METHOD ARTICLE

REVISED Quantification of polysaccharides fixed to Gram stained slides using lactophenol cotton blue and digital image processing [version 5; referees: 2 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.

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REVISED **Amendments from Version 4**

This version incorporates the suggestions of the second and third referees, including the removal or further clarification of speculative interpretations of the data, an explanation of the rationale for developing a polysaccharide stain, a brief description of the Virtual Colony Count assay procedure, additional methodological details of the Blue Gram Stain procedure, mentioning that the Gram stain kit and lactophenol cotton blue droppers produced a useful assay as is with no further method development required, and a more detailed presentation of regions of interest.

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 µL 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 (TSB).

In some instances, VCC experiments resulted in one or more turbid cross-contamination wells, necessitating an investigation as a microbiological quality control measure. It was hypothesized that microscopic cell clumps could have affected the aerosol properties of the pipette cell suspensions, causing cell clumps dispensed as droplets above the experimental (internal 60) wells of the plate to inoculate adjacent cross-contamination control wells (Ericksen, 2014b). This phenomenon may have resulted in more frequent cross-contamination than had been observed in previous experiments. 10 µL 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), Briefly, A 2-fold dilution series of antimicrobial peptides, ranging from 256 to 1 µg/mL, was incubated with a standard inoculum of ~5×10^5 virtual colony forming units (CFUv)/mL *E. coli* ATCC® 25922™ at 37°C for 2 h in 10 mM sodium phosphate buffer, pH 7.4, 1% tryptic soy broth (TSB), followed by addition of twice-concentrated Mueller-Hinton broth. Bacterial growth was measured kinetically at 650 nm every 5 minutes over 12 h using a Tecan Infinite M1000 plate reader set to shake 3s orbitally before each read. Sextuplicate calibration curves were measured at a threshold change in optical density at 650 nm of 0.02. The virtual LD50 (vLD50), vLD90, vLD99, and vLD99.9 were reported as the defensin concentration that resulted in survival rates of 0.5, 0.1, 0.01, and 0.001, respectively. Measurements were done in triplicate on three separate days. The method was modified from its previously published form by wrapping a rectangular piece of Parafilm M (6 × 0.25 squares) 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 MHB became more concentrated. This evaporation was too slight to affect experimental (inoculated) wells measurably or affect the linearity of the calibration curve.

After the end of the 12-hour outgrowth phase of the VCC procedure, 96 well plates were cooled to room temperature. 10 µL samples of cross-contamination 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 µL of cells sampled from broth cultures in 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. Smear preparations originating from colonies were not tested. 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, in some experiments a cover slip was added, and digital images were captured using an Amscope light microscope at 160×, 400× and 1600× magnification and Toupview

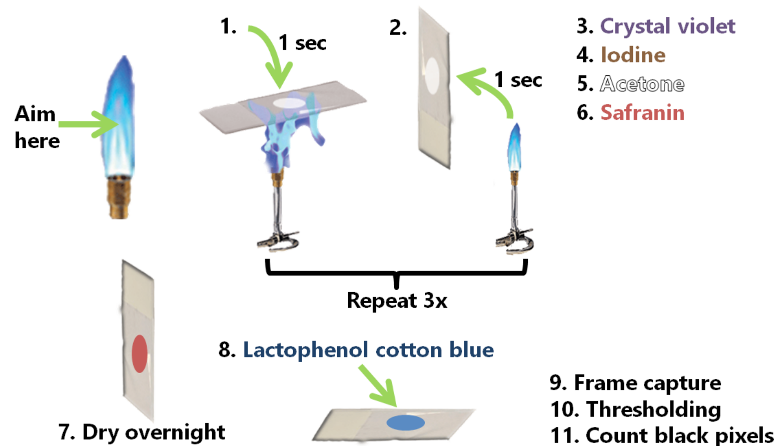

Figure 1. Lactophenol Cotton Blue Gram Stain Procedure. Overnight steps allowed for equilibration to the ambient humidity during summer months in the IHV building at UMB, which ranged from 40–60%. Water content and temperature may be important factors for the caramelization process to be quantitatively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

software. The Adobe Photoshop thresholding function was applied to the 400× 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 a small HEPA-filtered air purifier, 2. replacing in-house deionized Milli-Q water with purchased molecular biology grade water, 3. replacing TSB or 2×MHB prepared and autoclaved in-house using reusable jars with Teknova TSB or 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 (Ericksen, 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*.

