A Simple Assay To Quantify Polysaccharides Associated With Clumps Of *Escherichia coli* Cells Using Lactophenol Cotton Blue And Digital Image Processing

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Abstract

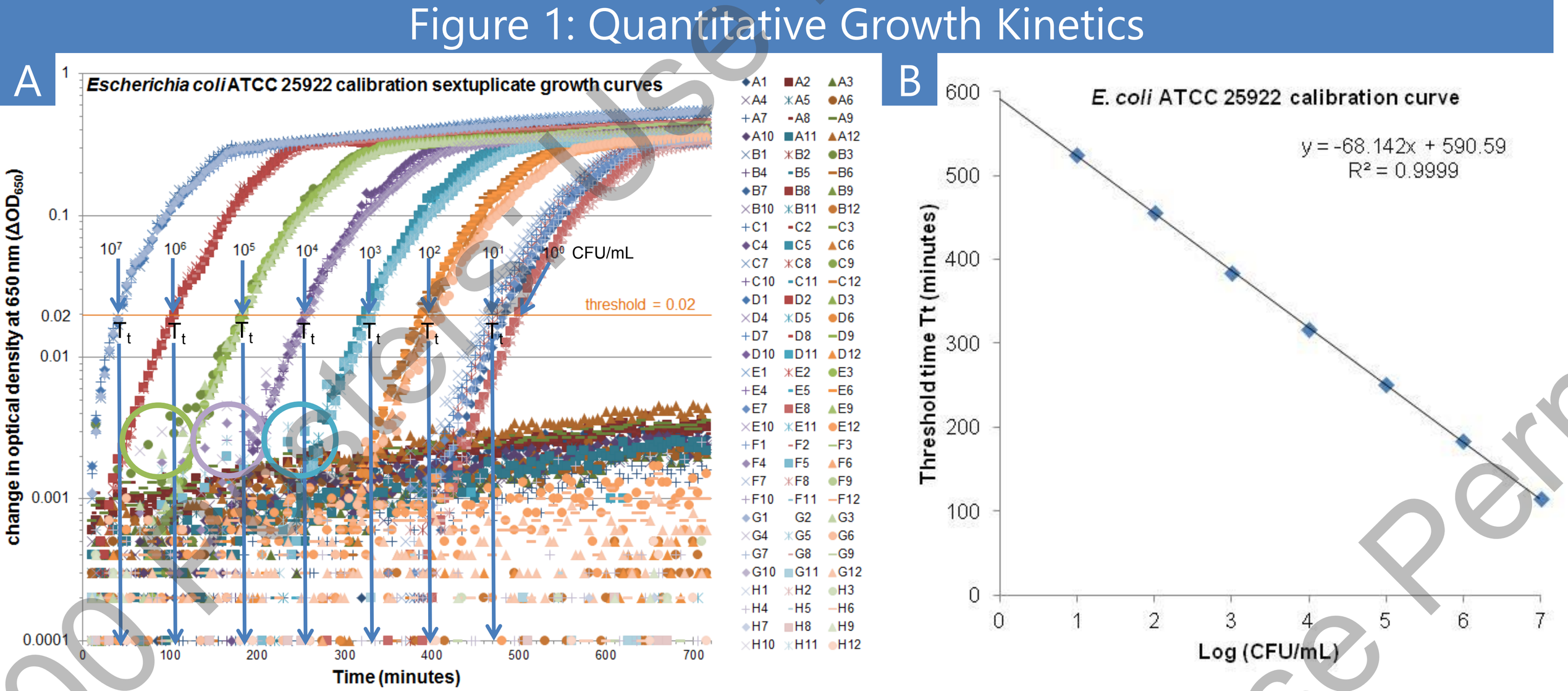
We discovered that *Escherichia coli* ATCC® 25922™ cells stain dark blue when the nonspecific polysaccharide stain lactophenol cotton blue is added to Gram stained slides. We attribute dark staining to the presence of polysaccharides that could contribute to the survival of persister cells, revealing the molecular basis of a mechanism of resistance to antimicrobial peptides such as defensins that are lectins with high affinity for polysaccharides. Since all bacterial cells are glycosylated, all cells stain light blue. The contrast between dark and light staining is sufficient to enable a digital image processing thresholding technique to be quantitative for circular or ring-shaped structures that imply the presence of capsular polysaccharides. We demonstrate the utility of the method by determining variation in dark staining observed among cells used for Virtual Colony Count antibacterial assays. This technique could potentially apply to a broad range of bacteria that secrete polysaccharide capsules and form clumps and biofilms.

