RAPID IDENTIFICATION OF *ACINETOBACTER* SPP. BY FLUORESCENCE *IN SITU* HYBRIDIZATION (FISH) FROM COLONY AND BLOOD CULTURE MATERIAL

H. Frickmann^{1,2*}, A. Essig³, R. M. Hagen¹, M. Riecker³, K. Jerke⁴, D. Ellison⁵ and S. Poppert⁶

¹Department of Tropical Medicine, Bernhard Nocht Institute for Tropical Medicine, Bundeswehr Hospital, Hamburg, Germany

²Institute for Medical Microbiology, Virology and Hygiene, University of Rostock, Rostock, Germany

³Institute for Medical Microbiology, University of Ulm, Ulm, Germany

⁴Virology Division, United States Army Medical Research Institute of Infectious Disease, Fort Detrick, MD 21702, USA

⁵Department of Pathology/Microbiology Division, Landstuhl Regional Medical Center (US Army), Landstuhl, Germany

⁶Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany

Received: August 16, 2011; Accepted: August 23, 2011

Multi-drug-resistant strains of the *Acinetobacter baumannii* complex cause nosocomial infections. Rapid identification of *Acinetobacter* spp. is desirable in order to facilitate therapeutic or hygiene decisions. We evaluated a newly designed DNA probe that can be used under standard conditions in both a microwave oven and a slide chamber for the rapid identification of *Acinetobacter* spp. by fluorescence *in situ* hybridization (FISH). Using FISH, the new probe correctly identified 81/81 *Acinetobacter* spp. isolates and excluded 109/109 tested non-target organisms from agar culture. Furthermore, the new probe correctly identified 7/7 *Acinetobacter* spp. in 214 blood cultures determined to contain Gram-negative bacteria by Gram staining. Using either the microwave oven or slide chamber technique, the new probe was able to identify *Acinetobacter* spp. in 100% of the samples tested. FISH used in conjunction with our newly designed probe provides an easy, cheap, precise, and rapid method for the preliminary identification of *Acinetobacter* spp., especially in laboratories where more sophisticated methods like matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are not available.

Keywords: fluorescence *in situ* hybridization, *Acinetobacter*, rapid diagnostics, molecular identification

Introduction

Opportunistic pathogens of the *Acinetobacter calcoaceticus*–*Acinetobacter baumannii* complex include *A. baumannii* and the genomic species 3 and 13TU [1]. These organisms are widely distributed in the hospital environment, and are responsible for a variety of nosocomial infections, especially in patients from intensive care units (ICUs) [2]. *Acinetobacter* spp. can harbor numerous antibiotic resistance genes [3]. The potential for horizontal gene transfer of resistance elements between co-circulating strains in epidemics has been described [4], as has the acquisition of resistance genes from other Gram-negative bacteria [5]. A rapid identification of *Acinetobacter* spp. from both plated media and blood culture materials is therefore needed for quick detection, prevention of outbreaks, and initiation of appropriate therapy for affected patients.

Traditionally, clinical laboratories have relied on classical biochemical identification systems, such as the API 20NE or the VITEK-II (bioMérieux, Nürtingen, Germany). These systems are quite reliable but time-consuming [6]. Matrix-assisted laser desorption ionization time-of-flight

mass spectrometry (MALDI-TOF-MS) and molecular methods such as NAAT (nucleic acid amplification technique) [1], amplified ribosomal DNA restriction analysis [7], 16S rRNA gene PCR-ligase detection reaction–capillary electrophoresis assays [8] and DNA microarrays [9] allow for rapid and reliable identification but are expensive, require sophisticated equipment and are not generally available in routine clinical laboratories. Fluorescence *in situ* hybridization (FISH) is an alternative molecular method for rapid identification of bacteria that is more cost-effective than automated NAAT platforms and requires less technical expertise to perform than most other available molecular methods. FISH works with fluorescence-labeled oligonucleotide probes, which bind to unique complementary target sites on ribosomal RNA [10–13]. The successful implementation of FISH has been described for detection and identification of various pathogens including *Acinetobacter* [10–17]. To date, the only published DNA probe [18] for the identification of *Acinetobacter* spp. is used under non-standard hybridization conditions and therefore cannot be used in concert with previously described diagnostic FISH-probes [10, 11, 13]. Another study describes

*Corresponding author: Hagen Frickmann, M.D., CPT (MC); Department of Tropical Medicine, Bernhard Nocht Institute for Tropical Medicine, Bundeswehr Hospital Hamburg, Bernhard-Nocht St 74, D-20359 Hamburg, Germany;
Phone: +49-40-6947-2862; Fax: +49-40-6947-2859; E-mail: Frickmann@bni-hamburg.de

the development of an *Acinetobacter*-FISH probe based on peptide nucleic acids (PNA). However, PNA probes cannot be used in conjunction with the previously published DNA probes, and they are considerably more expensive [19].

