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Utility of Peptide Nucleic Acid Fluorescence In Situ Hybridization for Rapid Detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa*▽

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The utility of peptide nucleic acid fluorescence in situ hybridization (PNA FISH) for the detection of *Acinetobacter* spp. and *Pseudomonas aeruginosa* was evaluated on broth suspensions and spiked blood cultures of ATCC strains and clinical isolates with select gram-negative rods. After testing 60 clinical isolates, PNA FISH had a sensitivity and specificity of 100% and 100%, respectively, for *Acinetobacter* spp. and 100% and 95%, respectively, for *P. aeruginosa*. PNA FISH was able to detect both pathogens simultaneously and directly from spiked blood cultures.

Pseudomonas aeruginosa and *Acinetobacter baumannii* have emerged as two of the most troublesome pathogens for health care institutions globally, contributing significantly to the morbidity and mortality of hospitalized patients (8, 9). Novel, rapid diagnostics are urgently required to assist in the epidemiological control and early treatment of infection caused by these organisms (10).

Fluorescence in situ hybridization (FISH) using labeled peptide nucleic acid (PNA) probes is a methodology that has been applied for the rapid diagnosis of infectious diseases (7, 11). Due to their neutral charge, PNA probes have more-robust hybridization characteristics than those of DNA probes. They target naturally abundant rRNA molecules and, thus, allow the detection of individual microorganisms without the need for an amplification step (5). Finally, and adding to its clinical applicability, PNA FISH is less susceptible to inhibition by impurities in clinical samples and has been used effectively for the direct testing of blood, sputum, and wound cultures (2, 6, 7). Thus far, this technology has not been applied to *A. baumannii* and has only recently been described for *P. aeruginosa* (7). In this study, we assessed the effectiveness of a new, genus-specific *Acinetobacter* sp. PNA probe (AdvanDx, Inc., Woburn, MA) by testing a range of reference and clinical isolates. Given the similarity of infectious syndromes caused by *A. baumannii* and *P. aeruginosa*, we also assessed the ability of using the *Acinetobacter* sp. and *P. aeruginosa* probes (AdvanDx, Inc., Woburn, MA) simultaneously. Currently, both probes are for research use only.

The PNA FISH assay was performed according to published methods (11, 12). In brief, a 10-μl aliquot from an overnight culture (grown in brain heart infusion agar at 37°C) was mixed with 1 drop of fixation solution on a slide. After fixation, 1 drop of the probe solution was placed on the slide and hybridized at 55°C for 90 min (12). The slides were then washed for 30 min and were read using a fluorescence microscope with a dual fluorescein isothiocyanate/Texas Red filter. The final PNA FISH result was available in ~2.5 h. Two independent investigators, who were blinded to the laboratory identification of the samples, examined all slides.

Initially, a group of ATCC reference strains was assessed using the genus-specific *Acinetobacter* sp. probe and included *A. baumannii* (ATCC 19606), *Acinetobacter calcoaceticus* (ATCC 14987), *Acinetobacter haemolyticus* (ATCC 19002), *Acinetobacter lwoffii* (ATCC 15309), *Acinetobacter* sp. (ATCC 49137, originally "*Acinetobacter anitratus*"), *Escherichia coli* (ATCC 35218), *Klebsiella pneumoniae* (ATCC 10031), *P. aeruginosa* (ATCC 10145), *Pseudomonas fluorescens* (ATCC 49838), *Pseudomonas putida* (ATCC 49128), and *Pseudomonas stutzeri* (ATCC 17588). PNA FISH testing with the *Acinetobacter* sp. probe correctly identified all *Acinetobacter* ATCC reference strains and no cross-hybridization with other ATCC reference gram-negative organisms were observed. We also assessed, in a separate experiment, the same group of ATCC strains with the previously designed *P. aeruginosa* probe (7). The *P. aeruginosa* probe correctly identified *P. aeruginosa* ATCC 10145 and showed no cross-hybridization with other *Pseudomonas* species or with other ATCC reference gram-negative organisms.

