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Rapid and Automated Specimen Preparation for Clinical Microbiology

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AMENDED ABSTRACT

Background: Pathogen-positive laboratory specimens contain many interferents such as proteins, mucus, nucleic acids, antibiotics, and multivalent ions that limit diagnostic testing. A lossless specimen preparation technique that concentrates bacteria and removes interferents could substantially improve detection capabilities.

Methods: Mock respiratory specimens containing 200 μg/mL of protein (BSA), 50 mM NaCl, and 0.003% w/v of ionic surfactant (SDS) were separately spiked with 10^3 CFU/mL cells from 23 Gram-negative and 8 Gram-positive isolates spanning 13 known MDRO-capable species. A remnant bronchoalveolar lavage (BAL) specimen and pooled 10 remnant CSF specimens were also spiked with isolates and processed. A buffer agent (final pH 7.3, 10 mM histidine) was added to all samples, and 50 µL was plated in duplicate to measure the input CFU. 60 µL was pipetted into a 3 mm long half-cylinder well (6 mm diam.) formed in a 0.5% (w/v) agarose gel slab for gel electrofiltration (GEF). The gel was electrophoresed (not submerged) at 250V for 10 minutes. The negatively charged bacterial cells migrated towards the positive electrode and were concentrated on the selectively permeable gel wall. An automated pipettor physically wiped the cells off the wall, aspirated and dispensed the volume to homogenize the suspension, and then recovered the volume for plating. Pre- and post-GEF conductivity and protein concentration (OD_{280}) measurements were used to assess prep cleanup. A 10-fold dilution series of mock specimens were Gram stained (commercial kit) with and without prep. Pooled and spiked CSF were compared between GEF and cytocentrifugation, using Gram stains.

Results: No significant CFU loss was detected: 111% isolate recovery, 90% recovery from $1.6x10^3$ CFU/mL in remnant BAL. The initial mock specimen OD_{280} and conductivity readings were 0.142 OD and 11 mS/cm respectively. In the recovered suspension, protein was at baseline (0.03 OD) and the conductivity was not significantly different than the ending run buffer (0.8 and 0.7 mS/cm respectively). The prepared specimen had a 10-fold lower Gram stain limit of detection (<10^4 CFU/mL) than the no-prep specimen.

Conclusions: Rapid specimen cleanup removed small ionic and large molecular interferents, while retaining a high percentage of the live input organisms. The process shows promise for methods requiring concentration prior to automated analyses such as MADM, PCR, MALDI-TOF, and also for routine manual procedures such as the Gram stain.

INTRODUCTION

A variety of new rapid and automated diagnostic methods require pathogen concentration in order to achieve adequate analytical sensitivity. Innovations such as multiplexed automated digital microscopy (MADM) of live cells require efficient recovery of viable cells. Traditional methods such as the Gram stain would also benefit from rapid concentration to improve sensitivity. Numerous and complex biological matrix components confound attempts to concentrate microbial pathogens. The purpose of our study was to characterize a novel method for electro-concentration and cleanup of complex clinical specimens with the potential for fast turnaround and in-line automation.

MATERIALS & METHODS

Testing used 23 Gram-negative and 8 Gram-positive bacterial isolates from 13 species able to acquire mechanisms for broad-spectrum multiple drug resistance (MDR). Aliquots of mock specimen solution containing 200 μg/mL protein (BSA), 50 mM NaCl, and 0.003% w/v of ionic surfactant (SDS) were spiked to 10^3 CFU/mL for each isolate.

Remnant bronchoalveolar lavage (BAL) specimen was spiked with *E. cloacae* to 10^3 CFU/mL for comparison with mock specimen results. 10 pooled CSF specimens were spiked to 10^5 CFU/mL with an equal mix of *P. aeruginosa* and *S. aureus* for cytocentrifuge comparison. A buffer agent (final pH 7.3, 10 mM histidine) was added to all samples. 50 µL of each sample was cultured (Cx) in duplicate to quantify input CFU.

Processing had two gel electrofiltration (GEF) pathways: concentrated capture on an excised gel patch; or recovery of the concentrated live organism suspension. Cytospin provided a comparator (***Fig. 1***).

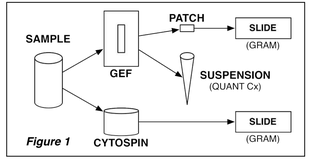

Figure 1

60 µL of sample was pipetted into a well cast in a 0.5% (w/v) agarose gel slab. Electrophoresis then followed, 10 minutes at 250V. Bacterial cells migrated toward the positive electrode. Small ions (salts, drugs, *etc.*), and large macromolecules entered the gel, but microbial cells concentrated against the gel wall. A robotic pipettor physically wiped the cells off the wall, homogenized the suspension, and recovered it for quantitative culturing. OD_{280} measured protein content. Electrical conductivity measured ionic content. Pre- and post-GEF measurements estimated sample cleanup and recovery efficiencies.

Gram stains using mock specimens were performed on a 10-fold dilution series for samples with and without GEF. Cytocentrifugation was performed by the University of Colorado Clinical Laboratory using a protocol appropriate for CSF Gram staining. An excised patch of the CSF post-GEF gel wall was directly Gram stained and compared with Gram stained cytocentrifuged slides.