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). The Gram Stain kit and lactophenol cotton blue stain droppers produced dark staining as is without the need for further method development. 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

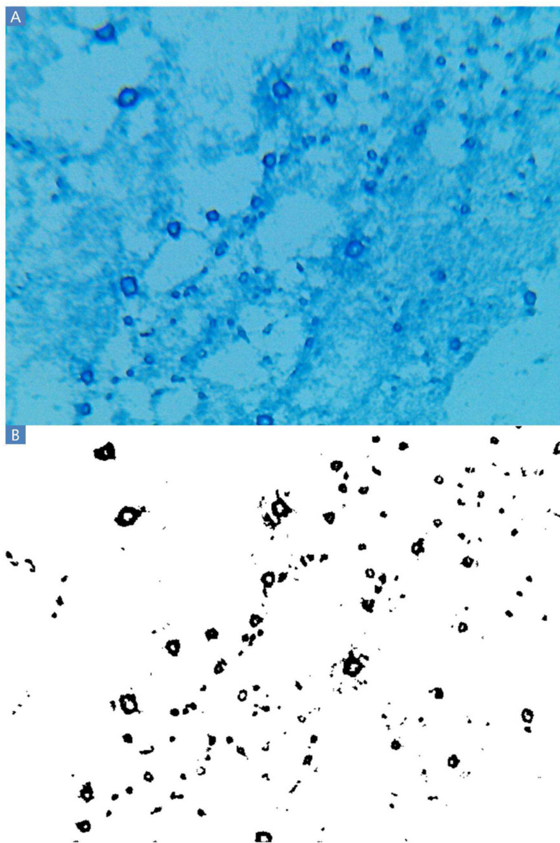

Figure 2. Blue Gram Stain and thresholding results at 400× magnification. A: Blue rings indicate the polysaccharide residue of clumps of cells presumably washed from the slides during the Gram stain procedure. These polysaccharides were invisible when inspected after Gram staining and before application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

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 carbohydrates other than 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.

Regions of interest

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 400×-8.bmp, (Ericksen, 2014a) which shows blue rings of varying sizes, which in all cases are substantially larger than a single cell. 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. However, cells stained light blue appeared to be planktonic. 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. 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. 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 400×-5.bmp, and a much smaller such region is apparent in the lower right quadrant of image 160×-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 160×-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 160×-2.bmp and 160×-3.bmp. Finally, black circles in images 160×-1.bmp, 160×-1.2.bmp, 160×-3.2.bmp and 160×-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.

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 (Ericksen *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

