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METHOD ARTICLE

REVISED Quantification of polysaccharides fixed to Gram stained slides using lactophenol cotton blue and digital image processing [version 3; peer review: 1 approved, 1 approved with reservations]

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Abstract

Dark blue rings and circles emerged when the non-specific polysaccharide stain lactophenol cotton blue was added to Gram stained slides. The dark blue staining is attributable to the presence of capsular polysaccharides and bacterial slime associated with clumps of Gram-negative bacteria. Since all bacterial cells are glycosylated and concentrate polysaccharides from the media, the majority of cells stain light blue. The contrast between dark and light staining is sufficient to enable a digital image processing thresholding technique to be quantitative with little background noise. Prior to the addition of lactophenol cotton blue, the Gram-stained slides appeared unremarkable, lacking ubiquitous clumps or stained polysaccharides. Adding lactophenol cotton blue to Gram stained slides is a quick and inexpensive way to screen cell cultures for bacterial slime, clumps and biofilms that are invisible using the Gram stain alone. The presence of cell clumping provides a possible explanation of the presence of persisters and paradoxical points observed in Virtual Colony Count antimicrobial assays, and suggests a phenotypic resistance mechanism to antimicrobial peptides involving capsular polysaccharides.

Keywords

Antimicrobials, Biofilms, E. coli, Environmental

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Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 2

This version provides additional detail and references related to the Virtual Colony Count antimicrobial assay, breaks the text into sections with headers, and removes Virtual Colony Count raw data from [Figure 1](#). The resulting paper focuses more closely on the Blue Gram Stain itself, while providing context for its development. Finally, clumping revealed by the Blue Gram Stain is mentioned as a possible explanation of the presence of persister cells, which in turn could be related to a mechanism of bacterial resistance to antimicrobial peptide activity.

See referee reports

Introduction

The virtual colony count (VCC) microbiological assay has been used for over a decade to measure the effect of antimicrobial peptides such as defensins and LL-37 against a variety of bacteria ([Ericksen et al., 2005](#); [Zhao et al., 2013](#)). It infers antimicrobial activity based on the quantitative growth kinetics of 200 μ L batch cultures of bacteria grown in 96-well plates using a method of enumeration of viable cells ([Brewster, 2003](#)) mathematically identical to the method of enumeration of amplicons utilized by quantitative real-time PCR ([Heid et al., 1996](#)). The originally published plate configuration included a ring of 36 wells containing uninoculated Mueller Hinton Broth (MHB) capable of detecting cross-contamination ([Ericksen, 2014b](#)). There was evidence that bacteria might form clumps and biofilms during the assay, including scatter detectable by the plate reader and the presence of ubiquitous macroscopic clumping in tryptic soy broth. 10 μ L samples of cross-contamination control wells that had become turbid after VCC experiments were Gram-stained, revealing few clumps. Apparently, most clumps were not retained on the glass during the Gram stain procedure ([Gram, 1884](#)), whether fixed to the slide by heat or methanol. The application of lactophenol cotton blue, ordinarily used to visualize fungi by staining cell wall polysaccharides such as chitin, revealed circles and rings consistent with the caramelized residue of polysaccharides, which presumably included capsular polysaccharides and slime secreted concomitantly with clump and biofilm formation. These dark blue circles and rings could be consistent either with a heterogeneous subpopulation of *E. coli* or with slight contamination with a second strain.

Materials and methods

Virtual colony count

The VCC assay was conducted using the 36 edge wells to detect contamination as originally described ([Ericksen et al., 2005](#)), except a rectangular piece of Parafilm M (6 $\times$ 0.25 squares) was wrapped around the 96-well plate before the start of the 2-hour and 12-hour plate reader runs. Parafilm strips remained almost entirely intact and in place throughout the 12-hour run at 37°C and resulted in the complete absence of dust large enough to be visible using an Olympus 8Z61 crystallographic microscope on the ledge between the 96 wells and the edge of the plate, except for a single speck in one experiment observed near a crack in the Parafilm. Parafilm also prevented the visible decrease in edge well volume due to evaporation that originally necessitated excluding these wells from the experimental portion of the assay ([Ericksen](#)

et al., 2005). This evaporation also caused a slight progressive increase in optical density to a maximum ΔOD_{650} of 0.004 among the edge wells over the course of the 12-hour experiment as the Mueller Hinton Broth became more concentrated. This evaporation was too slight to affect experimental (inoculated) wells measurably or affect the linearity of the calibration curve. 10 μ L samples of edge wells were added to droplets of sterile water or media and spread on Mueller Hinton Agar, Tryptic Soy Agar, and Sabouraud's Agar plates. Colonies were analyzed by morphology, wet mounts, Gram stains, and biochemical analysis using Becton Dickinson Enteropluri Product Number 261185.