In this paper, we describe the development and evaluation of a new DNA probe for the identification of *Acinetobacter* spp. by FISH using standard conditions.

Material and methods

Probe design

A new DNA probe with specificity for *Acinetobacter* spp. (Aci-16S 729: 5'-TTA-GGC-CAG-ATG-GCT-GCC-3') was designed with ARB software (www.arb-home.de) as previously described [20, 21] with sequence data extracted from the NCBI GenBank database. The probe was directly 5-labeled with the red sulfoindocyanine dye Cy3 (Thermo Fisher Scientific, Ulm, Germany and Eurogentec Deutschland GmbH, Köln, Germany). The probe was designed to optimally bind at a formamide concentration of 30% at 46 °C, which allows the Aci-16S 729 probe to be used in combination with other clinically relevant FISH probes that require similar conditions [10, 11]. The probe was used in combination with a green-fluorescing carboxyfluorescein-(FAM)-labeled eubacterial probe (EUB338: 5'-GCT-GCC-TCC-CGT-TAG-GAG-T-3') targeting nearly all bacteria [22].

In silico evaluation

An *in silico* evaluation of the new probe was performed using the program probeCheck (www.microbial-ecology.net/probecheck/) [23], which allows one to query sequence collections such as SILVA.

In vitro evaluation

Optimal hybridization conditions for the new probe were determined using the *A. baumannii* reference strain (ATCC 19606) with increasing formamide concentrations from 10% to 50% in the hybridization buffer.

The probe was then evaluated *in vitro* using 16 reference strains, 10 defined strains from German laboratory evaluations ("Ringversuche," later referred to as "laboratory control strains"), 164 clinical isolates (ci), and 214 blood culture samples in a multicentric approach. The clinical isolates included 20 *A. baumannii* isolates from Iraq, four *A. baumannii* isolates from Afghanistan, two well-defined strains of the worldwide multi-drug-resistant *A. baumannii* outbreak clones (WW1 and WW2, strains Dem1R and Berlin61, respectively) [24], and a well-defined multi-drug-resistant *A. baumannii* strain of a regional endemic clone from Rostock (Germany) [25].

Phenotypic characterization of *Acinetobacter* spp. isolates including blood culture isolates was performed

by Gram staining, colony morphology, negative cytochrome-oxidase reaction, API 20NE (bioMérieux, Nürtingen, Germany), and alternatively a VITEK-II (bioMérieux). Usually the results from a single biochemical identification system were sufficient to identify isolates from clinically relevant specimens in critically ill patients. However, in cases of unexpected or inconclusive biochemical results, the 16S rRNA gene was sequenced using primers AGA-GTT-TGA-TCM-TGG-CTC-AG and CCG-TCA-ATT-CMT-TTR-AGT-TT (bases 1–917 of the 16S rRNA gene, NCBI accession number NC_009085.1). This was done in only three instances. Clinical isolates of Gram-negative bacteria that were used as negative controls were phenotypically identified by Gram staining, cytochrome-oxidase reaction, API 20E (bioMérieux), API 20NE (bioMérieux), or VITEK-II (bioMérieux), respectively, or exceptionally by sequencing as described above.

Prior to FISH, clinical isolates were grown on Columbia agar enriched with 5% sheep blood (BD, Franklin Lakes, NJ, USA) and MacConkey-II agar (BD) at 37 °C for about 24 h and colony material was suspended in sterile 0.9% NaCl solution. Blood culture specimens (BacT/ALERT® 3D Microbial Detection System, bioMérieux, Nürtingen, Germany) that were positive for Gram-negative bacteria as determined by Gram staining (Table 1) were centrifuged and spotted onto multi-well glass slides [17].

