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Rapid Bacterial Identification Directly from Positive Blood Cultures Using Automated Sample Preparation and Multiplexed Fluorescence in situ Hybridization (FISH)



AMENDED ABSTRACT

Rapid identification of bloodstream pathogens can reduce delays in starting Background: adequate therapy, potentially decreasing morbidity and mortality in patients with sepsis. This study characterized a novel automated sample preparation method for positive blood cultures, and automated pathogen identification using multiplexed fluorescence in situ hybridization

Methods: A total of 204 known bacterial isolates were evaluated; 40 Gram-positives (16 Staphylococcus aureus, 8 S. lugdenensis, and 16 coagulase-negative staphylococci), 57 Gram-negatives (15 Acinetobacter baumannii, 12 Citrobacter spp., 14 Enterobacter spp., and 16 *Klebsiella* spp.), and 107 non-targets. Aliquots of BD BACTEC Standard Aerobic media containing healthy donor blood were seeded with 10-100 bacterial cells and incubated for 20-24 h. Culture aliquots were then processed using automated gel electrofiltration to reduce debris. Aliquots (20 µL) of each prepared sample were diluted in an electrokinetic buffer to approximately 5 x 10⁵ to 5 x 10⁶ CFU/mL. Independent flowcell channels in a disposable multichannel fluidic cassette were inoculated, and bacteria were immobilized. For FISH, each flowcell received an ATTO-647-labeled universal bacteria probe (EUB338) as well as 1 of 7 unique cocktails of ATTO-550 labeled probes for target groups. After FISH, an automated epifluorescence microscope acquired images and an automated image processor measured the intensity of detected signals in both fluorescent channels. Colocalized signals of EUB338 and the target specific probes identified target bacteria in each flowcell.

Results: The multiplexed FISH method agreed with known isolate identification in 200 of 204 tests (97% sensitivity and 99% specificity).

Conclusion: This novel method using automated sample preparation and multiplexed FISH identification, combined with automated microscopy and image analysis, accurately identified 7 clinically relevant Gram-positive or Gram-negative species directly from positive blood cultures. The platform simplifies sample preparation and allows multiple simultaneous FISH tests without manual interpretation. This method could be used to rapidly identify bloodstream pathogens, potentially allowing earlier determination of appropriate antimicrobial therapy.

INTRODUCTION

FISH is a diagnostic tool for bacterial identification. Current methods, however, require manual reagent addition and manual visual analysis of staining at high power, limiting the procedure to high concentration samples. Adding a sample preparation step allows automated image analysis as well as the ability to analyze lower concentration samples including lower respiratory bronchoalveolar lavage (BAL) specimens and blood culture. In this study, a novel automated gel electrofiltration method for sample preparation and a multiplex FISH method were characterized for bacterial identification directly from positive blood culture.

METHODS

Figure 1 summarizes the testing process. Simulated blood culture aliquots were diluted with lysis buffer to produce an inoculum containing approximately 1 x 10⁷ CFU/mL. A pipetting robot performed sample cleanup using automated gel electrofiltration. Briefly, 200 µL of inoculum were run on a 0.5% agarose gel for 20 min. Sample was recovered from the gel well and diluted to a final inoculum of 5 x 10⁵ to 5 x 10⁶ CFU/mL in L-DOPA buffer.



automated imaging, and automated data analysis.

Each sample (30 µL) was 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 peptidoglycan-targeting enzymes for 6 min and 80% ethanol for 5 min at 35°C. Samples were washed twice with ultrapure water.

Seven unique cocktails 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 3 fields of view (Figure 2) for 1 s per field of view at 550 nm and 647 nm and in dark-field.

fluorescent artifacts and exclude debris in this analysis. EUB338 and target probe signal levels and locations were compared to identify bacterial cells. An automated image processing algorithm counted labeled bacterial cells for each flowcell for identification.

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Each fluorescent object was referenced to its matching dark-field object to eliminate





Klebsiella spp. strain were not detected by their target probes (false-negative results), while one non-target strain was detected by the *Klebsiella* spp. probe (false-positive result), resulting in agreement with known isolate identity in 200 of the 204 tests.

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

Sensitivity	Specificity
16/16 (100%)	16/16 (100%)
8/8 (100%)	12/12 (100%)
14/16 (88%)	16/16 (100%)
15/15 (100%)	8/8 (100%)
12/12 (100%)	20/20 (100%)
14/14 (100%)	16/16 (100%)
15/16 (94%)	18/19 (95%)
94/97 (97%)	106/107 (99%)

CONCLUSION

A novel method using automated sample preparation by gel electrofiltration and identification by multiplexed FISH analysis accurately identified 7 bacteremiaassociated species of Gram-positive and Gram-negative bacteria directly from simulated blood cultures. Including the automated sample cleanup process and imaging multiple fields of view for each probe condition increases the sensitivity

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of the method for the analysis of low concentration samples such as respiratory specimens and blood cultures. The compatibility of this FISH method with automated robotic fluid handling, temperature control, imaging, and image analysis provides a clear path to fully automated multiplex FISH analysis from positive blood culture.

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