RESULTS

Fig. 2 shows a top-down view of the GEF fixture, with the red electrode (left) being the anode (positive) and black (right) the cathode (negative). The red dye in the lower well has a negative charge. Blue microspheres in the upper well also have a negative charge, resembling microbial cells that are too large to penetrate into the gel. The column shows time-lapse images after switching on the power supply. The small molecules of red dye rapidly migrate through the gel. The blue microspheres (blue arrows and ellipse) begin as a diffuse suspension, then quickly compress and concentrate against the gel wall.

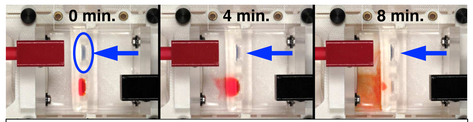

Figure 2: Time lapse GEF images. Blue microspheres (blue arrows) concentrate against the gel wall while smaller molecules (red dye) migrate into the gel.

GEL PATCH

Table 1 compares recovery from spiked CSF between cytocentrifugation and GEF (PA for *P. aeruginosa*, SA for *S. aureus*). Input spikes contained nominal 10^5 CFU/mL, and the first row of data shows the actual quantitative culture result of the spiked CSF. Average recovery counts per high-magnification field of view were converted to organisms/mm^2. ***Fig. 3*** shows Gram-stain fields for cytospin (top) and GEF direct gel slice (bottom).

TABLE 1

	PA	SA
Input CFU/mL	5.90E+04	4.50E+04
Cytospin recovery, cells/mm^2	51	26
GEF recovery, cells/mm^2	624	697
GEF/Cytospin Ratio	12.2-fold incr	27.2-fold incr

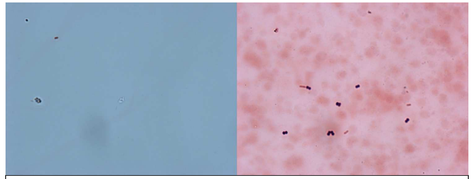

Figure 3: Gram stains for cytospin (top) and GEF gel slice (bottom). The GEF gel shows substantially greater numbers of clearly visible Gram-neg bacilli and Gram-pos cocci.

Table 2 compares direct Gram stain field counts with gel patch counts for log steps of sample content.

TABLE 2

% Positive Fields	7 Logs	6 Logs	5 Logs	4 Logs
Gel	100%	100%	100%	60%
Smear	100%	60%	20%	0%

ORGANISM SUSPENSION

Table 3 summarizes GEF recovery efficiency for mock specimens, organized to compare *Enterobacteriaceae* (eGNB, 9 species), non-fermenting Gram-negatives (NF-GNB: *P. aeruginosa* and *Acinetobacter baumannii*), *S. aureus* (SA), and coagulase-negative *Staphylococci* (CNS, 3 species).

TABLE 3

	eGNB	NF-GNB	SA	CNS	ALL
Strains	17	6	5	3	31
Recovery, mean	130%	117%	49%	70%	111%
Std Dev	62%	53%	20%	23%	60%
Range	45-313%	48-187%	21-76%	53-96%	21-313%

A BAL aliquot spiked with *E. cloacae* actually contained 188 CFU as determined by quantitative culture. GEF recovered 170 CFU, for a recovery efficiency of 90%.

Table 4 shows the GEF "cleanup" measurements for protein, charged molecules, and salts. Macromolecule removal and de-salting effectively reached the measurement limits of detection.

TABLE 4

	OD_{280}	CONDUCTIVITY
Initial mock specimen	0.142	11 mS/cm
Recovered suspension	0.03	0.8 mS/cm
Measurement baseline (detection limit)	0.03	0.7 mS/cm

Turnaround time from sample introduction to final pipette recovery averaged approximately 12 minutes.

DISCUSSION & CONCLUSIONS

Quantitative culturing using best practices to establish inoculum content has an estimated accuracy of ±0.5 $\log_{10}$ CFU (-68% +320%). This method therefore limits the accuracy of measuring recovery efficiency. Even so, there is some suggestion of higher apparent recovery for Gram-negative bacilli than Gram-positive cocci that deserves characterization. The mechanical homogenization that accompanies the GEF method may disrupt cell clusters. Slight growth during preparation may also occur. These factors may account for average recovery rates that exceed 100%. Nevertheless, the range of recovery rates falls within the tolerance of the quantitative culturing measurement method.

Although not directly measured, cytocentrifugation concentrates organisms by approximately 10-fold. GEF exceeds cytospin concentration another 10- to 25-fold. These results suggest that GEF could significantly increase sensitivity for Gram staining, and automate preparation for follow-on analyses.

Conclusions. This study demonstrated GEF feasibility for efficient recovery of live pathogen cells from mock specimens and spiked clinical specimens (BAL and CSF). The process shows promise for methods that require concentration prior to automated analyses (*e.g.* MALDI-TOF, FISH, MADM), and for routine manual procedures such as the Gram stain. It has excellent potential for automated in-line pathogen concentration and recovery without centrifugation.