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Abstract

Viable bacterial cell counting is fundamental to analytical microbiology and agar plate colony counting remains common yet laborious and slow. Here, we demonstrate two methods for counting bacteria using commercially available microfluidic devices. We show that accurate viable cell counting is possible using simple and easy 'dip and test' arrays of microcapillaries. Colorimetric and fluorescent growth detection both permit viable cell counting in microcapillaries either by limiting dilution into multiple microfluidic compartments using a single endpoint measurement, or alternatively by quantifying growth kinetics. The microcapillary devices are compatible with conventional 96 well plates and multichannel pipettes, expanding each microplate row into 120 individual 1 or 2 microlitre samples. At limiting dilution, counting the proportion of positive compartments permitted accurate

calculation of gram-negative and gram-positive bacteria (*E. coli* and *S. saprophyticus*) at concentrations down to as low as 10 CFU/mL with almost 1:1 agreement with agar plate colony counts over four orders of magnitude. A smartphone camera was sufficient to record endpoint images of resazurin growth detection both colorimetrically and fluorescently. Viable cell counting of *E. coli* and *S. saprophyticus* was also possible through recording growth kinetics and determining the time taken to detect resazurin conversion. However, only the limiting dilution method remained consistent in the presence of urine matrix, as some interference in growth rate was observed when bacteria were spiked into higher concentrations of normal urine to simulate urinary tract infection patient samples. However, with the limiting dilution counting method endpoint growth was always detected even in the presence of 90% urine matrix, suggesting that this method might permit bacterial pathogen counting directly in clinical samples without agar plating.

Introduction

Accurate counting of bacteria is a fundamental analytical microbiology technique which underpins many areas from research to clinical diagnosis of infection. Many important microbiological tests, such as antimicrobial susceptibility testing, rely on an accurate dilution of inoculum to a specific concentration to ensure reproducible results. Common methods for bacterial concentration determination are colony counting on solid media and turbidity measurement. Plating of bacteria can be laborious, and in spite of extensive automation, and refinements (e.g. spot titre method) that can increase throughput, they remain labour intensive and require overnight incubation. This method is also limited to microorganisms that form colonies on solid media (missing some viable non-culturable organisms). Turbidity measurements using a spectrophotometer provide a rapid estimate of bacterial density but cannot differentiate between live and dead cells and can vary between different bacterial species and growth conditions. A non-turbid sample matrix such as saline and broth is essential. Microscopy can be used to count bacteria at the time of sampling, but this method is also time-consuming and low throughput. Automated cell counters and cytometers use a

range of detection methods but often require expensive instrumentation. Cytometry methods (e.g. flow cytometry) have replaced colony counting in some specific applications where instrumentation is available, and these methods must be calibrated for the sample type and target organism against conventional colony counts. For example, the BactoScan FC is specifically designed to determine total bacterial counts in milk samples (Ramsahoi et al., 2011), a sample matrix that is incompatible with turbidity measurements. Automated cell counting relies on digitisation of the experiment, whether colonies on agar plates, single cells in flow cytometer/micrograph, or in microfluidic devices. While automated colony counting software for agar plates exist, there are still ongoing challenges with this method. Colony size, shape and clustering or growing at the edge of the plate, along with poor contrast of unstained colonies against agar, can lead to inaccuracies in software counting. These systems tend to focus on a single measurement after overnight incubation, however, kinetic analysis has also become available for standard microbiology applications, such as the Reshape Imaging system (reshapebiotch, Denmark). Non-specific colony staining dyes such as triphenyl tetrazolium chloride (TTC) have been used to increase the contrast of bacterial colonies (Putman et al., 2005) to obtain a cleaner image, but this does not address the other difficulties in automated colony counting.

Microfluidic devices are becoming increasingly applied to microbiological measurements, such as the detection of bacteria in clinical or environmental samples (Zhao et al., 2019). Small reaction volumes can be used to reduce time to result, increase throughput or allow for greater portability for field analysis or point of care diagnostics (Needs et al., 2020).