Following this initial validation, the *Acinetobacter* sp. and *P. aeruginosa* PNA probes were combined and used simultaneously to test 60 recent clinical isolates. Each probe had a different fluorescent marker as follows: green (fluorescein isothiocyanate) for *Acinetobacter* spp. and red (Texas Red) for *P. aeruginosa*. The clinical isolates included 20 *Acinetobacter* sp. strains, 20 *P. aeruginosa* strains, and 20 other strains with gram-negative rods (Table 1). All isolates were obtained from different patients and were resistant to at least three antimicrobial groups. The *Acinetobacter* sp. and *P. aeruginosa* isolates were confirmed to be of different genetic types, as determined by pulsed-field gel electrophoresis, which was performed according to previously described methods (4). Organism identification and susceptibility testing had been performed in a clinical microbiology laboratory according to CLSI standards (3). Using the multiprobe PNA FISH assay on the 60 clinical isolates individually, the sensitivity and specificity for *Acineto-*

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TABLE 1. Characteristics of the 60 clinical isolates tested using the *Acinetobacter* sp. and *P. aeruginosa* PNA probes simultaneously

Isolates (no. of organisms)[a]	PNA FISH results	
	Positive for *Acinetobacter* spp. (no. of organisms)	Positive for *Pseudomonas aeruginosa* (no. of organisms)
Acinetobacter spp. (20)	20	0
A. baumannii-calcoaceticus complex (18)		
A. lwoffii (1)		
Other *Acinetobacter* sp. (1)		
Pseudomonas aeruginosa (20)	0	20
Other isolates with gram-negative rods (20)		
Escherichia coli (5)	0	1
2 ESBL producers		
Enterobacter cloacae (2)	0	0
Klebsiella oxytoca (2)	0	0
1 ESBL producer		
Klebsiella pneumoniae (5)	0	0
3 ESBL producers		
Morganella morganii (1)	0	0
Proteus mirabilis (2)	0	0
Stenotrophomonas maltophilia (3)	0	1

[a] ESBL, extended-spectrum β-lactamase.

bacter spp. were 100% and 100%, respectively, and for *P. aeruginosa*, they were 100% and 95%, respectively (Table 1). Routine laboratory organism identification was used as the gold standard. The two false-positive results were from *Stenotrophomonas maltophilia* and *Escherichia coli*, which both produced a weak red signal identified as *P. aeruginosa*. After repeat testing, these results were negative. The positive and negative predictive values were 91% and 100%, respectively, for *P. aeruginosa* detection. There was no interobserver variability.

To determine the threshold of detection for the multiprobe PNA FISH assay, serial dilutions of an overnight culture of *A. baumannii* (ATCC 19606) and *P. aeruginosa* (ATCC 10145) were tested. The bacterial densities (CFU/ml) of the dilutions were confirmed by colony counts on brain heart infusion agar. We observed that the lower limit of detection for the multiprobe PNA FISH assay was 10^4 CFU/ml for both *Acinetobacter* spp. and *P. aeruginosa*. Given that hospital-acquired and ventilator-associated pneumonia is the most common infectious syndrome caused by these organisms, such a threshold is clinically applicable (1).

To assess whether *Acinetobacter* spp. and *P. aeruginosa* could be identified simultaneously, 10 mixed bacterial broth cultures were prepared. Each mixed culture consisted of a random selection of 3 isolates from the 60 clinical isolates described above. Each mixed culture was prepared by combining 1 ml of an overnight culture of each isolate. All mixed cultures, which were tested in a blinded fashion, were correctly identified by using the multiprobe PNA FISH assay. A total of 6 of these 10 mixed cultures contained both *Acinetobacter* spp. and *P. aeruginosa* (Fig. 1A).