Blue Gram stain

Glass slides were scrubbed with PCMX hand soap using a pipe cleaner. 10 μ L of cells sampled from 96-well plates after VCC assays using twice-concentrated MHB in the outgrowth step were added to the slides and equilibrated to ambient humidity overnight. The slides were heat-fixed by placing the sample at the point in space at the upper tip of the inner blue flame of a Bunsen burner three times for one second each, removing the slide for one second in between ([Figure 1](#)). Ambient relative humidity was 40–60%. The slides were stained with Fluka Analytical Gram Staining Kit Product Number 77730 and again equilibrated to ambient humidity overnight in a vertical position. Becton Dickinson Lactophenol Cotton Blue Stain Droppers Product Number 261188 were applied to the Gram stained sample and digital images were captured using an Amscope light microscope at 160 $\times$, 400 $\times$ and 1600 $\times$ magnification and Toupview software. The Adobe Photoshop thresholding function was applied to the 400 $\times$ digital images using a threshold of 100. Black pixels were enumerated using the histogram function.

Results

Clumps were observed in *E. coli* cultures and in open cuvettes

Macroscopic clumps were observed in 25 mL TSB batch cultures of *E. coli* ATCC® 25922™ grown at 37°C in early exponential phase to an expected optical density at 650 nm (OD_{650}) of approximately 0.3. A 1 mL uncovered sample placed in a cuvette and cooled to room temperature rapidly formed small macroscopic clumps (up to about 1 mm in diameter), some of which exhibited motility, swimming in a synchronized wave downward to form a single large macroscopic clump (up to 1 cm long, equal to the cuvette width) at the base of the cuvette. OD_{650} plummeted up to 2% per minute, reaching equilibrium after a 10–20% decrease when placed in a room temperature HPLC detector, as cells in suspension joined the clump beneath the light path. The optical density readings declined so rapidly that only the first two digits of the four reported by the Waters 600 detector could be recorded. Observing cuvettes containing such clumps, it was apparent that cohesion, rather than adhesion, was more important, since the clumps moved downward from one corner to the other corner of the cuvette as it was rotated by hand.

Remediation of clumping and use of an open cuvette as a biosensor

Macroscopic clumping in the batch culture or cuvette outside the detector was no longer observed after four changes: 1. using

Figure 1. Quantitative growth kinetics (QKG) and the lactophenol cotton blue Gram stain. Lactophenol Cotton Blue Gram Stain Procedure. Overnight steps allowed for equilibration to the ambient humidity during summer months in the IHV building at UMB, which ranged from 40–60%. Water content and temperature may be important factors for the caramelization process to be quantitatively reproducible.

a small HEPA-filtered air purifier, 2. replacing in-house deionized Milli-Q water with purchased molecular biology grade water, 3. replacing 2×MHB prepared and autoclaved in-house using reusable jars with Teknova 2× cation-adjusted MHB, and 4. filter-sterilizing phosphate buffers made near the portable air purifier, rather than autoclaving in reusable jars. Even after these remediation measures, uncovered 1 mL samples placed in the detector for 2 hours formed a macroscopic clump at the base of the cuvette accompanied by a decrease in optical density, suggesting that at least one clumping environmental factor (CEF) was concentrated by the fan and filter within detector acting as a dust trap. Thus, 1 mL samples of *E. coli* ATCC® 25922™ served as biosensors for CEFs, and the detector served as a biosensor positive control.

E. coli cells were also motile on plates

Corner-seeking motility of *E. coli* ATCC® 25922™ was also observed on MH agar plates wrapped in Parafilm and incubated at room temperature for 2–3 weeks, as indicated by the formation of a ~1 cm-wide confluent ring around the entire edge of the plate, even though confluent areas and single colonies that originally appeared after 1–2 days were separate from the edge.