Some portable or point-of-care tests for field use rely on the detection of by-products of bacterial growth such as the presence of nitrites in urine in dipsticks (Mambatta et al., 2015). Microfluidics are also facilitating the detection of microbes in rapid and portable devices including the detection of bacterial genes by nucleic acid amplification, and bacterial cells or antigens by antibody binding in immunoassays (Alves and Reis, 2019, Athamanolap et al.,

2018). Indirect quantitation via bacterial by-product, antigen or nucleic acid detection is therefore increasingly portable. Another approach to cell counting is directly sensing single cells (Song et al., 2010) or imaging single cells by digital microscopy (Yamaguchi et al., 2011). Distinguishing between living and dead cells can be achieved through differential staining (Düven et al., 2019) Although sensing or imaging may be the most rapid way to directly count bacterial cells, the enumeration of viable microbes, for example to determine colony forming units, still inherently requires cell growth. Cell growth can also be useful as an amplification for microbe detection with samples containing low cell concentrations. Furthermore, assays measuring cell growth can be adapted to functional measurement of bacteria in the presence of selective or growth modifying additives (restricted nutrients, selective media, antibiotics etc). Functional cell growth measurement remains an important objective for microfluidic microbiology methods.

Microfluidic devices can take advantage of bacterial segregation into microchambers or microdroplets to determine cell number at limiting dilution, with a digital readout of growth detected for an array of chambers which is used to calculate cell density (Hsieh et al., 2018, Cui et al., 2018, Lu et al., 2017). One study used an array of 600 picolitre chambers and monitored the fluorescent change in resazurin to determine bacterial growth in each chamber (Hsieh et al., 2018). Other studies use label-free detection means, measuring bacteria growth in microdroplets, quantifying *E. coli* using smartphone images of turbidity in samples ranging from 10^3 – 10^5 CFU/mL within 6 h (Cui et al., 2018). Microdroplets provide a way to produce thousands of compartments allowing enumeration of bacteria over a high dynamic range, however, monodisperse droplets are not trivial to produce and handle. Differences in droplet production can lead to polydisperse droplet sizes making analysis of the results a significant challenge (Kaminski et al., 2016). Using chambers with a fixed volume reduces the number of compartments analysed but can reduce error in compartment size and may be easier to produce for commercial uptake.

The use of chromogenic or fluorescent dyes in microfluidic devices can allow detection of bacteria using simple digital imaging setups. Smartphone cameras have been used to detect bacteria by light scattering, colorimetric or fluorescence detection in a range of microchannels and paper based microfluidics (Ding et al., 2019, Gopinath et al., 2014, Dönmez et al., 2020, Ma et al., 2020, Cui et al., 2018, Park et al., 2013, Alves and Reis, 2019). Alves *et al.* used an immunoassay with enzyme amplified fluorescent readout to detect 10^3 CFU/mL *E. coli* in microchannels. The detection system used a simple magnification lens with a smartphone adaptor with LED excitation and filter (Alves and Reis, 2019). Other studies have used smartphones to detect light scattering of particles using immunoagglutination in paper microdevices (Park et al., 2013) or light scattering of bacterial suspensions in microchannels (Dönmez et al., 2020). The use of smartphones in signal detection allows not only the digital capture of results but also the on-board analysis of results (Park et al., 2013). This illustrates the potential of smartphones and related low-cost digital imaging hardware to bring microfluidic devices to the point-of-care detection whilst avoiding instrument cost and equipment footprint (Ding et al., 2019). Resazurin dye is commonly used to detect bacterial metabolism and growth of a wide range of cells and can be monitored either by a colour change (from blue to pink) or from weakly fluorescent to a strong red fluorescence, and for this reason it has been incorporated in several microwell plate assays (Elshikh et al., 2016, Travnickova et al., 2019) and microfluidic systems to detect (Avesar et al., 2017, Elavarasan et al., 2013, Needs et al., 2019, Reis et al., 2016) or quantify bacterial growth (Hsieh et al., 2018).