Introduction

The Virtual Colony Count (VCC) antibacterial 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. Because these peptides are strongly inhibited by rich media, VCC differs from minimum inhibitory concentration (MIC) methods in which the assay consists of a single incubation of all cells and reagents added at the start of the assay. The VCC procedure first exposes bacterial cells to the active antimicrobial agent in 10 mM sodium phosphate pH 7.4 for two hours, then twice-concentrated broth is added to simultaneously inhibit antimicrobial activity and induce exponential growth. The growth kinetics of surviving cells is then monitored using a temperature-controlled plate reader. The method was initially developed by Robert I. Lehrer of the University of California Los Angeles (UCLA) using twice-concentrated tryptic soy broth (2XTSB) as the outgrowth media. However, the commonly used antimicrobial susceptibility testing (AST) positive control strain *E. coli* ATCC® 25922™ formed macroscopic clumps in TSB up to several millimeters in diameter when grown in the Institute of Human Virology building at the University of Maryland Baltimore (UMB). The same strain formed an apparently homogeneous suspension with no clumps visible to the unaided eye when Mueller-Hinton Broth (MHB) was substituted for TSB, so 2XMHB was chosen as the outgrowth media in VCC studies of *E. coli* ATCC® 25922™ at UMB published between 2005[1] and 2013[2].

The method of enumeration[3] of surviving cells used by VCC is termed quantitative growth kinetics (QGK). It relates the time taken for the turbidity of a bacterial batch microbiological culture in a well of a 96-well microplate to reach a threshold difference in turbidity to a 10-fold dilution series, often conducted as a separate sextuplicate calibration experiment (Figure 1A). Quantification of the number of viable cells follows an algorithm mathematically identical to quantitative real-time polymerase chain reaction (QPCR) as described by Heid et al. in 1996[4], except with QGK, cells, rather than copies of PCR products, grow exponentially. The time taken to reach the threshold is called the "threshold time", T_t, which is equivalent to the QPCR value "cycle time" or C_t. While calibration curve linear regression R^2 values were usually >0.999 (Figure 1B), scatter below a ΔOD_{650} of 0.01 (Figure 1A, green, lavender and blue points and circles) suggested the presence of microscopic bacterial clumps. Gram stains of both TSB and MHB cultures revealed far fewer clumps than expected, given that a broad size distribution should accompany the intermediate steps leading to macroscopic clumps. Apparently, clumps were washed off during the Gram stain procedure, whether fixed to the slide by heat or methanol.

