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An Automated Membrane Filtration System for Direct Gram Staining

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Abstract

Background: The POCARED™'s Sample Processor (SP), is a general purpose pre-analytical device, which has the ability to concentrate any volume of a liquid sample at any concentration down to 0.1ml. Its principle mode of action is based on filtration onto a membrane filter, followed by foam extraction. SP sample processing results in a cleaner, concentrated specimen by reducing background debris without impacting the integrity of the cellular elements. The SP has the ability to perform Gram stains as an integral part of the concentrating process. The purpose of this study was to evaluate the quality and advantages of the SP integrated Gram stain function.

Methods: *Enterococcus faecalis* (EF, ATCC 19433)*, Escherichia coli (*EC*,* ATCC 25922) and *Candida albicans* (CA, ATCC 90028*)* were used to compare unprocessed Gram stained samples to Gram stains of SP processed samples. All organisms were cultured onto 5% sheep blood agar (HyLabs, Rehovot, Israel). A solution of 10^8 CFU/ml from each organism was prepared in PBS (Sigma-Aldrich, USA). Two ml of each solution were processed in the SP. The microorganisms were Gram stained while in the SP using a modified Gram stain protocol. Ten (10) µl of SP processed Gram stained samples were fixed onto microscope slides and compared to 10µl of unprocessed sample, which was manually stained using the same reagents. A minimum of 30 replicas of unprocessed and processed samples were tested for each organism.

Results: Results of the staining quality of the gram-positive cocci (EF), gram-negative bacilli (EC) and yeast (CA) of the unprocessed and SP processed were similar. However, there were increased numbers of microorganisms observed after SP processing compared with the unprocessed samples.

Conclusions: POCARED™'s Sample Processor (SP) is capable of directly Gram staining the concentrated sample of microbial cells. This resulted in increased numbers of microorganisms compared to unprocessed specimens. The staining and cell morphology of the processed and unprocessed specimens were comparable. The advantage of the Gram stained, processed sample

is the increased numbers of microorganisms, which facilitates detection and differentiation while eliminating the need to manually perform a Gram stain.

Introduction:

Use of centrifugation to concentrate and stain microorganisms in liquid specimens may result in decreased recovery, especially when microbial concentrations are low. POCARED™'s SP (Figure 1) is a general purpose pre-analytical device, which was designed to overcome the major sample processing problems: a) the sampling issue – sampling utilizes only a fraction of the entire sample volume; b) the recovery and viability of low CFU/ml samples by concentrating any particles, such as microorganism and somatic cells from liquid samples and c) high quality Gram staining. The SP has the ability of concentrating any sample volume down to 0.1 ml. and the ability of purifying and performing matrix/buffer exchange into any chosen liquids/ buffers. The SP principle mode of action is based on sample filtration on any type and size of membrane and extracting the particles that lie on the membrane surface by washing the membrane with foam tangentially, created by a mixture of gas, usually CO_2, with any type of liquid/ buffer, e.g., water or PBS that contain surfactant. The SP has the ability to perform Gram stains as an integral part of the concentrating process. The purpose of this study was to evaluate the quality and advantages of the SP integrated Gram stain function.

Figure 1: POCARED™ SP

Methods:

