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RAPID DETECTION OF NON-ENTEROBACTERIACEAE DIRECTLY FROM POSITIVE BLOOD CULTURE USING FLUORESCENT IN SITU HYBRIDIZATION

EH Wong, G Subramaniam, P Navaratnam, *SD Sekaran

Abstract

Fluorescent in situ hybridization (FISH) was carried out using two different oligonucleotide probes specific for *Pseudomonas* spp. and *Acinetobacter* spp. These probes were tested against different organisms and were found to be highly specific. Sensitivity testing showed that the probes were able to detect as low as $10^3$ CFU/mL. In addition, FISH was carried out directly on positive blood culture samples and the detection of microorganisms took less than 2 h. We believe that FISH is a rapid method that can be used as a routine laboratory diagnostic technique for the detection of *Acinetobacter* spp. and *Pseudomonas* spp. in clinical samples.

Keywords: *Acinetobacter* spp., blood culture samples, fluorescently labeled probes

Nosocomial infections caused by gram negative bacilli have become an increasing problem worldwide.¹ This problem has escalated in tandem with the introduction and subsequent empirical administration of broad-spectrum antibiotics in hospitals. Multidrug resistant (MDR) *A. baumannii* and *P. aeruginosa* have been isolated as causative agents of a number of nosocomial infections including septicemia, ventilator- and catheter-associated pneumonia, urinary tract and wound infections.² Among these, bloodstream infections are one of the main causes of death in hospitalized patients with mortality rates between 30 and 70%.³ Thus, the rapid identification of the causative agent of septicemia is imperative in improving the overall prognosis of the patient. Routine laboratory diagnosis of positive blood cultures such as gram stain, biochemical tests and other standard bacteriological methods, all of which could take up to 48 h for an accurate identification of the pathogen to be made. In addition, culture and sensitivity assays to determine the antibiotic profile of the infecting agent could take up to an additional 24 h. Fluorescent in situ hybridization (FISH) has been used to detect various bacteria in clinical samples.⁴ The general principle of this method is the use of a fluorescently labelled oligonucleotide probe that specifically hybridizes to the target sequence of 16S rRNA, thereby enabling visualization of the whole bacterium with a fluorescence microscope. The aim of this study was to evaluate the specificity and sensitivity of FISH for the identification of *Acinetobacter* spp. and *Pseudomonas aeruginosa* directly from blood cultures.

Materials and Methods

Blood cultures positive for gram negative bacilli were obtained from the Microbiology Diagnostic Laboratory, University of Malaya Medical Centre, Kuala Lumpur, Malaysia.

A direct gram stain was performed and identification of the organisms was done using standard laboratory methods and the API20NE System (bioMérieux SA, Marcy-l’Etoile, France). An aliquot of blood was simultaneously used in a modified FISH assay.⁴ Briefly, 10-15 µL of aliquots from positive blood culture samples were applied onto glass slide, air-dried and fixed with 4% formaldehyde in 96% ethanol. The slides were dried and covered with 50 µL hybridization buffer [0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.1% SDS] containing labelled probes (10 ng/mL) prior to incubation at 50 °C for 40 min. The slides were then washed and dried prior to visualization using a fluorescence Carl-Zeiss microscope. Oligonucleotide probes, based on the 16S rRNA gene of the *Acinetobacter* spp. and *Pseudomonas* spp., were labelled with TAMRA and 6-FAM, respectively, at the 5’ end (Table). In addition, a universal eubacterial probe⁵ was used as a positive control.

The probes were tested individually against various gram negative bacteria (*Vibrio cholera*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Escherichia coli*, *Klebsiella pneumoniae*) to test their specificity. *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 15308 were included as positive controls. The sensitivity of *Acinetobacter* spp. and *Pseudomonas* spp. probes, respectively, were determined using a pure bacterial culture that was spiked into uninoculated blood culture bottles (Bectec Plus/Paeds Plus culture vial aerobic/anaerobic; Becton Dickinson, Heidelberg, Germany) in dilutions of between $10^5$ and $10^4$ CFU/mL, which were then incubated 16-20 h at 37 °C. Viable bacterial counts in the blood culture media was determined before and after incubation.
### Table: Probe sequences