Cell clumping accompanied cross-contamination in VCC edge wells

The UMB VCC procedure was sensitive to cross-contamination in the 36 uninoculated edge wells, possibly indicating that clumping affects the particle size distribution and adhesive properties of the cells, which in turn promotes aerosol formation during pipetting (Erickson, 2014b). Figure 2 depicts cells sampled from a cross-contaminated edge well after storage at 4°C. The UCLA VCC method, with cells in 10 µL pipetted beneath 90 µL rather than a 50 µL suspension added to 50 µL as droplets from above, (Welkos et al., 2011) minimizes the probability of cross-contamination and is a safer and more effective method of transferring bacteria such as the hazardous BSL-3 pathogen *Bacillus anthracis*.

Figure 2. Blue Gram stain and thresholding results at 400x magnification. A: Blue rings indicate the polysaccharide residue of clumps of cells presumably washed from the slides during the Gram stain procedure. These polysaccharides were invisible when inspected after Gram staining and before application of lactophenol cotton blue. Other experiments produced smaller dark blue circles rather than rings. B: Thresholding results. A large majority of black pixels are contained within the polysaccharide rings.

The blue Gram stain reveals polysaccharides that are invisible after Gram staining alone

The lactophenol cotton blue Gram stain (BGS) revealed ubiquitous circular or ring-shaped structures that stained dark blue (Figure 2A). All cells stained light blue because all cells are glycosylated and concentrate polysaccharides from the media as part of their metabolism. Rare regions of indistinct blue staining were also observed, probably resulting from starch and other polysaccharides present in MHB, suggesting that the intensity of blue staining could also arise from starch and other carbohydrates with the capsular polysaccharides. MHB contains 1.5 g/L starch, plus a variety of other carbohydrates contained in beef extract. Carbohydrates, which must have included Maillard reaction (Maillard, 1912) and caramelization products, adhered to the glass in the intense heat of the fixation steps and endured on the slide throughout the Gram stain procedure. These polysaccharide residues had been invisible when these same slides were observed after Gram staining and before application of lactophenol cotton blue. The intensity of dark blue staining suggests copious capsule and slime formation.

Polysaccharide staining can be readily quantified by thresholding

Applying the thresholding technique using a threshold of 100 differentiated the dark from the light staining with little apparent background noise (Figure 2B). Thresholding of BGS images captured at 160× and 1600× magnification (Figure 3) are also possible

using the Amscope microscope. However, pixelation could add imprecision at 160× and the large size of clumps would increase variability from field to field at 1600×. TSB or MHB cultures of *E. coli* ATCC® 43827™ (ML-35) produced no macroscopic clumps under any conditions in several experiments conducted in 2013 and 2014, indicating that the observed clumping is strain-dependent.

VCC cross-contamination is ordinarily a rare event

The history of hundreds of VCC experiments at UMB between 2003 and 2014 (Erickson *et al.*, 2005; Pazgier *et al.*, 2012; Rajabi *et al.*, 2012; Wei *et al.*, 2009; Wei *et al.*, 2010; Wu *et al.*, 2005; Wu *et al.*, 2007; Xie *et al.*, 2005a; Xie *et al.*, 2005b; Zhao *et al.*, 2012; Zhao *et al.*, 2013; Zou *et al.*, 2008) clearly shows that edge wells are almost always clear, not turbid, after the 12h outgrowth phase of VCC experiments. In a 1-month period in August and September 2013, 13 quadruplicate calibration experiments were conducted using the same pipetting technique as the sextuplicate calibration experiments in the original VCC publication (Erickson *et al.*, 2005). However, in the 2013 experiments, four, rather than six, calibration curves were confined to 32 internal wells (C3-F10). These experiments used the rich media MHB, TSB or slight variations thereof. The external 64 wells (rows A, B, G and H and columns 1, 2, 11 and 12) contained two rings of contamination control wells rather than the single ring of 36 wells originally used. In these experiments conducted just outside a biosafety cabinet used for VCC experiments, none of the 832

Figure 3. Blue Gram stain results at 160×, 400× and 1600× magnification. A–C: 160×. D–F: 400×. G–I: 1600×. Cells were sampled from the edge wells of a different virtual colony count experiment than Figure 2.

