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Rapid Identification of Gram-negative Bacteria in Positive Blood Culture Broth Using a Multiplex Fluorescence *in situ* Hybridization (FISH) Assay and Automated Microscopy

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AMENDED ABSTRACT

Background: In this study, the accuracy of an automated microscopy-based multiplex FISH assay to rapidly identify Gram-negative bacteria (GNB) in positive blood culture (BC) specimens was evaluated.

Methods: Clinical specimens (64) and spiked BC (16) with GNB were tested. Cultures were performed using the VERSATrek BC system, with both aerobic (46) and anaerobic (34) bottles analyzed. A 1 mL aliquot of positive specimen was centrifuged, washed, diluted 1:50, then inoculated into multiple channels of a disposable fluidic cassette. Bacteria were immobilized on the lower surface of each channel then permeabilized with ethanol. One of seven unique cocktails of ATTO-550 fluorescently labeled FISH probes for targeted species groups (Table 1) were added to separate channels as well as a universal bacterial probe. After a wash step, automated epifluorescence microscopy detected the fluorescence intensity of labeled bacteria. An acridine orange control channel detected the presence of microorganism(s). Colocalization of universal bacterial probe signal to target probe signal identified target bacteria. Results were compared to VITEK MS as the reference standard.

Results: The FISH assay exhibited 95% overall agreement with the reference method for monomicrobial cultures (Table 1), and correctly detected ≥1 organism in 7 of 7 polymicrobial samples. Eleven specimens were not included in the analysis: Four were below the detection limit, four had inconsistent counts due to operator or cassette error, two had too high background, and one was out of focus.

Conclusions: The automated microscopy-based multiplex FISH method shows promise for identification of GNB directly from positive BC broth in approximately 1 h, but further method refinements are needed.

INTRODUCTION

For patients with sepsis, the most favorable outcomes are achieved with rapid initiation of optimized antimicrobial therapy. Traditional methods for bacterial identification require overnight culturing prior to organism identification. In this study, we evaluate the performance of a novel multiplex FISH method for the expedited identification of Gram-negative bacteria directly from positive blood culture broth without the need for subculture.

METHODS

Blood culture broth from positive blood cultures obtained as part of routine clinical care and/or spiked blood cultures were evaluated (Figure 1). From each culture, a 1 mL aliquot of broth was centrifuged, washed, then diluted 1:50 with L-DOPA buffer for manual sample cleanup.

Each sample (30 µL) was manually pipetted into individual flowcell channels of a 32-channel disposable cassette. A 5-min low-voltage electric field immobilized bacteria on a poly-cationic coating on the lower surface of each flowcell. Immobilized bacterial cells were washed twice with Tris buffered saline (TBS), pH 7.2, then permeabilized by treatment with 80% ethanol for 5 min at 35°C. Samples were washed twice with ultrapure water.

Seven unique cocktails (Table 1) of ATTO-550 fluorescently labeled probes for targeted species groups and hybridization buffer (10-55% formamide, 0.01% SDS, 0.9 M NaCl, 20 mM Tris, pH 7.2) were added to separate flowcells and incubated 10 min at 52°C. All cocktails also contained an ATTO-647-labeled universal bacterial probe (EUB338). Flowcells were rinsed twice with wash buffer (0.01% SDS, 50 mM EDTA, 46-450 mM NaCl, 20 mM Tris, pH 8.2) for 5 min at 52°C. Flowcells were washed twice with TBS at room temperature, then imaged using a custom engineering prototype epifluorescence microscope with camera (Accelerate Diagnostics Inc., Tucson, AZ USA). Images were taken in 5 fields of view (Figure 2) for 100 ms and 1 s per field of view at 550 nm and 647 nm and in dark-field. Each fluorescent object was referenced to its matching dark-field object to eliminate fluorescent artifacts and exclude debris in this analysis. EUB338 and target probe signal levels and locations were compared to identify bacterial cells. Expert manual review for identification was assisted by an automated image processing algorithm that counted labeled bacterial cells for each flowcell.

