [30.48–100]	100 [96.34–100]	100 [30.48–100]
True positive	100				
True negative	3				

PPV, positive predictive value; NPV, negative predictive value.

B. fragilis. These bacteria are rarely present in blood cultures and are not included in the panels.

Polymicrobial growth was detected in 4.9% (5/103). In general, the detection of the microorganisms showed the same performance than in monomicrobial samples (Table 1). The limitation to detect one or more of the microorganisms in a polymicrobial sample depended on the bacterial species involved, especially, when they belonged to the same Gram-staining category.

Although this evaluation showed a sensitivity and specificity of 100% (95% CI, 96.34%−100% and 30.48%−100%, respectively), we had to exclude three samples because of control errors. Moreover, in 11 cases we had difficulties in the microscopic examination; therefore, consulting a second investigator was necessary. These problems may be a result of poor sample condition, especially, when many debris of blood cells influenced the judgment. We recommend the addition of a second positive control for the FAM probe to further validate the results from the green channel.

Although no concrete susceptibility information can be obtained with the hemoFISH, it offers the precise detection of a broad range of microorganism, including the most frequently found pathogens in positive blood cultures useful because of the knowledge of intrinsic resistance patterns of the microorganisms.[15] Furthermore, the identification to species level together with the patient's history and susceptibility patterns in the hospital environment may help to support the decision on the empirical antibiotic therapy until the result of the susceptibility testing is available.[3,4]

In conclusion, the hemoFISH assay that uses the novel bbFISH technology is an easy-to-handle, rapid, reliable tool for the identification of a broad range of microorganisms and facilitates the decision of antimicrobial therapy in septic patients.

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