contamination control wells turned turbid after the 12h incubation. Assuming clumping is caused by an environmental factor, these experiments strongly suggest that CEFs present in the laboratory environment are overwhelmingly non-culturable in rich media such as MHB or TSB. An alternate explanation of infrequent cell clumping and rare paradoxical points is that bacterial cells have a mechanism to induce clumping and biofilm formation infrequently and constitutively even in the absence of any causative agent or contaminant. If cell clumping is caused by a contaminant, several possible sources are present in the laboratory environment. In addition to viable contamination, unculturable bacteria could exert an influence upon rapidly growing *E. coli* cells. Furthermore, nucleic acids are known to cause cells to coalesce into clumps over a broad size distribution in both bacterial and mammalian cell culture. Airborne CEFs smaller than a bacterial cell could pass through the HEPA filters with little or no resistance, meaning that these molecules could have affected experiments conducted both inside and outside biosafety cabinets. Measures such as trypsinization, treatment with other proteases, and treatment with nucleases such as benzonase are commonly employed to reduce or eliminate clumping (Kruse & Patterson, 1973). For the same purpose, shear was employed in VCC calibration curves by placing pipette tips in contact with the cross-sectional corner of each well when pipetting up and down 15 times to mix (Ericksen, 2014b), although growth curves showed evidence of clumps large enough to produce measurable differences in optical density that preceded exponential growth. Clumping had no effect on the linearity of the calibration curve, possibly indicating that a small fraction of cells routinely grow as clumps and biofilms in the absence of antimicrobial agents.

Discussion

Clump formation could lead to persisters that are resistant to antimicrobial peptides

The presence of polysaccharides associated with *E. coli* ATCC® 25922™ cohesion suggests that in the conditions studied at UMB, this strain employs clumping, possibly as a defense mechanism. Forming a clump surrounded by polysaccharides could contribute to resistance to antimicrobial lectins such as defensins (Wang *et al.*, 2003) that would be bound and inhibited at the surface, limiting further inward diffusion and protecting persister cells (Ericksen *et al.*, 2005) at the center of the clump. These survivors could contribute to the deviation from simple exponential killing (Luria &atarjet, 1947) observed throughout all VCC studies at UMB of defensin activity against *E. coli*. They could also explain the presence of paradoxical data points observed occasionally throughout the history of VCC experiments at UMB. For example, the defensin HNP1 at the highest concentration of 256 µg/mL caused greater survival than 128 µg/mL in the initial VCC study (Ericksen *et al.*, 2005) MHB contains a considerable amount

(1.5 g/L) of added starch. Polysaccharides in rich media could contribute to the complete inhibition of antimicrobial peptides, which is essential for VCC assays to be capable of enumerating surviving bacteria by the QGK data analysis method. Qualitative defensin lectin activity generally follows the hierarchy: glycosylated proteins > branched polysaccharides > linear polysaccharides > oligosaccharides > monosaccharides. (Lehrer, R. I., personal communication) Bacterial slime and capsules are highly branched and contain glycosylated proteins (Wilkinson, 1958). If inhibition follows the same qualitative pattern as binding, bacterial capsular polysaccharides would be potent defensin inhibitors. Clump, biofilm and capsule formation may have evolved partially as resistance mechanisms to the ancient selection pressure exerted throughout the tree of life by antimicrobial peptides in the environment.

Clump formation suggests that glycosidase activity is essential for efficacy against persisters

A possible consequence of the inhibition of defensins by polysaccharides could be that therapies with lectin antimicrobial peptides as active ingredients would not be effective against clumps or biofilms in the absence of at least one other active ingredient that degrades the polysaccharide capsule, such as a glycosidase. Because polysaccharide structures in capsules and slime vary widely, as do glycosidase substrate specificities, any given enzyme might be active against only a narrow range of bacteria. In the absence of *in vivo* glycosidases, activity against a broad spectrum of pathogenic bacteria would therefore require an enzyme cocktail of glycosidases accompanying the lectin antimicrobial peptide or a glycosidase with unusually promiscuous substrate specificity.

Data availability

figshare: Blue Gram Stain images from three cross-contamination edge wells of a Virtual Colony Count assay at 160x, 400x, or 1600x, doi: <http://dx.doi.org/10.6084/m9.figshare.1269193> (Ericksen, 2014a).