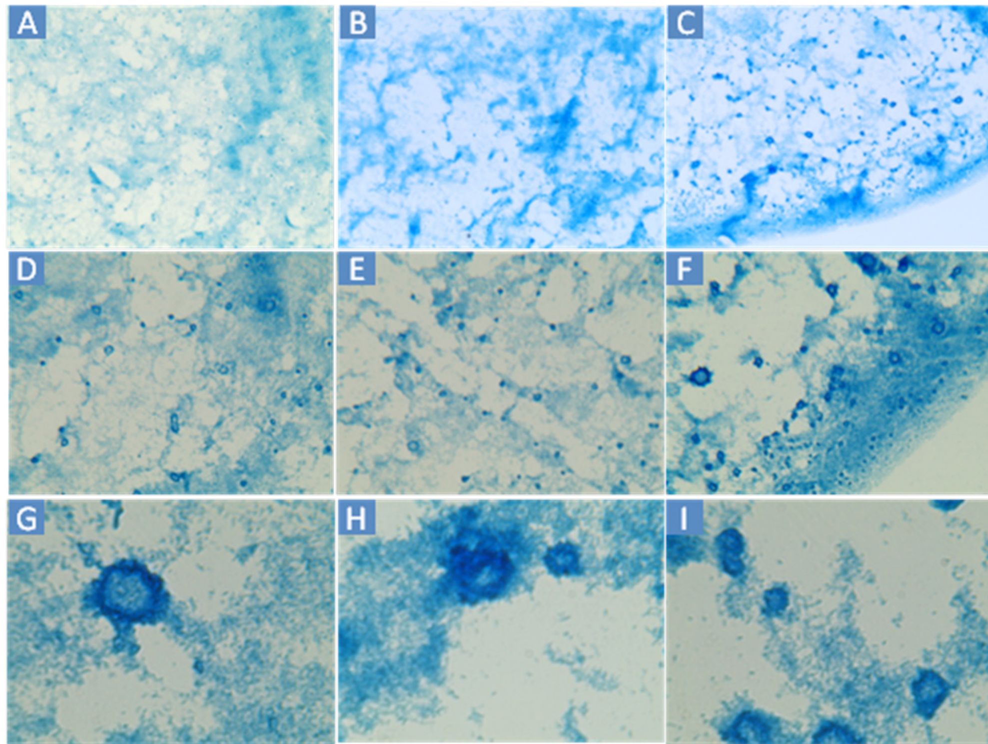

Figure 3. Blue Gram Stain results at 160×, 400× and 1600× magnification. A–C: 160×. **D–F**: 400×. **G–I**: 1600×. Cells were sampled from the edge wells of a different Virtual Colony Count experiment than Figure 2.

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 (Ericksen *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 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

Validity of the thresholding step

The image thresholding step resembles other segmentation techniques commonly employed to analyze images in the field of histopathology. 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 image segmentation. The more complex algorithms referenced in the histopathology digital image processing literature would not be applicable to the Blue Gram Stain, in which dark staining highlights relatively amorphous chemical residues, not spatially organized biological structures. Simply counting black pixels to obtain a rough measure of the quantity of darkly stained polysaccharides in an image, however, is a method of quantification that remains valid even where the spatial organization of black pixels is irrelevant.

Clump formation could protect cells from 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 protect cells from antimicrobial lectins such as defensins (Wang *et al.*, 2003) that would be bound and inhibited at the surface, limiting further inward diffusion and protecting cells (Ericksen *et al.*, 2005) at the center of the clump. These survivors could contribute to the deviation from simple exponential killing (Luria & Latarjet, 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 quantitative growth kinetics 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 a response to the ancient selection pressure exerted throughout the tree of life by antimicrobial peptides in the environment. Although this relationship between capsular polysaccharides and antimicrobial peptide activity remains speculative at this point, deviations from simple exponential killing such as bimodal survival curves and paradoxical points have been unequivocally demonstrated repeatedly throughout over a decade of VCC experiments measuring the antimicrobial activity of lectin antimicrobial peptides such as the defensin HNP1.

Data availability

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

Competing interests

No competing interests were disclosed.

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Version 5

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I am satisfied with the changes the authors made to the manuscript in this version and would recommend it for publication.

Competing Interests: No competing interests were disclosed.

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

Version 4

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William R. Jacobs
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The manuscript entitled "Quantification of polysaccharides fixed to Gram stained slides using lactophenol cotton blue and digital image processing" is a potentially interesting story that may have promise in identifying persister organisms, but the work is far too premature for acceptance. The author states in the abstract that "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." This statement suggests that the author is observing the presence of persister cells that were first described by Gladys L. Hobby when she treated *Streptococci* with penicillin in which a subpopulation of a cells that were phenotypically resistant to penicillin action. Kim Lewis and others have shown that penicillin will reveal the presence of such persisters in *E. coli* as well. The author could get persistent mutants of *E. coli* from Kim Lewis that would allow them to explore their stain in a much more quantitative and rigorous way. It would be of interest to demonstrate if the microbial peptides reveal the presence of persisters in a fashion similar to penicillin. None of these experiments are included. Moreover, the conclusion in the discussion quotes that "clump formation suggest that glycosylate activity is essential for efficacy against persisters" which is clearly not substantiated by any of the data provided. Again, the author would benefit greatly by including

mutants defective in capsule formation or defective in entering into the persister state to test if their blue stain is detecting persisters.

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

Is the description of the method technically sound?
Partly

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

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?
No

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 02 Dec 2017

Bryan Ericksen, University of Maryland, USA

Thank you for your thoughtful review and for mentioning the fascinating work of microbiologists who have studied persisters. Your comments indentify three shortcomings of Version 4 of this Method Article: the work is too premature for publication, speculation regarding persisters, and speculation regarding glycosidase activity. I have addressed all three shortcomings in Version 5, and I would like to respond to your comments in reverse order.