FISH was performed as previously described at a formamide concentration of 30% in a microwave oven [11] at the Institute for Microbiology of the University Hospital Ulm (Germany) or in a slide chamber (ThermoStat, Eppendorf, Hamburg, Germany) [12, 16, 17, 26] at the Institute for Microbiology of the University Hospital Rostock (Germany) and the Bernhard-Nocht Institute in Hamburg (Germany). In total, seven reference strains, 114 clinical isolates, and 214 blood culture samples were tested using the microwave-based method, while colonies of nine reference strains, 10 defined strains from laboratory evaluations and 50 clinical isolates were investigated using the slide chamber. A direct comparison of the two well-established and well-described procedures was not conducted.

Results

In silico evaluation

The Aci-16S 729 probe targets all species in the genus *Acinetobacter*, but we failed to design a probe with specificity to those in the *A. calcoaceticus*–*A. baumannii* complex alone. The *in silico* evaluation of the new Aci-16S 729 probe using the software probeCheck in July 2011 resulted in a total of 2619 hits within the zero-mismatch range and 2923 hits within the one-mismatch range. In the case of a single mismatch, probe binding is still expected. Out of the total, 1796 cultured and uncultured *Acinetobacter* strains were detected within the zero-mismatch range and altogether 2016 *Acinetobacter* strains within

Table 1. Number of positive results by *Acinetobacter* FISH using different procedures with different samples

Species	Reference strains*		Clinical isolates		Blood cultures
	Microwave	Slide chamber	Microwave	Slide chamber	Microwave
<i>Achromobacter denitrificans</i>				0/1	
<i>Acinetobacter anitratus</i>		1/1			
<i>Acinetobacter baumannii</i>		4/4	1/1	32/32	1/1
<i>Acinetobacter calcoaceticus</i>	1/1	1/1		1/1	
<i>Acinetobacter haemolyticus</i>				3/3	
<i>Acinetobacter johnsonii</i>				1/1	
<i>Acinetobacter junii</i>	1/1				
<i>Acinetobacter lwoffii</i>	1/1	2/2	7/7	1/1	2/2
<i>Acinetobacter radioresistens</i>				2/2	
<i>Acinetobacter</i> spp.			22/22		4/4
<i>Bacteroides caccae</i>			0/1		
<i>Bacteroides fragilis</i>			0/3		0/6
<i>Bacteroides vulgates</i>			0/1		
<i>Bordetella avium</i>	0/1				
<i>Bordetella hinzii</i>	0/1				
<i>Bordetella holinesii</i>	0/1				
<i>Burkholderia cepacia</i>		0/1			
<i>Burkholderia gladioli</i>				0/1	
<i>Burkholderia multivorans</i>				0/1	
<i>Burkholderia vietnamensis</i>		0/1			
<i>Citrobacter freundii</i>					0/4
<i>Citrobacter koseri</i>			0/1		0/2
<i>Citrobacter</i> spp.			0/1		0/1
<i>Comamonas acidovorans</i>		0/1			
<i>Escherichia coli</i>			0/20		0/106
<i>Enterobacter amnigenus</i>					0/1
<i>Enterobacter aerogenes</i>					0/7
<i>Enterobacter cloacae</i>					0/12
<i>Enterobacter</i> spp.			0/4		
<i>Flavimonas oryzae</i>		0/2			
<i>Haemophilus parainfluenzae</i>			0/1		
<i>Haemophilus</i> spp.			0/1		
<i>Klebsiella oxytoca</i>					0/8
<i>Klebsiella pneumonia</i>			0/7		0/22
<i>Moraxella catarrhalis</i>				0/1	
<i>Morganella morganii</i>			0/1		0/1
<i>Pantoea</i> spp.					0/1
<i>Proteus mirabilis</i>			0/2		0/1
<i>Proteus vulgaris</i>			0/1		
<i>Pseudomonas aeruginosa</i>	0/1	0/2	0/15	0/1	0/23
<i>Pseudomonas alcaligenes</i>				0/1	
<i>Pseudomonas fluorescens</i>		0/1			
<i>Pseudomonas luteola</i>		0/1			
<i>Pseudomonas mendocina</i>				0/1	
<i>Pseudomonas putida</i>		0/1		0/1	
<i>Ralstonia pickettii</i>				0/1	