Miniaturised detection places fundamental lower limits of cell density that can be detected, since at least 1 single colony forming unit (CFU) must be present in the test sample volume (Needs et al., 2020). Samples containing lower cell concentrations can be quantified using larger compartment volumes, or exploiting capture or concentration by flow through the device (Yamaguchi et al., 2011). However, devices using flow systems may require additional equipment that is not always accessible to non-microfluidic based laboratories

which constructs a barrier to implementation in many laboratories (Mohammed et al., 2015, Streets and Huang, 2013). Therefore, simple to use, scalable and cost-effective devices that maintain the benefits of microfluidic platforms, i.e. portability and high analytical sensitivity, which are compatible with existing microbiological lab infrastructure, are still needed.

Previously we demonstrated a simple dip and test format for microfluidic microbiology using microcapillary film (MCF) (Reis et al., 2016, Needs et al., 2019). Here, we describe a method that combines these dip-strips with ubiquitous microtitre plates to permit simple viable cell quantitation. Minimal hands-on time is required when combined with common multichannel pipettes and microwell plates to make serial dilutions of bacterial samples. An internal hydrophilic coating draws the sample up into multiple MCF test strips, each an array of 10 capillaries. This results in a simple and rapid method for determination of bacterial cell count using either growth kinetics (time taken for resazurin conversion) or by limiting dilution (proportion of positive vs negative growth per capillary). The mass-manufactured fabrication permits bulk purchase of MCF as a consumable costing around £10/meter representing 29 test strips i.e. 290x 1ul or 2ul chambers, significantly lower than other microfluidic devices currently on the market. ChipShop microfluidic chips cost approximately £35 per chip using a similar arrangement. To use this method, either bulk material can be purchased and the system re-created or ready-to-use kits can be purchased (CFT, UK).