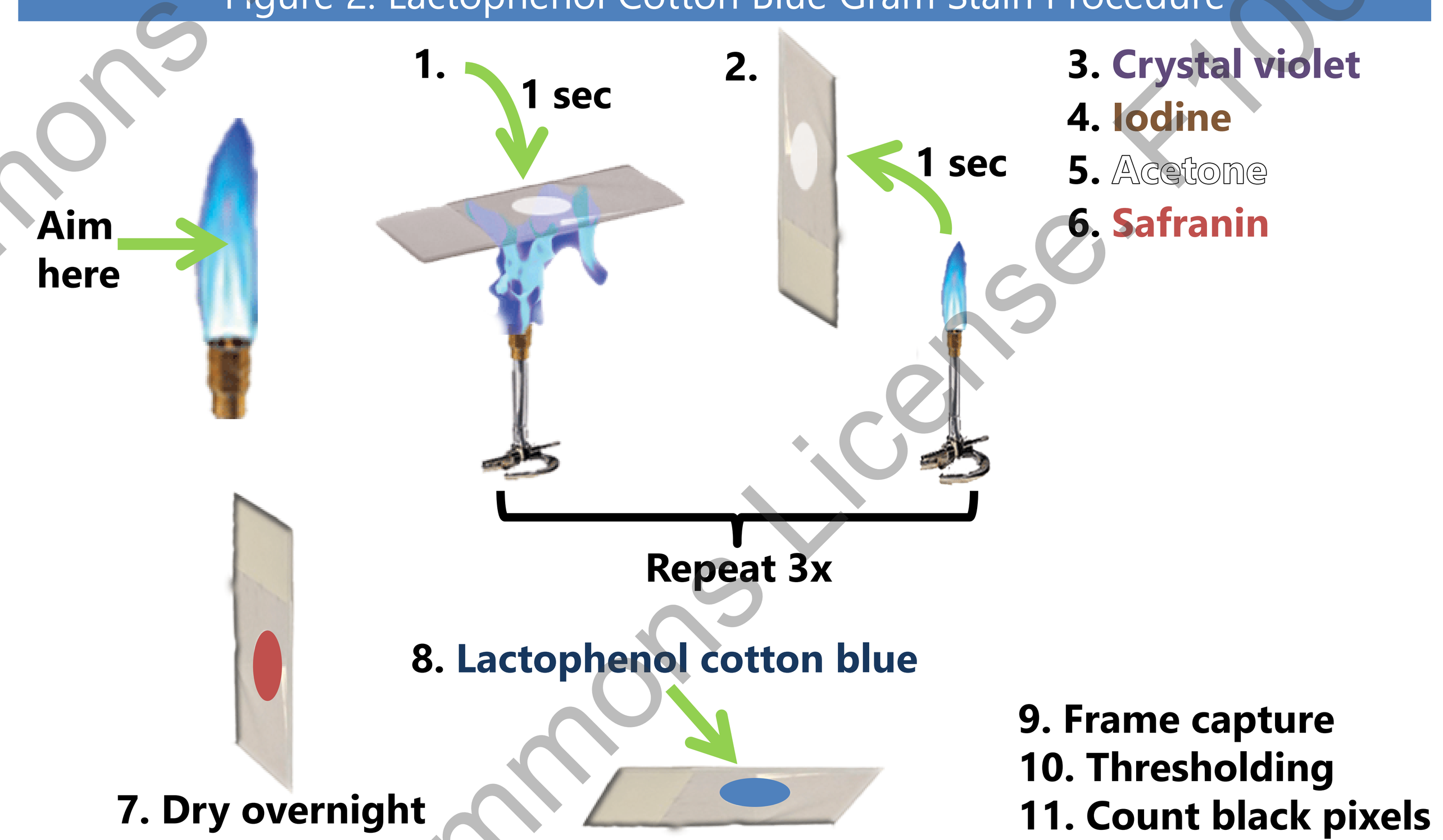
Figure 1: Quantitative Growth Kinetics



Methods

Glass slides were scrubbed with PCMX hand soap using a pipe cleaner. 10 uL of cells sampled from 96-well plates after VCC assays that used twice-concentrated MHB in the outgrowth step were added to the slides and dried overnight. 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 2). Ambient relative humidity was 40-60%. Slides were stained with Fluka Analytical Gram Staining Kit Product Number 77730 and dried overnight. 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 400X and 1600X magnification and Toupview software. The Adobe Photoshop thresholding function was applied to the 400X digital images using a threshold of 100. Black pixels corresponding to polysaccharides fixed to the slide were enumerated using the histogram function.