Table 1. Continued

Species	Reference strains*		Clinical isolates		Blood cultures
	Microwave	Slide chamber	Microwave	Slide chamber	Microwave
<i>Salmonella</i> Bredeney					0/1
<i>Salmonella</i> Enteritidis			0/1		0/3
<i>Salmonella</i> Paratyphi			0/1		
<i>Salmonella</i> Typhi					0/1
<i>Salmonella</i> spp.			0/1		
<i>Serratia liquefaciens</i>					0/1
<i>Serratia marcescens</i>			0/1		0/3
<i>Stenotrophomonas maltophilia</i>		0/1	0/21	0/1	0/2
<i>Yersinia enterocolitica</i>					0/1
Total numbers	7	19	114	50	214
Analytic sensitivity	3/3 (100%)	8/8 (100%)	30/30 (100%)	40/40 (100%)	7/7 (100%)
Analytic specificity	0/4 (100%)	0/11 (100%)	0/84 (100%)	0/10 (100%)	207/207 (100%)

*Including reference strains ATCC 19606 *Acinetobacter baumannii*, ATCC 23055 *Acinetobacter calcoaceticus*, ATCC 17908 *Acinetobacter junii*, ATCC 15309 *Acinetobacter lwoffii*, ATCC 25416 *Burkholderia cepacia*, ATCC 27853 *Pseudomonas aeruginosa*, ATCC 9027 *Pseudomonas aeruginosa*, ATCC 13637 *Stenotrophomonas maltophilia*, the NCTC: NCTC 7844 *Acinetobacter anitratus*, NCTC 5866 *Acinetobacter lwoffii*, NCTC 10618 *Pseudomonas aeruginosa*, the DSMZ (Germany): DSM 30008 *Acinetobacter baumannii*, DSM 30006 *Acinetobacter calcoaceticus*, DSM 11332 *Bordetella avium*, DSM 11333 *Bordetella hinzii*, DSM 13416 *Bordetella holnisei*; and including well-defined strains from laboratory elevations ("Ringversuche") *Acinetobacter baumannii* (2×), *Acinetobacter lwoffii*, *Burkholderia vietnamsensis*, *Comamonas acidovorans*, *Flavimonas oryzihabitans* (2×), *Pseudomonas fluorescens*, *Pseudomonas luteola*, *Pseudomonas putida*.

the one-mismatch-range. A comparison with the two-mismatch-range, where probe binding is less reliable, indicated that 1 out of 79 *Acinetobacter johnsonii* strains, 1 out of 35 *Acinetobacter junii* strains, 1 out of 25 *Acinetobacter radioresistens* strains, 3 out of 505 *Acinetobacter* spp. strains and 38 out of 1071 uncultured *Acinetobacter* spp. strains would have been missed. However, all available sequences from the clinically relevant *A. calcoaceticus*–*A. baumannii* complex were detected within the one-mismatch range (Table 2).

In addition to *Acinetobacter*-specific binding, the new probe also matched to some non-*Acinetobacter* sequences. In the zero-mismatch range 823 sequence matches and in the one-mismatch range, 907 sequence matches were identified. Most of these matches were to environmental species without clinical relevance such as; uncultured bacteria, symbionts, or various Gram-negative bacteria (e.g. the Gram-negative soil bacterium *Aeromonas punctata*, *Alkanindiges illinoisensis*, *Alkanindiges hongkongensis*, *Caenorhabditis remanei*, and *Perluclidibaca piscinae*). Matches with individual isolates of *Enterobacter* spp., *Arthrobacter* spp., and non-pathogenic Moraxellaceae within the zero-mismatch range were troublesome (Table 2). While *Arthrobacter* spp. can easily be excluded because of their positive reaction in Gram staining, cross reactions with isolates of clinically relevant *Enterobacter* spp. and the pathogenic *Moraxella catarrhalis* had to be experimentally excluded *in vitro*.

Table 2. *In silico* evaluation using the software probeCheck (July 2011)*

Species	2-base mismatch-range	1-base mismatch-range	0-base mismatch-range
<i>Acinetobacter baumannii</i>	109	109	107
<i>Acinetobacter baylyi</i>	14	14	13
<i>Acinetobacter beijerinckii</i>	3	3	3
<i>Acinetobacter bouvetii</i>	1	1	1
<i>Acinetobacter brisouii</i>	1	1	1
<i>Acinetobacter calcoaceticus</i>	99	99	96
<i>Acinetobacter calcoaceticus</i> ssp. <i>anitratus</i>	2	2	2
<i>Acinetobacter</i> genomospecies 3	8	8	8
<i>Acinetobacter</i> genomospecies 13	1	1	1
<i>Acinetobacter</i> genomospecies 13 TU	5	5	5
<i>Acinetobacter</i> genomospecies C1	1	1	1
<i>Acinetobacter gernerii</i>	1	1	1
<i>Acinetobacter grimontii</i>	2	2	2
<i>Acinetobacter guillouiae</i>	1	1	1
<i>Acinetobacter gyllenbergii</i>	3	3	3