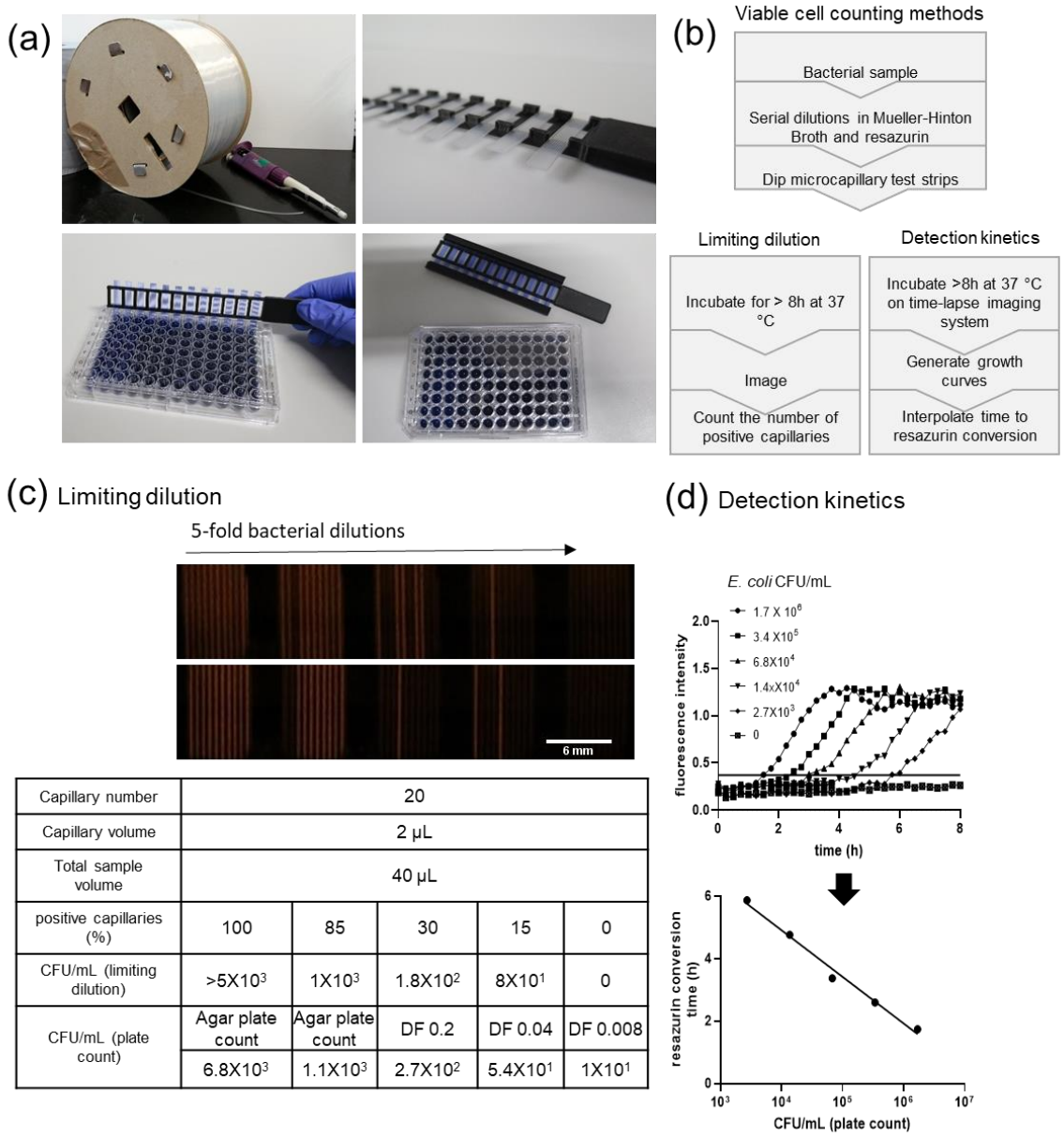
Materials and Methods

Microwell-Compatible Microcapillary Dip -strip Arrays

The fluorinated ethylene propylene microcapillary film (MCF) was manufactured by melt-extrusion by Lamina Dielectrics Ltd (Billingshurst, West Sussex, UK) from a highly transparent fluorinated ethylene propylene co-polymer (FEP-Teflon®) (figure 1a).The fluoropolymer MCF ribbon contained an array of 10 capillaries along its length, incorporated during extrusion (Barbosa et al., 2015, Reis et al., 2016, Dönmez et al., 2020). 1 to 5m lengths of MCF were given an internal hydrophilic coating by incubation with a 5 mg/mL

158 solution of polyvinyl alcohol (PVOH) in water (MW 146,000-186,000, >99% hydrolysed,
159 Sigma-Aldrich, UK) at room temperature for 2h (Pivetal et al., 2017, Reis et al., 2016).
160 Coated strips were washed with 5 ml of PBS with 0.5 % Tween 20 (Sigma-Aldrich, UK) to
161 remove residual PVOH, and dried on a vacuum manifold for 20 minutes per metre using a
162 SLS Lab Basics Mini Vacuum Pump with PTFE Coated Diaphragm (Scientific Laboratory
163 Supplies, UK). Two sizes of capillary diameter test strip were compared, with final total
164 capillary volume of either 1 or 2 µL and thus a total sampling volume of 10 or 20 µL per test
165 strip respectively (inside the 10 microcapillaries). The mean internal diameter of the 1 µL test
166 strips was 200 µm and the internal diameter for the 2 µL test strips was 280 µm and outer
167 diameter of 4.3 and 6 mm wide respectively and were cut to 33 mm in length. The
168 hydrophilic coated test strips are vacuum sealed and stored at room temperature until use.
169 An array of up to 12 test strips were clipped into a reusable 'ladder' holder with a 9mm pitch
170 (figure 1a). Test strip arrays could then be dipped directly into a row of a 96-well microtitre
171 plate allowing the sample to be drawn up by capillary action. 3D printed reusable end covers
172 were filled with Dow Corning vacuum grease (Sigma-Aldrich, UK) and slid over the ends of
173 the test strips, with the grease sealing the capillary ends and preventing evaporation during
174 incubation. The models for the ladder combs and end covers can be accessed here:
175 https://gitlab.com/sneeds/bacterial_testing. The components described here: hydrophilic
176 coated microcapillary film, test strip holders and end covers are available from Capillary Film
177 technology Ltd.