1. The following strains were purchased from the American Type Culture Collection: *Enterococcus faecalis* (EF, ATCC 19433), *Escherichia coli (*EC, ATCC 25922) and *Candida albicans* (CA, ATCC 90028*)* for the purpose of demonstrating Gram staining during the SP process*.* All ATCC strains were cultured onto 5% sheep blood agar.
2. A solution of 0.5 McFarland standard (10^8 CFU/ml) for each of the ATCC strains, EF, EC, and CA was prepared in PBS. Two (2) ml of each solution were processed in the SP using a cassette with 0.4µm PCTE filter membrane.
3. The microorganisms were Gram stained during SP processing using a modified Gram stain since the concentration of the acetone reagent in the BD Gram stain kit dissolved the filter membrane. Safranin was also modified as described below. All reagents were filter sterilized with 0.2µm Acrodisc® syringe filters (Pall Corp. Port Washington, NY) prior to use.
4. The filter membranes were washed with a series of solutions and type 1 water obtained from the laboratory's Mill-Q water purification system (Merck Millipore, Billerica, MA, USA), in the following order: 1 ml of Gram Crystal Violet, 50 ml water, 1 ml Gram Iodine, 20 ml water, 50 ml Decolorizer (90% EtOH, 10% acetone V/V, (Sigma-Aldrich), 20 ml water, 2 ml Gram Safranin that contained Safranin O powder 5g (TCI, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan); Ethanol 150 ml; 50 ml Methanol (Sigma-Aldrich) and 800 ml water, and last wash with 50 ml water. Subsequently the filter membranes were washed with PBS containing 0.05% Polysorbate 20 mixed with CO_2 gas in order to extract the microorganisms.
5. A 10 µl aliquot of each processed, Gram stained sample was placed on a microscope slide. For comparison, a 10µl aliquot of the unprocessed solution was fixed on a microscope slide and was Gram stained with the above reagents as well.
6. A minimum of 30 replicas of unprocessed and processed samples were tested for each organism.

Results:

To demonstrate the effectiveness of Gram staining during SP processing, three different ATCC microorganisms, which included EF, EC and CA, were tested. These organisms were selected to represent gram-positive cocci, gram-negative bacilli and yeast. The staining was done before the last stage of the SP processing, which was washing of the filter membranes with PBS containing 0.05% Polysorbate 20 mixed with CO_2 gas in order to extract the microorganisms as described above. The concentration used for each microorganism was 10^8 CFU/ml in PBS. These samples were Gram stained before and after SP processing. Ten (10) µl of each type of microorganism of

unprocessed and SP processed samples were placed on glass slides, air dried and fixed.

Results of the stained unprocessed and SP processed organisms are shown in Figure 2. Images 2A, 2C and 2E are unprocessed and Gram stained samples of EF, EC and CA, respectively. In comparison, images 2B, 2D and 2F represent EF, EC and CA, respectively, which were Gram stained during SP processing.

These images demonstrate that Gram staining during SP processing does not alter the morphology of the organisms or the Gram reaction. Although the Gram stain was modified to avoid dissolution of the membrane filter, the quality of the Gram reactions was excellent compared to the organisms stained with the unmodified, BD gram stain kit (data not shown). The SP concentrating effect is also observed since there are fewer organisms in the unprocessed samples compared to the processed samples.

Conclusions:

1. POCARED™'s Sample Processor (SP) is capable of directly Gram staining the concentrated sample of microbial cells. This resulted in increased numbers of microorganisms compared to unprocessed samples.
2. Results of the SP processed and Gram stained cells compared favorably to the unprocessed, manually Gram stained cells.
3. Images of the SP processed samples (Figures 2B, 2D and 2F) demonstrate that Gram staining during SP processing does not alter the morphology of the organisms or the Gram reaction when compared to the unprocessed samples (Figures 2A, 2C and 2E), while centrifugation has been shown to effect Gram stain results.[2]
4. The advantage of the Gram stained, processed sample is the increased numbers of microorganisms, which facilitates detection and differentiation while eliminating the need to manually perform a Gram stain.
5. A recent study reported that the clinical utility of urine Gram stain does not warrant the time or cost it requires compared to results from urinalysis.[3] However, filtration, followed by automated Gram staining, may enhance the value of the procedure as well as the results as demonstrated in this study.