Competing interests

No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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References

- Brewster JD: **A simple micro-growth assay for enumerating bacteria.** *J Microbiol Methods.* 2003; **53**(1): 77–86.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Ericksen B: **Blue Gram Stain images from three cross-contamination edge wells of a Virtual Colony Count assay at 160x, 400x, or 1600x.** *figshare.* 2014a.
[Data Source](#)
- Ericksen B: **Safety, efficacy and utility of methods of transferring adhesive and cohesive *Escherichia coli* cells to microplates to avoid aerosols [v2; ref status: indexed, <http://f1000r.es/4yt>].** *F1000Res.* 2014b; **3**: 267.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Ericksen B, Wu Z, Lu W, *et al.*: **Antibacterial activity and specificity of the six human (alpha)-defensins.** *Antimicrob Agents Chemother.* 2005; **49**(1): 269–275.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Gram C: **The Differential Staining of Schizomycetes in Tissue Sections and in Dried Preparations.** *Fortschritte der Medicin.* 1884; **2**: 185–189.
[Reference Source](#)
- Heid CA, Stevens J, Livak KJ, *et al.*: **Real time quantitative PCR.** *Genome Res.* 1996; **6**(10): 986–994.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kruse PF, Patterson MK: **Tissue culture: methods and applications.** Academic Press, New York. 1973.
[Reference Source](#)
- Luria SE, Latarjet R: **Ultraviolet Irradiation of Bacteriophage During Intracellular Growth.** *J Bacteriol.* 1947; **53**(2): 149–163.
[PubMed Abstract](#) | [Free Full Text](#)
- Maillard LC: **Action of Amino Acids on Sugars. Formation of Melanoidins in a Methodical Way.** *Compt Rend.* 1912; **154**: 66.
[Reference Source](#)
- Pazgier M, Wei G, Ericksen B, *et al.*: **Sometimes it takes two to tango: contributions of dimerization to functions of human α -defensin HNP1 peptide.** *J Biol Chem.* 2012; **287**(12): 8944–53.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Rajabi M, Ericksen B, Wu X, *et al.*: **Functional determinants of human enteric α -defensin HD5: crucial role for hydrophobicity at dimer interface.** *J Biol Chem.* 2012; **287**(26): 21615–27.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wang W, Cole AM, Hong T, *et al.*: **Retrocyclin, an antiretroviral theta-defensin, is a lectin.** *J Immunol.* 2003; **170**(9): 4708–4716.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wei G, de Leeuw E, Pazgier M, *et al.*: **Through the looking glass, mechanistic insights from enantiomeric human defensins.** *J Biol Chem.* 2009; **248**(42): 29180–92.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wei G, Pazgier M, de Leeuw E, *et al.*: **Trp-26 imparts functional versatility to human alpha-defensin HNP1.** *J Biol Chem.* 2010; **285**(21): 16275–85.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Welkos S, Cote CK, Hahn U, *et al.*: **Humanized theta-defensins (retrocyclins) enhance macrophage performance and protect mice from experimental anthrax infections.** *Antimicrob Agents Chemother.* 2011; **55**(9): 4238–4250.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wilkinson JF: **The extracellular polysaccharides of bacteria.** *Bacteriol Rev.* 1958; **22**(1): 46–73.
[PubMed Abstract](#) | [Free Full Text](#)
- Wu Z, Li X, de Leeuw E, *et al.*: **Why is the Arg⁶-Glu¹³ salt bridge conserved in mammalian alpha-defensins?** *J Biol Chem.* 2005; **280**(52): 43039–47.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wu Z, Li X, Ericksen B, *et al.*: **Impact of pro segments on the folding and function of human neutrophil alpha-defensins.** *J Mol Biol.* 2007; **368**(2): 537–49.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Xie C, Prahl A, Ericksen B, *et al.*: **Reconstruction of the conserved beta-bulge in mammalian defensins using D-amino acids.** *J Biol Chem.* 2005a; **280**(38): 32921–9.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Xie C, Zeng P, Ericksen B, *et al.*: **Effects of the terminal charges in human neutrophil alpha-defensin 2 on its bactericidal and membrane activity.** *Peptides.* 2005b; **26**(12): 2377–83.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Zhao L, Ericksen B, Wu X, *et al.*: **Invariant gly residue is important for α -defensin folding, dimerization, and function: a case study of the human neutrophil α -defensin HNP1.** *J Biol Chem.* 2012; **287**(23): 18900–12.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Zhao L, Tolbert WD, Ericksen B, *et al.*: **Single, double and quadruple alanine substitutions at oligomeric interfaces identify hydrophobicity as the key determinant of human neutrophil alpha defensin HNP1 function.** *PLoS One.* 2013; **8**(11): e78937.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Zou G, de Leeuw E, Lubkowski J, *et al.*: **Molecular determinants for the interaction of human neutrophil alpha defensin 1 with its propeptide.** *J Mol Biol.* 2008; **381**(5): 1281–91.
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Open Peer Review

Current Peer Review Status:

Version 3

Reviewer Report 10 July 2017

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Venkataramana Kandi

Department of Microbiology, Pratima Institute of Medical Sciences, Karimnagar, India

This technical report is very interesting. Although the author used a simple experiment to demonstrate slime production, further researches need to be done to evaluate such studies. In a clinical microbiology laboratory, this technique might well be of great use to preliminarily identify, if a microorganism has the potential to form slime or bio-film. This may help us to analyze the reason, if any, in case the patient is not responding to the antibiotic (s) currently being used.