Table 2. Continued

Species	2-base mismatch-range	1-base mismatch-range	0-base mismatch-range
<i>Acinetobacter haemolyticus</i>	9	9	9
<i>Acinetobacter johnsonii</i>	79	78	78
<i>Acinetobacter junii</i>	35	34	33
<i>Acinetobacter lwoffii</i>	41	41	41
<i>Acinetobacter marinus</i>	1	1	1
<i>Acinetobacter parvus</i>	2	2	2
<i>Acinetobacter psychrotolerans</i>	1	1	1
<i>Acinetobacter radioresistens</i>	25	24	24
<i>Acinetobacter rhizosphaerae</i>	4	4	4
<i>Acinetobacter schindleri</i>	6	6	6
<i>Acinetobacter seohaensis</i>	2	2	2
<i>Acinetobacter septicus</i>	5	5	5
<i>Acinetobacter soli</i>	4	4	4
<i>Acinetobacter</i> spp.	505	502	490
<i>Acinetobacter tandoii</i>	1	1	0
<i>Acinetobacter tjernbergiae</i>	2	2	2
<i>Acinetobacter townneri</i>	3	3	3
<i>Acinetobacter ursingii</i>	3	3	3
<i>Acinetobacter venetianus</i>	7	7	7
<i>Acinetobacter xiamenensis</i>	2	2	0
Uncultured <i>Acinetobacter</i> spp.	1071	1033	836
<i>Arthrobacter</i> spp.	2	2	1
<i>Enterobacter</i> spp.	1	1	1
<u>Phillipines-An1</u>	1	1	1
<i>Moraxella</i> spp.	1	0	0
<u>Moraxellaceae</u>	2	2	2
Other bacteria (uncultured and/or without clinical relevance)	1156	900	817

*Probe binding to be expected within the zero-mismatch range and one-mismatch range. Bold type indicates single isolates that would have been missed. Underlining indicates potential cross reaction of clinical relevance.

Hybridization conditions

Optimal binding conditions for the newly designed *Acinetobacter* probe were 30% formamide and 46 °C (data not shown) for both the microwave and the slide chamber-based procedures. Both procedures produced high to very high fluorescence intensities as subjectively determined by the investigators (Fig. 1). Although 40% formamide yielded fluorescence intensities that were sufficient for discrimination, optimal fluorescence was adjusted at 30% formamide.

In vitro results from colony material

All eight *Acinetobacter* spp. reference strains and three laboratory control strains were correctly identified by either the microwave oven or slide chamber-based FISH procedure using the Aci-16S 729 probe, while all eight non-*Acinetobacter* reference strains and seven laboratory control strains were excluded. FISH further identified all 70 clinical isolates of *Acinetobacter* without cross reacting with any of the 94 other Gram-negative clinical isolates (Table 1). Following the successful evaluation of the Aci-16S 729 FISH probe with reference strains and clinical isolates, it was used in combination with standard microbiological procedures as part of the diagnostic laboratory testing algorithm for specimens including blood cultures.

In vitro results from blood culture materials

Seven blood culture specimens that were later determined to harbor *Acinetobacter* spp. were correctly identified using the microwave oven-based FISH procedure. Additionally, the new probe did not cross react with any of the 207 blood cultures that were negative for *Acinetobacter* infection (Table 1). In three cases, *Acinetobacter* spp. were identified as a component of mixed bloodstream infections comprising *Acinetobacter* spp., *Bacteroides* spp., *Corynebacterium* spp., and *Stenotrophomonas maltophilia*, respectively. There were only one or two isolation events per year of *Acinetobacter* spp. from blood culture materials in Rostock or Hamburg, where the slide chamber-based FISH was established. Hence this particular procedure was not evaluated for blood cultures.