Figure 2: Lactophenol Cotton Blue Gram Stain Procedure

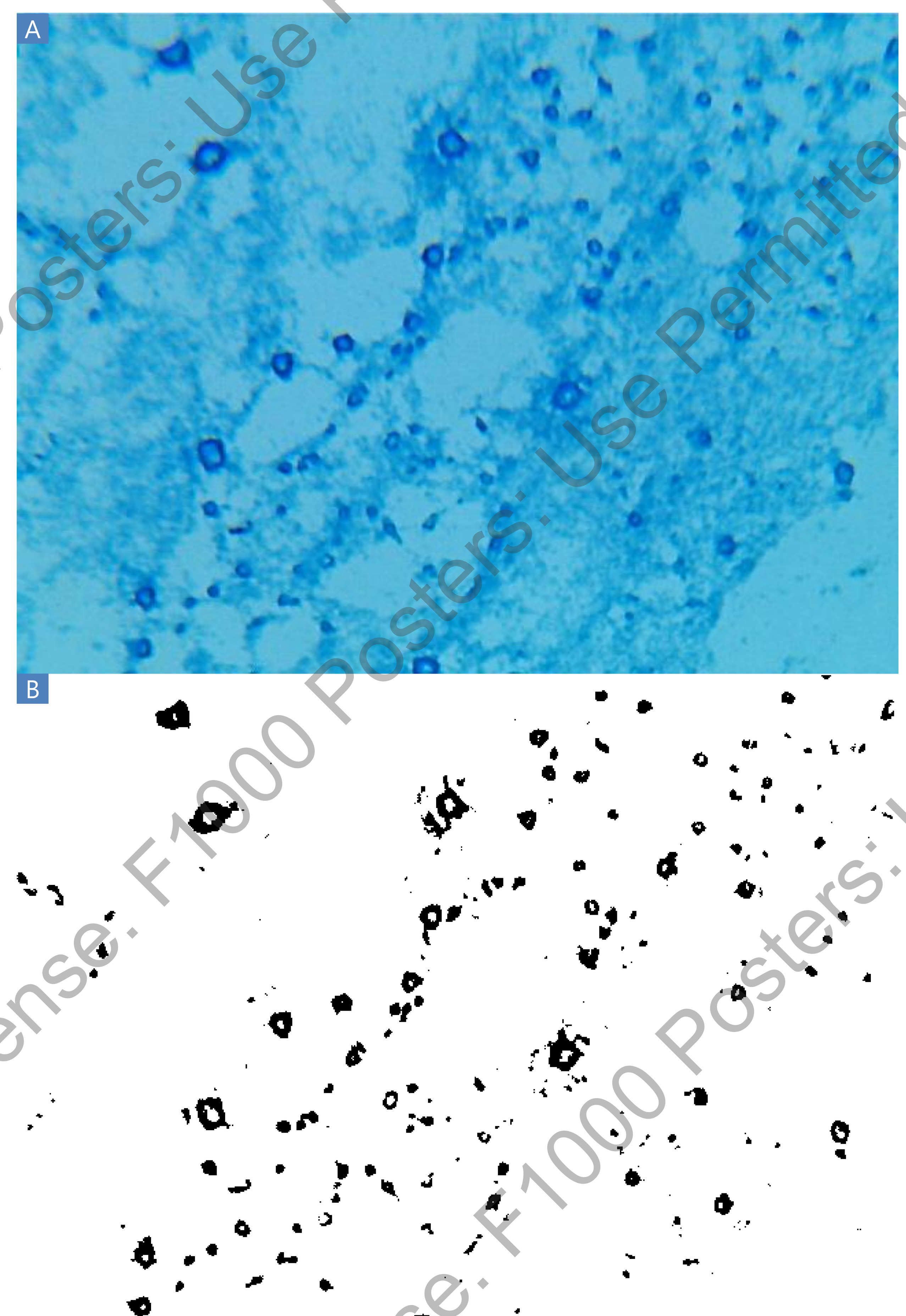


Results

Macroscopic clumps were observed in 25 mL TSB batch cultures of *E. coli* ATCC® 25922™ grown at 37° C 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 (< 1 mm in diameter) that exhibited motility, swimming in a synchronized wave downward along a chemotactic gradient 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. Macroscopic clumping in the batch culture or cuvette outside the detector was no longer observed after four changes: 1. using a HEPA-filtered air purifier, 2. replacing in-house deionized Milli-Q water with purchased molecular biology grade water, 3. replacing 2XMHB prepared and autoclaved in-house using reusable jars with Teknova 2X cation-adjusted MHB, and 4. filter-sterilizing phosphate buffers made near the portable air purifier, rather than autoclaving in reusable jars. Even after these measures, uncovered 1 mL samples placed in the detector for 2 hours formed a macroscopic clump at the base of the cuvette, suggesting that at least one clumping environmental factor (CEF) is concentrated by the detector acting as a dust trap. Thus, 1 mL samples of *E. coli* ATCC® 25922™ serve as biosensors for CEF, and the detector serves as a biosensor positive control. 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. The UMB VCC procedure was sensitive to cross-contamination in the 36 uninoculated edge wells, possibly indicating that clumping affects the particle size distribution, which in turn promotes aerosol formation during pipetting. Figure 3 depicts cells sampled from an 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, prevents cross-contamination and is preferred for hazardous bacteria such as the BSL-3 pathogen *Bacillus anthracis*.[5]