Drawback of the study includes, uncertainties about exactly what causes such observations under this special staining modification. Also it is unknown if the smear preparation has to be taken from broth or from the colonies. In case of colonies, what should be exact method to pick, since while routine smear preparation might interfere in the demonstrability of the slime, and you can almost never see biofilm,

This character is demonstrated by bacteria only when there is a demand, example, while the bacteria is inside human, fighting immune system to establish and cause infection.

I appreciate the attempt of the author, though!

References

1. Oleksy M, Klewicka E: Capsular Polysaccharides of *Lactobacillus* spp.: Theoretical and Practical Aspects of Simple Visualization Methods. *Probiotics Antimicrob Proteins*. 2017. [PubMed Abstract](#) | [Publisher Full Text](#)
2. Kandi V: Bacterial capsule, colony morphology, functions, and its relation to virulence and diagnosis. *Annals of Tropical Medicine and Public Health*. 2015; **8** (4). [Publisher Full Text](#)

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 02 Dec 2017

Bryan Ericksen, University of Maryland School of Medicine, Baltimore, USA

Thank you for approving this brief Method Article and for your thoughtful comments. You mentioned several drawbacks of the study. I hope the revisions in Version 5 have addressed each of them.

Regarding uncertainty about what causes these observations under this special staining modification, although it seems a safe assumption that polysaccharides are being stained, it is unclear why the staining is ring-shaped. In the caption to Figure 2, I mentioned that staining produced “smaller dark blue circles” in other experiments; I have added the detail “filled-in circles” to Version 5. I also added to the “Regions of interest” section, “It is unclear why staining appeared as rings in some experiments and filled-in circles in other experiments. Perhaps subtle variations in the heat fixation or Gram stain steps resulted in clumps carrying polysaccharides from the center of each ring with them as they are washed away in some cases but not others.” This is a question that should be addressed further should these same two staining morphologies be discovered by other researchers who attempt to repeat these results.

Regarding whether smear preparations need to be taken from broth cultures or colonies, I only tested broth cultures, since my purpose was to investigate cross-contamination in VCC assays, in which cells are grown in 200 microliter batch cultures on 96-well plates. I emphasized this detail in Version 4, and it is still reflected in Version 5.

You mentioned that bacteria typically secrete slime only when there is a demand for such slime, such as in the human host fighting the immune system. I agree that slime formation may help bacteria evade the innate immune system, and it would be tempting to suggest

that one of the ways that they do so is by secreting capsular polysaccharides to bind and inhibit lectin antimicrobial peptides such as the defensin HNP1. However, since I have not demonstrated this effect in the laboratory I would prefer not to mention it in the main text of this brief Method Article, which has already been criticized by another referee as being too speculative. I did, however, cite Robert I Lehrer's studies of the lectin activities of defensins as a personal communication in the discussion. It would be helpful to emphasize that the cells in VCC cross-contamination control wells were not exposed to antimicrobial agents, and therefore the ubiquitous nature of the polysaccharide rings is somewhat surprising. I have added a sentence to this effect in the "Regions of interest" section of the results, and also mentioned that cells stained light blue appear to be planktonic.

Competing Interests: No competing interests were disclosed.