178 The bacterial samples were prepared in 96 well plates in Mueller-Hinton broth with resazurin
179 by serial dilution using a multichannel pipette over a range of $10^6 > 0$ CFU/mL. The sample
180 was taken up into the capillaries by dipping test strips into each well and the sample rises by
181 capillary action to fill the capillaries. After overnight incubation at 37 °C the endpoint change
182 in resazurin fluorescence or colour was imaged using a smartphone or digital camera (figure
183 1c). The growth kinetics was also monitored over time using time lapse imaging of resazurin
184 fluorescence (figure 1d).



Capillary number	20				
Capillary volume	2 µL				
Total sample volume	40 µL				
positive capillaries (%)	100	85	30	15	0
CFU/mL (limiting dilution)	>5X10^3	1X10^3	1.8X10^2	8X10^1	0
CFU/mL (plate count)	Agar plate count	Agar plate count	DF 0.2	DF 0.04	DF 0.008
	6.8X10^3	1.1X10^3	2.7X10^2	5.4X10^1	1X10^1

Figure 1. Viable cell counting using MCF Dipstick combs. (a) 1-5 m lengths of microfluidic ribbon are cut from the bulk material and functionalised with polyvinyl alcohol. Hydrophilic dipstick test strips are cut to 33 mm and clipped into a 3D printed microtitre plate compatible “comb”. When strips are dipped into samples in resazurin growth indicator medium in 96-well plates, the sample is drawn into capillaries. End covers are used to stop evaporation. Each MCF test strip takes 10 replicate 1uL or 2uL samples, with a12-strip comb taking a total of 120 samples from 12 microtitre plate wells. **(b)** Bacterial concentration can then be measured based on either an endpoint measurement of the number of capillaries that have observed bacterial growth by smartphone or digital camera or by quantifying bacterial kinetics of growth. (c) Example endpoint fluorescent images of dilutions of 5

dilutions of *E. coli* in test strips comparing agar plate counts and limiting dilution counts demonstrating limiting dilution calculations **(d)** Growth curves of *E. coli* 25922 were plotted against the starting CFU/mL and the time taken for resazurin conversion, it is clear that starting cell concentration can be determined simply by determining the time to detect increased resazurin fluorescence.

Resazurin growth detection and growth kinetics measurement in microcapillary arrays

ATCC reference strains *Escherichia coli* 25922 and *Staphylococcus saprophyticus* 15305 were purchased from LGC Standards (Middlesex, UK). The bacterial strains were cultivated on Mueller-Hinton agar and diluted in Mueller-Hinton broth with resazurin sodium salt (Sigma Aldrich, UK). Bacterial reference strains from a cell bank were cultured on LB agar overnight at 37 °C. A single colony was taken and grown for several hours in Mueller-Hinton broth until turbid. The bacteria were normalised to 0.5 McFarland standard and diluted to cover the range of 10^6 – 10^1 CFU/mL in Mueller-Hinton broth with 0.06 mg/mL resazurin solution for fluorescence detection and 0.25 mg/mL for colorimetric detection, followed by serial five-fold dilutions in a 96 well plate . Microcapillary film test strips in combs were dipped into wells, and after fully filled with sample end caps added followed by incubation at 37 °C for 12 h. In parallel to the microcapillary bacterial colony forming units were determined by overnight colony counting on LB agar (Fisher, UK) using the spread plate method. To ensure accurate counting between the bacteria in the capillaries and plate counting, capillary test strips were dipped and immediately 100 μL from a duplicate well was spread onto an LB agar plate for the first two dilutions. At least two dilutions and three replicates were plated and the final three dilutions were calculated by dilution factor.