Results

Figure 3: Blue Gram Stain and Thresholding Results



Microscopic clumping was observed after VCC experiments using *E. coli* ATCC® 25922™ and twice-concentrated MHB, as observed using the Gram stain. The lactophenol cotton blue Gram stain (BGS) revealed ubiquitous circular or ring-shaped structures that stained dark blue (Figure 3A). All cells stained light blue because all cells are glycosylated to some degree. Carbohydrates, possibly including Maillard reaction 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, indicating the residues of clumps of cells that were washed off the slide and were missed by Gram staining alone. The processes of adhesion, cohesion and biofilm formation. *E. coli* cells secrete polysaccharides and concentrate polysaccharides such as starch from the media. The intensity of dark blue staining suggests copious capsule and slime formation in response to CEF.

Applying the digital image processing thresholding technique differentiated dark from light staining with little apparent background noise (Figure 3B). Setting the threshold to 100 (on a pixel intensity scale of 0-255) identified rare dimmer images with greater background noise, which could be corrected for by modulating the threshold based on background pixel intensity or the gamma curve. VCC results using *E. coli* ATCC® 25922™ and 2XMHB fluctuated over a 23-fold range as measured by the virtual lethal dose $vLD_{99.9}$ using HNP1 as the positive control before remediation efforts to remove CEF. After those efforts, $vLD_{99.9}$ values generally fell near the low end of that range, suggesting less adhesion/cohesion, shorter lag phases, and/or improved defensin potency. TSB or MHB cultures of *E. coli* ATCC® 43827™ (ML-35) produced no macroscopic clumps under any conditions.

Discussion

E. coli ATCC® 25922™ is one of the most commonly used AST quality control strains because it is susceptible to many major classes of small molecule antibiotics. Over the decades that it has been used for this purpose, to the best of our knowledge neither the rapid motility and macroscopic clumping in TSB nor the microscopic clumping in MHB revealed by BGS has previously been described, suggesting that CEF is rare. However, one laboratory uses this strain as a biofilm formation positive control[6], and another laboratory observed clumping preceding exponential growth by mass resonance when *E. coli* was sampled from a stationary phase culture and used to inoculate fresh Luria-Bertani (LB) broth at 37 °C.[7] Further work is needed to determine what environmental factors these three laboratories have in common, and whether this strain also forms adhesive and cohesive clumps and biofilms in other laboratories and AST studies. Currently, the identity of CEF is unknown, but any airborne particulate present in the laboratory environment could have entered the reagents used in VCC assays or the 96-well plates themselves. Possible sources of laboratory aerosols include personnel, ceiling tile degradation products, and ambient microorganisms that are unculturable in MHB. Combinations of more than one CEF may be required to produce activity resulting in the hypermotility and dramatic rapid clumping observed in open cuvettes of TSB cultures at room temperature. The absence of rapid clumping outside the detector after attempts to remove airborne contaminants from reagents and the laboratory environment, followed by slow clumping in the uncovered cuvette within the detector, illustrate the utility of TSB cultures of *E. coli* as biosensors and suggest that CEF activity is dose-dependent.

The presence of polysaccharides associated with *E. coli* ATCC® 25922™ cohesion suggests that in the conditions studied at UMB, this strain employs cohesion as a defense mechanism in response to the presence of CEF. Forming a clump surrounded by polysaccharides could contribute to resistance to antimicrobial lectins such as defensins[8] that would be bound and inhibited at the surface, limiting further inward diffusion and protecting persister cells at the center of the clump. These survivors could contribute to the deviation from simple exponential killing observed throughout all VCC studies at UMB of defensin activity against *E. coli*.[1] Mueller Hinton Broth 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. Bacterial slime and capsules are highly branched and contain glycosylated proteins. 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.

Acknowledgments

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