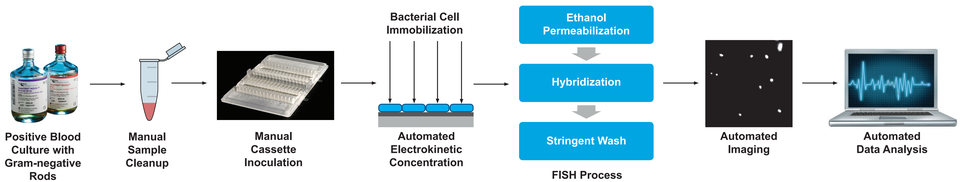

Figure 1: *Process flow including positive blood culture, manual sample cleanup, manual cassette inoculation, bacterial immobilization by automated electrokinetic concentration, FISH, automated imaging, and automated data analysis.*

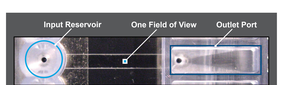

Figure 2: *Closeup of one flowcell in 32-channel flowcell cassette.*

Table 1: *FISH Assay Target Species*

FISH Assay	Target Species
Escherichia coli	*Escherichia coli*
Klebsiella spp.	*Klebsiella oxytoca + K. pneumoniae*
Enterobacter spp.	*Enterobacter aerogenes + E. cloacae*
Proteus spp.	*Proteus mirabilis + P. vulgaris*
Citrobacter spp.	*Citrobacter freundii + C. koseri*
Serratia marcescens	*Serratia marcescens*
Pseudomonas aeruginosa	*Pseudomonas aeruginosa*
Acridine orange	All bacterial DNA

For Research Use Only. Not for use in diagnostic procedures.

RESULTS

Table 2: *Monomicrobial Blood Culture Results (n=73)*

Target Species	n	Aerobic Media (n)	Anaerobic Media (n)	Clinical Specimen (n)	Spiked Specimen (n)	Detected	Not Detected
Escherichia coli	17	8	9	15	2	16	1*
Klebsiella oxytoca + K. pneumoniae	18	11	7	14	4	18	0
Enterobacter aerogenes + E. cloacae	7	5	2	5	2	6	1*
Proteus mirabilis + P. vulgaris	4	1	3	3	1	4	0
Citrobacter freundii + C. koseri	2	2	0	0	2	1	1*
Serratia marcescens	3	2	1	1	2	3	0
Pseudomonas aeruginosa	10	7	3	8	2	10	0
Non-target**	12	5	7	11	1	1[†]	11

*False negative result
**2 *Bacteroides fragilis*, 3 *Bacteroides thetaiotamicron*, 1 *Bacteroides vulgatus*, 1 *Morganella morganii*, 1 *Prevotella oris*, 1 off-panel *Proteus* spp., 3 *Stenotrophomonas maltophilia*
[†]False positive result. *Bacteroides vulgatus* detected by *Serratia marcescens* probe.

The FISH method showed an overall sensitivity and specificity of 95% and 92%, respectively for monomicrobial specimens (Table 2). One *E. coli*, one *E. cloacae*, and one *C. freundii* strain were not detected by their target probes (false-negative results), while one non-target strain was detected by the *S. marcescens* probe (false-positive result), resulting in agreement with known isolate identity in 69 of the 73 tests.

Figure 3 shows examples of images and automated analysis histograms of universal bacterial probe signal and target probe signal compared to a dark-field image from the same channel sites for a *P. aeruginosa* isolate. The universal bacterial probe detects all bacteria while the target probes detect individual bacterial species or groups of species by targeting ribosomal RNA.