Regarding the suggestion that glycosidase activity could be essential for the activity of a lectin antimicrobial peptide therapy to be effective against persisters, that paragraph was entirely speculative and has been removed from the discussion. I should explain that I did demonstrate in the laboratory that RNA inhibits antimicrobial peptide activity and the addition of ribonuclease to the VCC assay increased activity, as I mentioned in a poster presented at the 2014 ICAAC meeting and published on this website (https://f1000research.com/posters/1097133), but I did not have time to demonstrate the analogous effect with polysaccharides and glycosidases. I believe such experiments would be of interest to the antimicrobial peptide field in the future, but including these ideas in the main text of this article is admittedly premature.

Regarding persisters, Robert I. Lehrer and I speculated in the initial VCC paper (Ericksen *et al.,* 2005) that bimodal survival curves were consistent with a small subpopulation of phenotypically resistant cells. I cited a publication entitled, “Bacterial persistence as a phenotypic switch” by

Balaban et al. (Science 2004 Sep 10;305(5690):1622-5). That article cited Kim Lewis ("Persister cells and tolerance to antimicrobials", FEMS Microbiol Lett 2004 Jan 15;230(1):13-8). The abstract of that article begins, "Bacterial populations produce persister cells that neither grow nor die in the presence of microbicidal antibiotics." Dr. Lehrer showed that *E. coli* cells that do not grow are not killed by defensins. (J Clin Invest 1989 Aug;84(2):553-61.) Therefore, since persisters do not grow, one would expect persisters to be phenotypically resistant to defensins. Had I asked Dr. Lewis for mutant persister *E. coli* strains in 2004, I would have had time to address the issue in the laboratory, but I doubt the Blue Gram Stain procedure would have occurred to me then. In this article I wish to suggest that persisters might also be responsible for paradoxical data points in VCC assays. Because the involvement of persisters is speculative, I am tempted to remove all mention of them from Version 5 as I did with glycosidases above. However, in this case, I see little harm in citing and slightly extending speculation that has already been published in 2005, especially since paradoxical data points have been unequivocally demonstrated repeatedly in that VCC publication and others cited in this article. Doing so might benefit the antimicrobial peptide field, because it identifies a remaining poorly studied phenomenon that has emerged from VCC studies: the presence of paradoxical data. Doing so also partially explains my motivation for seeking to stain polysaccharides in the first place. However, in order to underscore that this discussion of persisters is speculative, I have removed the mention of them from the abstract. I have also changed the title of the relevant section of the discussion from "Clump formation could lead to persisters that are resistant to antimicrobial peptides" to "Clump formation could protect cells from antimicrobial peptides", since the resistance mechanism is also speculative at this point. I have also removed speculative terms from the remainder of that paragraph. Finally, to clearly identify this line of thinking as speculation, I have added the sentence, "Although this relationship between capsular polysaccharides and antimicrobial peptide activity remains speculative at this point, deviations from simple exponential killing such as bimodal survival curves and paradoxical points have been unequivocally demonstrated repeatedly throughout over a decade of VCC experiments measuring the antimicrobial activity of lectin antimicrobial peptides such as the defensin HNP1." It is that long history of VCC experimentation that should give me the ability to speculate in an informed and appropriate manner along these lines.

Regarding your suggestion that publishing this article is premature, I fully understand and agree with your reservations. I wish I had time for further experiments, but I left the University of Maryland in 2014 and I am no longer able to conduct the interesting experiments using bacterial mutants that you proposed. However, I should emphasize that this article is not a Research Article, but rather a Method Article. It is not intended as a thorough study of the phenomenon or persistence, antimicrobial peptide resistance mechanisms, or any other research topic in the field of microbiology, but rather it is a brief report of a novel microbiological method. Strictly as a description of a new way to visualize and quantify polysaccharides, this paper is complete as is because the invention it describes has already been reduced to practice. Utilizing the Gram stain kit and lactophenol cotton blue droppers in succession produced useful dark polysaccharide staining with no further modifications. To emphasize this fact, I have added the sentence "The Gram Stain kit and lactophenol cotton blue stain droppers produced dark staining as is without the need for further method development" to the Results section. That is not to say there are no possible avenues for improvement of the method. The main purpose of phenol in lactophenol cotton blue is to kill fungi that come in contact with the stain; this feature is unnecessary when studying bacteria heat-fixed to a glass slide, which have already been killed. Replacing lactophenol with lactoglycerol might work just as well. Also, it is unclear which if any of the four Gram stain steps in Figure 1 (steps 3-6) are essential for the stain to result in dark blue polysaccharide staining. Growing cells in TSB or other media without a high concentration of

starch (unlike MHB) might alleviate some of the artifacts of nonspecific blue staining mentioned in the “Regions of interest” section of the article. However, testing all possible permutations of a new invention should certainly not be required in order to publish that invention in the peer-reviewed literature. This article presents a novel useful method for polysaccharide staining that can be performed easily using materials already present in many microbiology laboratories. As such, the Blue Gram Stain should be publishable in this form. I look forward to reading further method articles published by others that improve upon this simple assay.