Finally, to determine whether this multiprobe PNA FISH assay could be used directly on positive blood cultures, we prepared artificially spiked BacT/Alert SA blood culture bottles (bioMerieux, Inc., Durham, NC), which contained 10 ml of seeded human blood (Research Blood Components, Brighton, MA). One colony of *A. baumannii* (ATCC 19606) or *P. aeruginosa* (ATCC 10145) from a fresh agar plate was used to inoculate two bottles each, and they were incubated aerobically at 37°C for 3 to 6 h before testing. Two further bottles were also inoculated with the two organisms combined. We observed

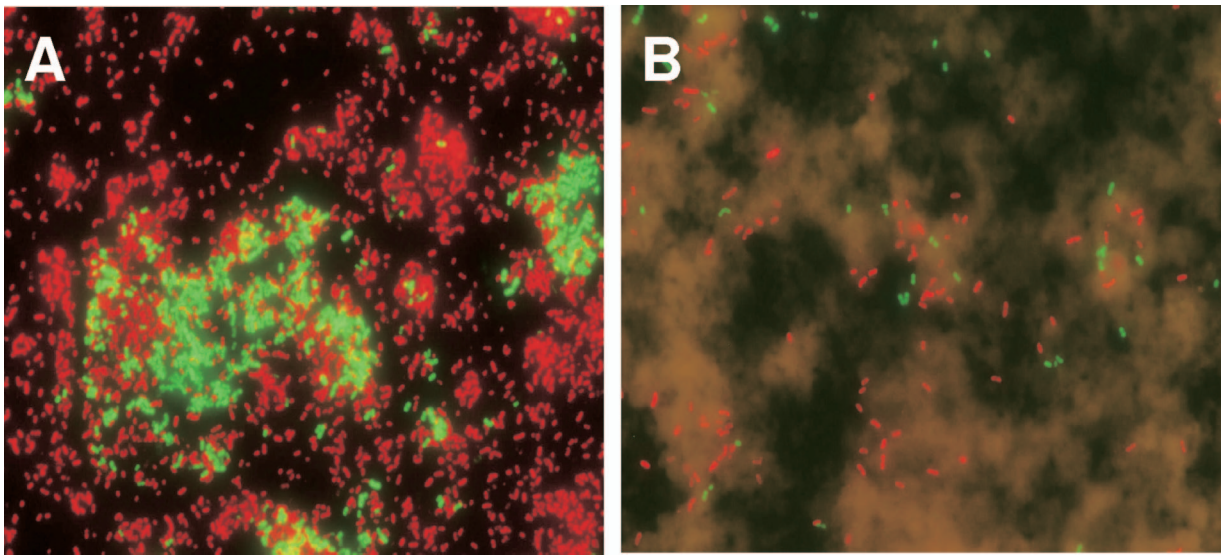

FIG. 1. FISH of a mixed culture of *Acinetobacter baumannii* (ATCC 19606) and *Pseudomonas aeruginosa* (ATCC 10145) using PNA probes specific for these organisms. Green fluorescence signifies hybridization with *Acinetobacter* spp., and red signifies hybridization with *P. aeruginosa*. A sample from an overnight broth culture (A) and a sample from a spiked blood culture bottle taken 3 h after inoculation (B) are shown, presenting evidence of both microbes and overlying red cells.

that the multiprobe PNA FISH assay was able to detect *A. baumannii* and *P. aeruginosa* from blood culture bottles either alone or when inoculated simultaneously (Fig. 1B).

PNA FISH testing is a rapid and highly sensitive and specific method for the detection of troublesome gram-negative pathogens such as *P. aeruginosa* and *Acinetobacter* spp. Importantly, the methodology has the potential to be used on direct clinical samples, and we have demonstrated its potential for pathogen detection from blood cultures. The commonality of infection types caused by these organisms, particularly pneumonia and bloodstream infection, makes simultaneous detection clinically useful. Such rapid diagnostics have the potential to not only improve therapeutic decision making but may also help optimize infection control interventions by more rapidly identifying patient or environmental reservoirs. Before the full utility of this new technology can be determined, it is important for further clinical studies to be performed.

AdvanDx, Inc., Woburn, MA, provided supplies for this project.

Potential conflicts of interest are that Y.T. and M.J.F. were employees of AdvanDx, Inc. at the time of the study.

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