Discussion

In total, 404 distinct analyses were performed using FISH with the Aci-16S 729 probe and in all cases the correct result was obtained, giving a sensitivity and specificity of 100%. Frequently isolated Gram-negative bacteria that may be easily confused with *Acinetobacter* spp. were chosen as negative controls and were reliably excluded by the new probe. In particular, the Aci-16S 729 probe did not cross react with any of the 24 tested *Enterobacter* spp. or the clinically relevant *M. catarrhalis* as might have been expected from the *in silico* evaluation.

According to our data, the Aci-16S 729 probe has both excellent sensitivity and specificity in a clinical context. However, the FISH procedure using the Aci-16S 729 probe was only evaluated for medical routine diagnostics, so analytic problems in environmental samples harboring non-pathogenic *Acinetobacter* spp. cannot be excluded. According to the *in silico* evaluation, environmental *Acinetobacter* spp. strains or *Acinetobacter* spp. strains of minor clinical relevance such as *A. johnsonii*, *A. junii* or *A. radioresistens* might occasionally be missed, although such misidentifications were not observed in the *in vitro* evaluation.

Fig. 1. (A) *Acinetobacter baumannii* stained with the specific, Cy3-labeled Aci-16S 729 probe. (B, D) *Acinetobacter baumannii* stained with the FAM-labeled EUB338 probe with specificity to virtually all eubacteria as internal hybridization control. (C) *Acinetobacter baumannii* stained with a Cy3-labeled probe with specificity to *Salmonella* spp. to exclude relevant autofluorescence. (E) *Klebsiella pneumoniae* stained with the specific, Cy3-labeled Aci-16S 729 probe to demonstrate the probe's specificity. (F) *Klebsiella pneumoniae* stained with the FAM-labeled EUB338 probe with specificity to virtually all eubacteria as internal hybridization control

The Aci-16S 729 probe is genus-specific, covering all *Acinetobacter* spp. including non-pathogenic strains. Thus a preliminary identification of *Acinetobacter* spp. with the Aci-16S 729 probe has to be carefully interpreted considering the type and quality of the diagnostic specimen, (semi-)quantification on the agar plate, and the clinical state of the patient in order to avoid unnecessary treatments.

Our data strongly suggest that the Aci-16S *Acinetobacter* probe may be used for the identification of *Acinetobacter* spp. isolates directly from blood culture materials, even in blood cultures containing mixed bacteria. The rapid hybridization of *Acinetobacter* spp. and other Gram-negative bacteria in 60 min by the microwave-FISH-procedure allows for fluorescence analysis after no more than 90 min. This may result in improved clinical outcomes by allowing clinicians to initiate appropriate treatment earlier considering that multi-drug-resistant *Acinetobacter* clones are endemic worldwide [24]. The hybridization time may be reduced even further to 20 min using the slide chamber method, leading to a result in only 60 min. This is faster than biochemical methods and other molecular identification methods [1, 7–9], including the previously described *Acinetobacter*-FISH procedures [18, 19]. The newly developed probe may be used on multi-well glass slides in combination with a broader panel of previously published probes including probes for non-fermentative bacteria and Enterobacteriaceae [10, 11]. Thus it supplements the panel described by Kempf et al. [11] including Gram-negative pathogens of major relevance for blood culture diagnostics such as *Escherichia coli* and *Pseudomonas aeruginosa*.

However, prior to employing this technique as a tool for the detection of various Gram-negative bacteria in blood cultures, further evaluation with all of the respective probes is required, because many other probes have not yet been evaluated with suitably large sample panels. Further validation studies with other clinical specimens such as sterile body fluids other than blood, and even more difficult matrices such as respiratory secretions, could be conducted on an as-needed basis.

The same advantages that qualify our FISH procedure for the diagnosis of *Acinetobacter* spp. from blood cultures – its rapidity and the possibility of simultaneous use of our newly designed probe with previously described probes for easily confusable non-fermentative bacteria such as *P. aeruginosa*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Burkholderia* spp. or *Burkholderia cepacia* complex [10, 11] in one assay under standard conditions – make it suitable for the identification of Gram-negative non-fermentative bacteria from agar cultures, particularly in cases where optimized therapeutic decisions are needed in life-threatening situations.

