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Note to Authors

As of the 1st January 2016, the Clinical Microbiology Newsletter will be changing reference style. All submissions from this date should adhere to the new reference style. Please refer to the *Guide for Authors* for additional information: www.cmnewsletter.com/content/authorinfo.

Multiplexed Automated Digital Microscopy for Rapid Identification and Antimicrobial Susceptibility Testing of Bacteria and Yeast Directly from Clinical Samples

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Abstract

Traditional methods for identification and antimicrobial susceptibility testing (AST) of organisms from clinical samples typically require overnight subculturing to isolate individual species prior to phenotype-based identification (for example, biochemical testing), followed by growing isolated organisms in the presence of various antimicrobials to determine susceptibilities. Molecular and mass spectrometry identification methods can provide organism identification in a few hours directly from clinical samples, as well as resistance marker detection, but these methods do not provide the antimicrobial susceptibility information required by clinicians to inform treatment decisions. Multiplexed, automated digital microscopy is a fully automated method that can perform bacterial/yeast identification in 1 hour and AST in 5 hours directly from clinical samples, such as a positive blood culture or bronchoalveolar lavage fluid.

Basic Principles of Multiplexed Automated Digital Microscopy

Multiplexed automated digital microscopy uses a multichannel test cassette and cell immobilization to enable microscopy-based, single-cell analysis for organism identification in 1 hour and antimicrobial susceptibility testing (AST) in 5 hours directly from clinical samples. Bacterial and yeast cell-by-cell identification is performed using fluorescence *in situ* hybridization (FISH). Susceptibility reports are generated by digital microscopic observation of individual, live, growing, immobilized bacterial cells in near real time (approximately every 10 minutes) in the presence of antimicrobial agents. Antimicrobials for susceptibility testing are selected based on the organism identification result. Organisms that are not identified by a specific FISH assay (non-target organisms) are reported as detected but not identified, and susceptibility testing is not performed on these microbes. The technology enables the analysis of polymicrobial samples, and an integrated, automated sample preparation process has been developed for certain sample types. The general process flow for a fully automated system is shown in Fig. 1.

Automated Sample Preparation—Gel Electrofiltration

Automated sample preparation is performed using a process called gel electrofiltration, which is based on gel electrophoresis principles (Fig. 2). Clinical samples are automatically transferred to an agarose gel containing pores smaller than bacterial and yeast cells. The gel is immersed in an electrokinetic buffer that causes bacterial and yeast cells to carry a negative charge. When a voltage is applied, sample impurities, such as lysed blood cells and debris, pass into the gel, while the larger bacterial/yeast cells remain trapped in the well. At the end of the process, the voltage is briefly reversed to liberate the bacterial/yeast cells from the wall of the well. The purified inoculum is then pipetted into individual flow cells of a multichannel test cassette for cell immobilization and identification or AST.

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Cell Immobilization via Electrokinetic Concentration

The multichannel test cassette is composed of a transparent glass bottom and plastic top that are molded to form parallel flow cell channels. The top and bottom surfaces of each flow cell channel are coated with a layer of conductive indium tin oxide that serves as electrodes. The bottom surface has an additional cationic poly-L-lysine layer that acts as a capture surface. When inoculum is added to the flow cell, a low voltage is briefly applied that causes negatively charged bacterial/yeast cells to migrate to the lower surface, where they are immobilized and ready to undergo identification or AST (Fig. 3).

Identification by Fluorescence *in situ* Hybridization

Once cells are immobilized, a FISH assay is performed for identification. Following permeabilization and washing steps, cocktails of ATTO-532 (green) fluorescently labeled DNA probe(s) designed to bind to the rRNA of each identification target are added to different flow cells. Each cocktail also contains an ATTO-647 (red) labeled universal bacterial probe or universal eukaryotic probe for bacterial or yeast target cocktails, respectively. The universal bacterial probe binds to rRNA of all bacteria, while the universal eukaryotic probe binds to rRNA of all yeast cells. Each flow cell is imaged using an epifluorescence microscope with a camera at 532 nm and 647 nm and in dark field.

After image collection, custom image analysis software measures the signal-to-background ratio for each fluorescent and dark-field object in each flow cell. To exclude debris, only dark-field objects colocalized with universal probe signal are included in the analysis. Colocalization of target probe signal and universal probe signal identifies a target organism (Fig. 4). The software can also quantitate the number of objects in a flow cell. A universal nucleic acid stain is added to an additional flow cell as a control in order to quantitate the total number of organisms present per flow cell in the sample. Comparing the relative numbers of each target organism to objects lit up with universal bacterial, yeast, or nucleic acid probes allows the detection of non-target organisms and identification of polymicrobial samples.