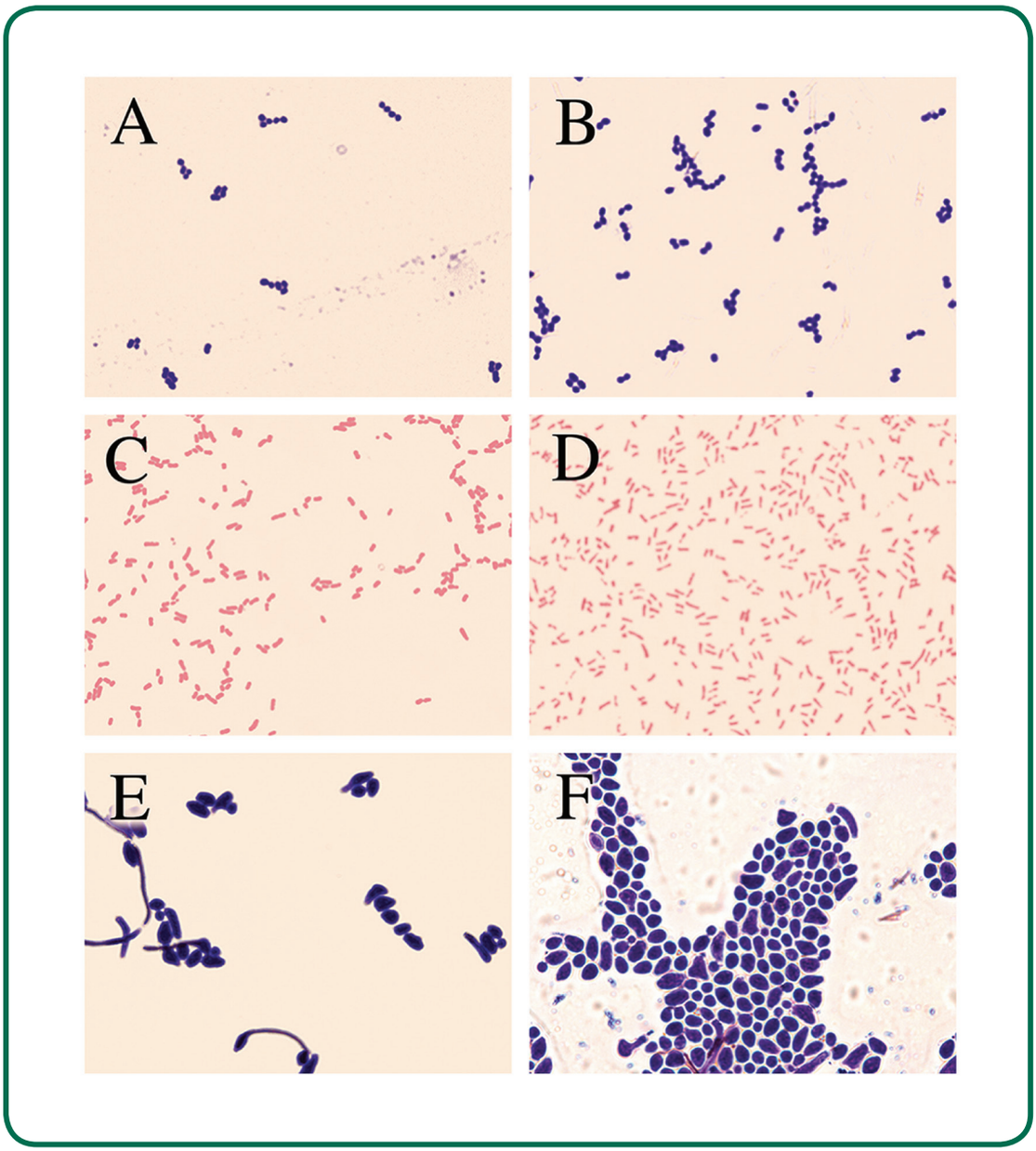

Figure 2: Gram staining of gram-positive bacteria, gram-negative bacteria and yeast. Images 2A, 2C and 2E are unprocessed samples, respectively; 2B, 2D and 2F are the same organisms following SP processing.

References:

1. Bartholomew JW, Mittwer T. 1952. The Gram stain. Bacteriol Rev 16:1-29.
2. Gauthier DW, Torres W, Meyer WJ, Lewis BG, Vernon MO, Janda WM. 1994. In vitro study of amniotic fluid Gram stains: effect of centrifugation. Infect Dis Obstet Gyn 1:282-284.
3. Cantey JB, Gaviria-Agudelo C, TeKippe EM, Doern, CD. 2015. Lack of clinical utility of urine Gram stain for suspected urinary tract infections in pediatric patients. J Clin Microbiol 53:1282-1285.