Furthermore, detection of *Acinetobacter* by FISH may be applied in a single-probe assay for rapid discrimination of potentially multi-drug-resistant *Acinetobacter* spp. from other Gram negative, non-fermentative bacteria during suspected outbreaks. Although our FISH protocol cannot discriminate resistant and sensitive *Acinetobacter* strains, even the mere identification on genus level may strengthen the suspicion of a transmission event in the setting of an outbreak. Under outbreak conditions, a positive *Acinetobac-*

ter-FISH result may hasten the implementation of elevated infection control measures (e.g. contact precautions), preventing or stopping the spread of outbreaks. As a further benefit, if suspicious colonies grow from relevant materials such as broncho-alveolar lavage or blood culture specimens under the selective pressure of broad-spectrum antibiotic therapy in the critically ill patient on an ICU, treatment with infrequently administered antibiotic drugs such as polymyxin B can be initiated earlier in outbreak scenarios with multi-drug-resistant *Acinetobacter* spp.

Other significant advantages of FISH, in addition to the short time required for identification, are the low cost of less than 1 euro, and the simple and convenient execution, which requires neither highly trained personnel nor extensive equipment except for a fluorescence microscope. While fluorescence microscopy of blood culture material requires some experience, the analysis of colony material is simple and demands no more than a few seconds. The assay may therefore be especially useful in smaller clinical laboratories, particularly those in non-industrialized countries, where sophisticated molecular methods and MALDI-TOF-MS technology are not available.

In conclusion, due to the specificity and reliability of the Aci-16S 729 probe, FISH can be applied to blood culture materials or isolates from critical samples for the detection of *Acinetobacter* spp. The rapid nature of FISH compared with biochemical or other molecular testing makes it a potentially valuable part of the laboratory testing algorithm, particularly when time is important and the need for therapeutic decisions is urgent. FISH is a valuable supplement to more time-consuming or more expensive identification methods, especially in cases of outbreaks or septicemia.

Acknowledgements

The authors are grateful to Danuta Stefanik and Harald Seifert for providing *Acinetobacter baumannii* strains Dem1R (worldwide clone 1) and Berlin61 (worldwide clone 2). We thank the directors of our institutions Andreas Podbielski, Reinhardt Marre, Steffen Stenger, Egbert Tanich, and Bernhard Fleischer for their support.

References

- Higgins PG, Wisplinghoff H, Krut O, Seifert H: A PCR-based method to differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species 13TU. Clin Microbiol Infect 13, 1199–1201 (2007)
- Hammami S, Ghazzi R, Ben Redjeb S: Spread of multidrug resistant *Acinetobacter baumannii* in a teaching hospital. Tunis Med 86, 312–315 (2008)
- Mak JK, Kim MJ, Pham J, Tapsall J, White PA: Antibiotic resistance determinants in nosocomial strains of multidrug-resistant *Acinetobacter baumannii*. J Antimicrob Chemother 63, 47–54 (2009)
- Valenzuela JK, Thomas L, Partridge SR, van der Reijden T, Dijkshoorn L, Iredell J: Horizontal gene transfer in a polyclonal outbreak of carbapenem-resistant *Acinetobacter baumannii*. J Clin Microbiol 45, 453–460 (2007)
- Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P, Weissenbach J, Raoult D, Claverie JM: Comparative genomics of multidrug resistance in *Acinetobacter baumannii*. PLoS Genet 2, 62–72 (2006)
- Ferreira L, Vega S, Sánchez-Juanes F, González M, Herrero A, Muñoz MC, González-Buitrago JM, Muñoz JL: Identifying bacteria using a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer. Comparison with routine methods used in clinical microbiology laboratories. Enferm Infecc Microbiol Clin 28, 492–497 (2010)
- Vaneechoutte M, Dijkshoorn L, Tjernberg I, Elaichouni A, de Vos P, Claeys G, Verschraegen G: Identification of *Acinetobacter* genomic species by amplified ribosomal DNA restriction analysis. J Clin Microbiol 33, 11–15 (1995)
- Pingle MR, Granger K, Feinberg P, Shatsky R, Sterling B, Rundell M, Spitzer E, Larone D, Golightly L, Barany F: Multiplexed identification of blood-borne bacterial pathogens by use of a novel 16S rRNA gene PCR-ligase detection reaction-capillary electrophoresis assay. J Clin Microbiol 45, 1927–1935 (2007)
- Park TJ, Yoo SM, Keum KC, Lee SY: Microarray of DNA-protein complexes on poly-3-hydroxybutyrate surface for pathogen detection. Anal Bioanal Chem 393, 1639–1647 (2009)
- Hogardt M, Trebesius K, Geiger AM, Hornef R, Rosenecker J, Heesemann J: Specific and rapid detection by fluorescent in situ hybridization of bacteria in clinical samples obtained from cystic fibrosis patients. J Clin Microbiol 38, 818–825 (2000)
- Kempf VA, Trebesius K, Autenrieth IB: Fluorescent in situ hybridization allows rapid identification of microorganisms in blood cultures. J Clin Microbiol 38, 830–838 (2000)
- Peters RP, van Agtmael MA, Simoons-Smit AM, Danner SA, Vandenbroucke-Grauls CM, Savelkoul PH: Rapid identification of pathogens in blood cultures with a modified fluorescence in situ hybridization assay. J Clin Microbiol 44, 4186–4188 (2006)
- Wellinghausen N, Köthe J, Wirths B, Sigge A, Poppert S: Superiority of molecular techniques for identification of gram-negative, oxidase-positive rods, including morphologically nontypical *Pseudomonas aeruginosa*, from patients with cystic fibrosis. J Clin Microbiol 43, 4070–4075 (2005)
- Jansen G, Mooibroek JM, Idema J, Harmsen HJM, Welling GW, Degener JE: Rapid identification of bacteria in blood cultures by using fluorescently labeled oligonucleotide probes. J Clin Microbiol 38, 814–817 (2000)
- Poppert S, Essig A, Stoehr B, Steingruber A, Wirths B, Juretschko S, Reischl U, Wellinghausen N: Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. J Clin Microbiol 43, 3390–3397 (2005)
- Poppert S, Riecker M, Essig A: Rapid identification of *Propionibacterium acnes* from blood cultures by fluorescence in situ hybridization. Diagn Microbiol Infect Dis 66, 214–216 (2010)
- Poppert S, Riecker M, Wellinghausen N, Frickmann H, Essig A: Accelerated identification of *Staphylococcus aureus* from blood cultures by a modified fluorescence in situ hybridization procedure. J Med Microbiol 59(Pt 1), 65–68 (2010)
- Wong EH, Subramaniam G, Navaratnam P, Sekaran SD: Rapid detection of non-enterobacteriaceae directly from positive