Antimicrobial Susceptibility Testing

The results of the identification assay drive antibiotic selection for AST. In order to prepare for AST, the remaining sample undergoes a pre-growth step to normalize growth rates during the 1-hour FISH identification assay. Then, the concentration of organisms in the purified inoculum is determined by repeating the quantitation process with the universal nucleic acid stain described above. Based on these results, additional flow cells are filled with the purified inoculum automatically diluted to the appropriate target range for AST. Following cell immobilization, antimicrobial solutions in Mueller-Hinton agar are dispensed into the flow cells. Different antimicrobials are tested in separate flow cells, and only a single concentration of each antimicrobial is used. A growth control consisting of Mueller-Hinton agar without any antimicrobial is included for each run. A dark-field microscope and camera take time-lapse images approximately every 10 minutes of progenitor cells growing into clones of daughter cells in each flow cell. The agar ensures the daughter cells stay localized to each growing clone. Resistant clones grow, while susceptible clones arrest or lyse over time (Fig. 5). Custom image analysis software assigns unique spatial *x*-*y* coordinates to individual progenitor cells (Fig. 6), allowing it to identify each growing clone across a series of time-lapse images. The intensity of each clone can be used as a metric of clone mass in each image. By measuring over time, this

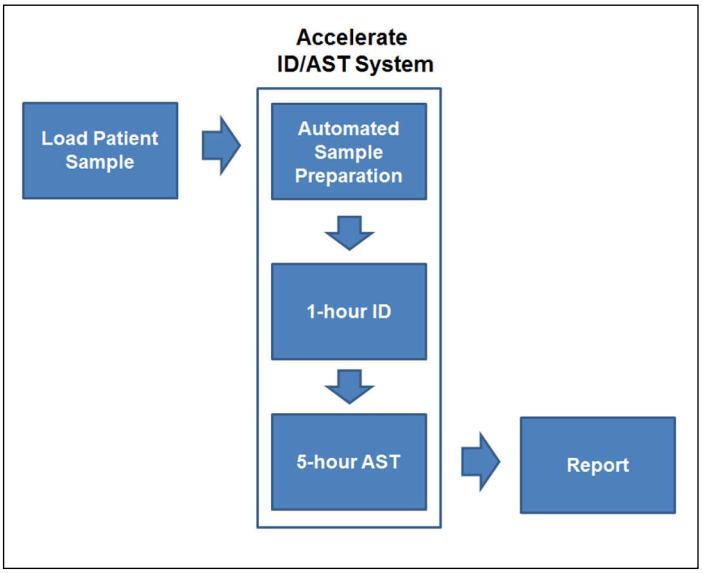

Figure 1. Fully automated multiplex digital microscopy system workflow. ID, identification.

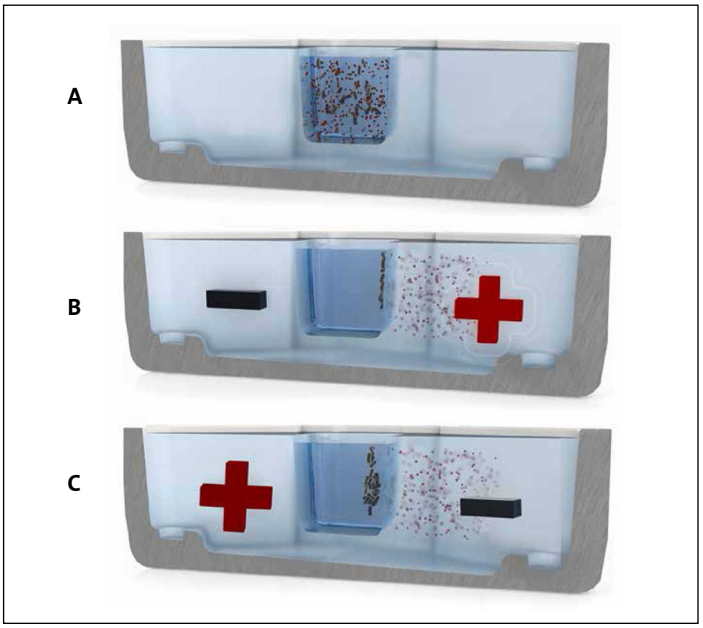

Figure 2. Sample impurities, such as lysed blood cells and debris, are separated from bacterial/yeast cells using automated gel electrofiltration. (a) Sample is loaded onto a gel with pores smaller than bacterial/yeast cells. (b) When a voltage is applied, debris migrates into the gel, leaving bacteria/yeast behind. (c) Voltage is briefly reversed to allow negatively charged bacteria/yeast to move to the center of the well for ease of retrieval.