<table>
<thead>
<tr>
<th>Microorganism (probe name)</th>
<th>Gene</th>
<th>Gene accession number</th>
<th>Nucleotide sequence</th>
<th>Probe size</th>
<th>Nucleotide position</th>
<th>Fluorescent tag (5' end)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acinetobacter spp.</strong></td>
<td>16S rRNA</td>
<td>Z93435</td>
<td>GCTTGCTACCGGACCTAGCGGC</td>
<td>22 bp</td>
<td>62-83</td>
<td>TAMRA</td>
</tr>
<tr>
<td><strong>Pseudomonas spp.</strong></td>
<td>16S rRNA</td>
<td>AF448034</td>
<td>ATGAAGGAGCTTGCTCCTGGATTCAG</td>
<td>26 bp</td>
<td>14-39</td>
<td>6-FAM</td>
</tr>
</tbody>
</table>

![Figure: Specificity testing of Acinetobacter spp. probe using different types of organisms. Fluorescence microscopy (×40 magnification) of gram negative bacilli from pure cultures and blood culture smears](image)

**Figure**: Specificity testing of *Acinetobacter* spp. probe using different types of organisms. Fluorescence microscopy (×40 magnification) of gram negative bacilli from pure cultures and blood culture smears.

EUB - eubacterial probe; A spp. - *Acinetobacter* species-specific probe; S. maltophilia - *Stenotrophomonas maltophilia*; V. cholera - *Vibrio cholera*; C. violaeceum - *Chromobacterium violaeceum*; B. cepacia - *Bukholderia cepacia*; P. aeruginosa - *Pseudomonas aeruginosa*
Results

Both the probes were highly specific and hybridized to the respective target genus only and not to other bacterial genera (Figure). All the bacterial strains tested could be detected using the universal eubacterial probe. Microscopic sensitivity testing with serially diluted bacterial suspension revealed a limit of detection by FISH at 10^3 CFU/mL. Thus, in our study, the sensitivity and specificity of FISH for the detection of Acinetobacter spp. and Pseudomonas spp. were 100%, respectively.

Discussion

Pseudomonas aeruginosa and Acinetobacter baumannii are the most frequently isolated non-fermentative gram negative species from critically ill and immunocompromised patients in intensive care units. Early detection of these microorganisms in clinical samples and blood can result in more definitive antimicrobial therapy. FISH is a suitable method for rapid and specific detection of pathogenic bacteria in clinical samples without time-consuming cultivation. The entire assay took less than 2 h compared to the conventional laboratory methods that require 1-3 days, resulting in a time gain of almost 70 h. Besides that, this technique delivers additional information concerning cell count and cell morphology and is an *in situ* means of differentiation of mixed infections. This could allow for an early detection of microorganisms and thus more definitive antimicrobial treatment of the infected patients could be adjusted 1 or 2 days earlier. This in turn could reduce the overall mortality among patients with gram negative bacteraemia as has been documented in previous studies.10

FISH has proven to be a powerful molecular method for identification, visualization and quantification of organisms of interest in microbial communities.11 Several reports show that FISH has already been successfully applied for the detection of *E. coli*, *H. pylori*, *Staphylococcus aureus* and *Brucella* spp.5-8 However, this is the first report on detection of acinetobacters and *Pseudomonas aeruginosa* using FISH in Malaysia.

Several studies have documented the value of molecular techniques, including PCR for amplification and detection of microbial DNA or RNA in order to identify bacteria in clinical specimens.12,13 Although PCR is a highly sensitive technique that can be used in direct identification of bacteria in blood,12,13 it may not be appropriate for daily routine work as it is time-consuming, expensive and expertise demanding compared to FISH, which is rapid and inexpensive. Furthermore, the added advantage of FISH over PCR is that extraction of DNA from bacteria is not required in the former.

In conclusion, the ability of rapid and simultaneous detection of non-*Enterobactericeae* in clinical samples within 2 h without time-consuming cultivation and identification by standard bacterial techniques may suggest FISH as an alternative method in routine diagnostic laboratory.

Acknowledgement

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