- blood culture using fluorescent in situ hybridization. *Indian J Med Microbiol* 25, 391–394 (2007)
19. Peleg AY, Tilahun Y, Fiandaca MJ, D'Agata EMC, Venkataraman L, Moellering RC Jr, Eliopoulos GM: Utility of peptide nucleic acid fluorescence in situ hybridization for rapid detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa*. *J Clin Microbiol* 47, 830–832 (2009)
 20. Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadukumar, Buchner A, Lai T, Steppi S, Jobb G, Förster W, Brettske I, Gerber S, Ginhart AW, Gross O, Grumann S, Hermann S, Jost R, König A, Liss T, Lüssmann R, May M, Nonhoff B, Reichel B, Strehlow R, Stamatakis A, Stuckmann N, Vilbig A, Lenke M, Ludwig T, Bode A, Schleifer KH: ARB: a software environment for sequence data. *Nucleic Acids Res* 32, 1363–1371 (2004)
 21. Kumar Y, Westram R, Kipfer P, Meier H, Ludwig W: Evaluation of sequence alignments and oligonucleotide probes with respect to three-dimensional structure of ribosomal RNA using ARB software package. *BMC Bioinformatics* 7, 240 (2006)
 22. Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, Stahl DA: Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 56, 1919–1925 (1990)
 23. Loy A, Arnold R, Tischler P, Rattei T, Wagner M, Horn M: probeCheck – a central resource for evaluating oligonucleotide probe coverage and specificity. *Environ Microbiol* 10, 2894–2898 (2008)
 24. Higgins PG, Dammhayn C, Hackel M, Seifert H: Global spread of carbapenem-resistant *Acinetobacter baumannii*. *J Antimicrob Chemother* 65, 233–238 (2010)
 25. Frickmann H, Crusius S, Walter U, Podbielski A: Management of an outbreak with cases of nosocomial pneumonia caused by a novel multi-drug-resistant *Acinetobacter baumannii* clone. *Pneumologie* 64, 1–8 (2010)
 26. Wellinghausen N, Bartel M, Essig A, Poppert S: Rapid identification of clinically relevant *Enterococcus* species by fluorescence in situ hybridization. *J Clin Microbiol* 45, 3424–3426 (2007)