Reviewer Report 14 June 2017

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Klaus Kayser

Institute of Pathology, Charité-Universitätsmedizin Berlin, Berlin, Germany

This is a good article that describes a new method to demonstrate E. coli cultures. The physical (laboratory) technique is well described and can easily reproduced. The virtual images, segmentation methods and colony identifications are good in principle, however, they could be explained more in detail. Especially detailed discussions of Regions of Interest (ROIs), segmentation algorithms, and potential expansion to improved interpretation could remarkably improve the reader's interest. Here are some articles mentioned that describe and discuss these aims and algorithms:

Kayser, K., B. Molnar, and R. Weinstein, 2006¹

Kayser, K., *et al.*, 2016²

Sharma, H. *et al.*, 2015³

References

1. Kayser K, Molnar B, Weinstein R: Virtual Microscopy: Fundamentals, Applications, Perspectives of Electronic Tissue-based Diagnosis. *Berlin: VSV Interdisciplinary Medical Publishing*. 2006.
2. Kayser K, Borkenfeld S, Carvalho R, Djenouni A, et al.: How to analyze Structure and Function in Tissue – based Diagnosis?. *The Diagnostic Pathology Journal*. 2016; **2** (1).
3. Sharma H, Zerbe N, Lohmann S, Kayser K, et al.: A review of graph-based methods for image

analysis in digital histopathology. *The Diagnostic Pathology Journal*. 2015.

Is the rationale for developing the new method (or application) clearly explained?

Yes

Is the description of the method technically sound?

Yes

Are sufficient details provided to allow replication of the method development and its use by others?

Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?

Yes

Competing Interests: Klaus Kayser is the Editor-in-Chief for The Diagnostic Pathology Journal, where two of the recommended articles are published.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 15 Jun 2017

Bryan Ericksen, University of Maryland School of Medicine, Baltimore, USA

Thank you for your references to several interesting articles in the field of histopathology that describe sophisticated algorithms for image segmentation. The Blue Gram Stain, however, is not a histologic method, and it is important to make the distinction between a microbiological culture where objects are allowed to float freely relative to one another in solution and a histologic slide that is the result of paraffin embedding and thin sectioning, where geometry is much more relevant. It is also important to make a distinction between a region that stains dark blue as the result of the Blue Gram Stain, which presumably indicates polysaccharides such as bacterial slime, and cellular structures such as nuclei that stain darkly in histologic stains such as hematoxylin and eosin. Thresholding is the simplest form of segmentation. The more complex algorithms referenced in *The Diagnostic Pathology Journal* articles would not be applicable to the Blue Gram Stain, in which dark staining highlights relatively amorphous chemical residues, not spatially organized biological structures.

Thank you also for suggesting the addition of a detailed discussion of regions of interest. I will do so here, referring to the figshare image names. Many of the slides depict similar

fields, with rings of dark blue staining indicating polysaccharide residues fixed to the slides. A clear example of such an image is 400x-8.bmp, which shows blue rings of varying sizes, which in all cases are substantially larger than a single cell. The ring shape could indicate that a clump of bacteria had been present at that position, surrounded by a slime layer. During the subsequent steps of the Gram stain procedure, each clump was washed from the slide, carrying capsular polysaccharides in the center of the clump with it and leaving only a ring-shaped residue of slime behind on the slide. Several artifacts of the procedure are also apparent from these images. A large dark blue object is present in the lower right quadrant of image 400x-5.bmp, and a much smaller such region is apparent in the lower right quadrant of image 160x-3.bmp. These objects are the result of contamination that results from the manufacture of the glass slides used for this study, which necessitated scrubbing the slides with soap and a pipe cleaner before use. This contaminant was present in slides purchased from all five different manufacturers tested, even though the slides were marketed as “prewashed”. Scrubbing greatly reduced the frequency of this type of contamination. On rare occasion, staining appeared somewhat purple rather than blue, such as in image 160x-1.2.bmp, which presumably was the result of color distortion introduced by the microscope frame capture hardware. It is noted that the background of the slides is light blue, not white, indicating some very light staining due to the starch and other polysaccharides present in Mueller Hinton Broth; starch may also cause intermediate blue staining that does not appear to correspond to cell clumping, such as in images 160x-2 and 160x-3. Finally, black circles in images 160x-1.bmp, 160x-2.bmp, 160x-3.2.bmp and 160x-4.bmp are the result of air bubbles trapped beneath the coverslip. These can be avoided by omitting the coverslip, and must be absent from images used for quantitative digital image processing by thresholding.

Finally, when asked whether all source data was available underlying the results to ensure full reproducibility, you responded “partly”. I assure the reader that the figshare contains a comprehensive set of images. All representative images were included, even those showing experimental artifacts, and the figshare includes a fairly large set of images (51). Also, as you mentioned, the laboratory technique can be easily reproduced from the description in this article. Therefore, this set of source data should be regarded as complete.

Competing Interests: No competing interests were disclosed.

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