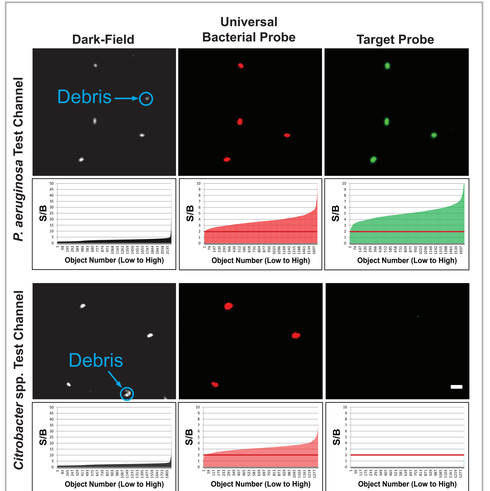

Figure 3: *Representative images and signal-to-background (S/B) intensity histograms of a P. aeruginosa sample in two different test channels with probes targeting P. aeruginosa and Citrobacter spp. Images were taken in dark-field, 647 nm (universal bacterial probes), and 550 nm (target probes). Each probe cocktail has different positive threshold criteria (red horizontal lines) for the universal bacterial probe and target probe scores. Universal bacterial probe binds to all bacterial cells to differentiate bacteria from debris. Colocalization of universal and target probe signals identifies target bacteria. Images zoomed in to view individual cells. Scale bar in lower right image is 5 µm.*

Polymicrobial results are summarized in Table 3. Each of the polymicrobial specimens contained two organisms. FISH detected 2/2 target organisms in 3/7 polymicrobial samples and 1/2 target organisms in the remaining 4 samples. The second target organism was below the detection limit in two of the polymicrobial samples, while high background prevented detection in the *Enterobacter* spp. channel, resulting in the two runs being disqualified. Figure 4 shows an example of images from a polymicrobial sample containing *K. pneumoniae* and *E. faecalis*.

Table 3: *Polymicrobial Blood Culture Results (n=7)*

Sample	VITEK MS Result	FISH Result
1	1) *Pseudomonas aeruginosa* 2) *Staphylococcus auricularis*	80% *Pseudomonas aeruginosa* 20% Non-Target
2	1) *Pseudomonas aeruginosa* 2) *Staphylococcus epidermidis*	86% *Pseudomonas aeruginosa* 14% Non-Target
3	1) *Klebsiella pneumoniae* 2) *Enterococcus faecalis*	57% *Klebsiella spp.* 43% Non-Target
4	1) *Enterobacter cloacae/asburiae* 2) *Pseudomonas aeruginosa*	100% *Enterobacter* spp.*
5	1) *Klebsiella pneumoniae* 2) *Escherichia coli*	100% *Klebsiella* spp.*
6	1) *Klebsiella pneumoniae* 2) *Enterobacter cloacae/asburiae*	100% *Klebsiella* spp.**
7	1) *Klebsiella pneumoniae* 2) *Enterobacter cloacae/asburiae*	100% *Klebsiella* spp.**

*Second target organism below detection limit
**Background too high in *Enterobacter* spp. channel to make a call. Run disqualified.

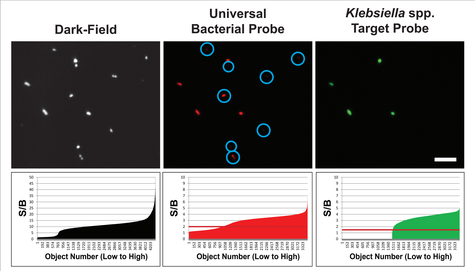

Figure 4: *Representative images of a polymicrobial sample containing E. faecalis and K. pneumoniae. E. faecalis (dim cocci, circled in blue) are stained lightly by universal bacterial probe, but do not appear in Klebsiella spp. channel. Universal bacterial probe histogram shows two populations that are stained differently, and Klebsiella spp. channel histogram shows many objects stained by the universal probe are not stained by the Klebsiella spp. probe. Images zoomed in to view individual cells. Scale bar in lower right image is 10 µm.*

CONCLUSION

A novel multiplexed FISH method accurately identified 7 bacteremia-associated species of Gram-negative bacteria directly from clinical and spiked blood cultures. This methodology has the potential to expedite the identification of pathogens causing bloodstream infections.