When asked whether the rationale for developing the new method was clearly explained, you responded “Partly”. To provide a fuller explanation, the introduction has been split into two paragraphs, the second of which beginning with, “In some instances, VCC experiments resulted in one or more turbid cross-contamination wells, necessitating an investigation as a microbiological quality control measure. It was hypothesized that microscopic cell clumps could have affected the aerosol properties of the pipette cell suspensions, causing cell clumps dispensed as droplets above the experimental (internal 60) wells of the plate to inoculate adjacent cross-contamination control wells. This phenomenon may have resulted in more frequent cross-contamination than had been observed in previous experiments.” I mentioned this hypothesis in another F1000Research paper (https://f1000research.com/articles/3-267/v2), which has been cited in this paper. I should note that my curiosity regarding persisters and paradoxical points was also a motivating rationale for developing the new method, as I mentioned above, and that is another reason why I have left this rationale as part of the discussion section in Version 5.

When asked whether the description of the method is technically sound, and whether sufficient details are provided to allow replication of the method development and its use by others, you responded “Partly” to both questions. I feel the method can be easily followed from the clear depiction in Figure 1. Further instructions are contained in the product literature of the Gram stain kit and lactophenol cotton blue droppers. I have rarely seen product instructions quoted in the peer-reviewed literature, so I would prefer not to repeat that information here. To improve the technical quality of the description of the method, I have modified the Blue Gram Stain portion of the Results section as mentioned above, which states that no method development was required; the two products used in succession worked as is. I mentioned that in some experiments, a cover slip was added. I also realize that most readers would not be familiar with the Virtual Colony Count method; therefore, I have added a more thorough description of it to the Materials and Methods section, which is roughly twice as long in Version 5 compared to Version 4.

When asked whether the conclusions about the method and its performance are supported adequately by the findings presented in the article, you responded “no”. It appears that your answer results from my inclusion of speculation regarding persisters, glycosidases, and resistance mechanisms that has been removed from Version 5, as detailed above. Strictly speaking, the conclusion that this method can be used to identify and quantify polysaccharides as described by the title and abstract of Version 5 is adequately supported for a Method Article.

Relevant to these comments, I feel I should explain my choice of journal for this work. F1000Research differs from traditional journals in many respects, but one of the most important is its stated purpose to publish results from conferences that would otherwise never be found in the peer-reviewed literature, that may in some sense be preliminary in nature. This article is a more thorough description of the “simple assay” that I presented as a poster at the asm2014 conference, which is published elsewhere on this website (https://f1000research.com/posters/1096823). I am grateful for the opportunity to share the poster in this form. You will notice that it is packed densely

with small text to give a thorough description of the method, but I could only do so much with the space available. Expanding the description of the method to the length of this Method Article has benefitted it greatly, and the quality of the article has also benefitted from the many suggestions of the three referees. Another advantage of F1000Research is the requirement to publish all raw data, which gave me the ability to be unusually thorough by submitting to the figshare a collection of 51 images at full resolution, far too large a body of work to publish as figures in most traditional journals. I hope you agree that the unique features of F1000Research make it an ideal journal for this Method Article.

Competing Interests: No competing interests were disclosed.

Version 3

Referee Report 10 July 2017

doi:10.5256/f1000research.12462.r23949

Venkataramana Kandi
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This technical report is very interesting. Although the author used a simple experiment to demonstrate slime production, further researches need to done to evaluate such studies. In a clinical microbiology laboratory, this technique might well be of great use to preliminary 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!

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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 have read this submission. I 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, 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.

Referee Report 14 June 2017

doi:10.5256/f1000research.12462.r22739

Klaus Kayser
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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, 2006[1]

Kayser, K., *et al*., 2016 [2]

Sharma, H. *et al.,* 2015 [3]

References

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3. Sharma H, Zerbe N, Lohmann S, Kayser K, Hellwich O, Hufnagl P: 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 have read this submission. I 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, 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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