For urine tests, urine from healthy volunteers was tested with Uritest 10V Urinalysis strips and Quantofix Ascorbic Acid test (Sigma Aldrich, UK), filter sterilised and stored at -20 °C within 4 h of donation. Urine was diluted to 90 % with 10X concentrated Mueller-Hinton broth and resazurin to a final concentration of 60 μg/mL. Ethical consent for the collection of urine

from healthy donors was received from the University of Reading, reference code 19/59. Informed written consent was obtained from all participants.

Data analysis

Two recordings of the test strips were made. A single endpoint was recorded by either fluorescence or colorimetric measurement after overnight incubation at 37 °C using a smartphone camera or digital camera. For fluorescence imaging a flight case was used as a dark box with two strips of green LEDs (RS Components, Catalogue Number: 855-5943) illuminating the test strips from above. A small hole was cut in the box and a 570 nm longpass glass filter (Edmund Optics) was placed over the opening allowing an image to be taken. For colorimetric images, the colour change can be observed by eye on a plain white background (figure S1). For imagin,g the strips were placed on an even white light illumination screen. Growth and no-growth can be scored by eye.

For timelapse imaging, resazurin conversion to the fluorescent resorufin was recorded every 15 minutes using the POLIR robot (Needs et al., 2019) configured for resazurin fluorescence using green LED illumination and 570 nm long pass emission filter (Edmund optics, Filter Reference: SCHOTT OG570), with 3280 x 2464 resolution images taken with a Raspberry Pi v2 camera (figure S2). MatLab scripts were used to analyse time-lapse image series of bacterial growth in MCF, and the code can be accessed here: https://gitlab.com/sneeds/code-repository. Briefly, colour images were split into red, blue and green (RGB) channels and the red channel analysed for fluorescent intensity. The fluorescent intensity across the capillaries was calculated and normalised to a reference fluorescent strip of plastic. Time for resazurin conversion was calculated based on fluorescence intensity reaching a threshold: (mean signal for no bacteria control) + 3*(standard deviation of control signal). Statistical analysis comparing linear regression was performed in GraphPad Prism using ANOVA.

To calculate the number of bacteria per capillary in each test the following equation was used (Hsieh et al., 2018):

$$bacteria\ per\ capillary = -Ln\left(\frac{total\ capillaries - positive\ capillaries}{total\ capillaries}\right)$$

Which can then be converted to CFU/mL.

Results and Discussion

Compartmentalisation of bacteria in capillaries allows accurate viable cell counting of gram-negative and gram-positive bacteria from endpoint growth recorded with a smartphone camera

Compartmentalisation of bacteria in microfluidic devices in individual chambers has been used to count the number of bacteria cells present in a sample, usually using a fluorescent or colorimetric substrate to detect bacterial growth (Hsieh et al., 2018, Lu et al., 2017, Matuła et al., 2020). Here, we describe two methods for microfluidic bacterial cell counting using 96-well compatible arrays of 'dip and test' microcapillary strips. We first evaluated the accuracy of these methods for counting two common bacterial species. Reference strains of *E. coli* and *S. saprophyticus* were selected as they represent important examples of gram-negative and gram-positive uropathogenic species associated with uncomplicated urinary tract infections (Bitew et al., 2017). The 9mm pitch array consists of 12 test strips, each of which contains 10 parallel microcapillaries, such that a rack of 12 test strips can be dipped into a full row of a 96 well plate and perform 120 parallel microfluidic measurements.

The random uptake of sample into the arrays of capillaries should lead to a distribution of bacteria – and subsequently growth – that follows Poisson statistics (Collins et al., 2015). We found as expected that as concentrations were reduced by serial dilution to 1 CFU/capillary and below, an increasing proportion of capillaries showed no fluorescence indicating no growth (0 CFU), and fewer capillaries showed red fluorescence indicating bacterial growth (≥1 CFU).

The proportion of capillaries showing bacterial growth for both *E. coli* and *S. saprophyticus* were counted and the cell concentration CFU/mL calculated by assuming a Poisson distribution of cells, and from the device volume. This count was plotted against the CFU/mL measured in parallel by conventional colony counting on agar plates (Figure 2a-b).

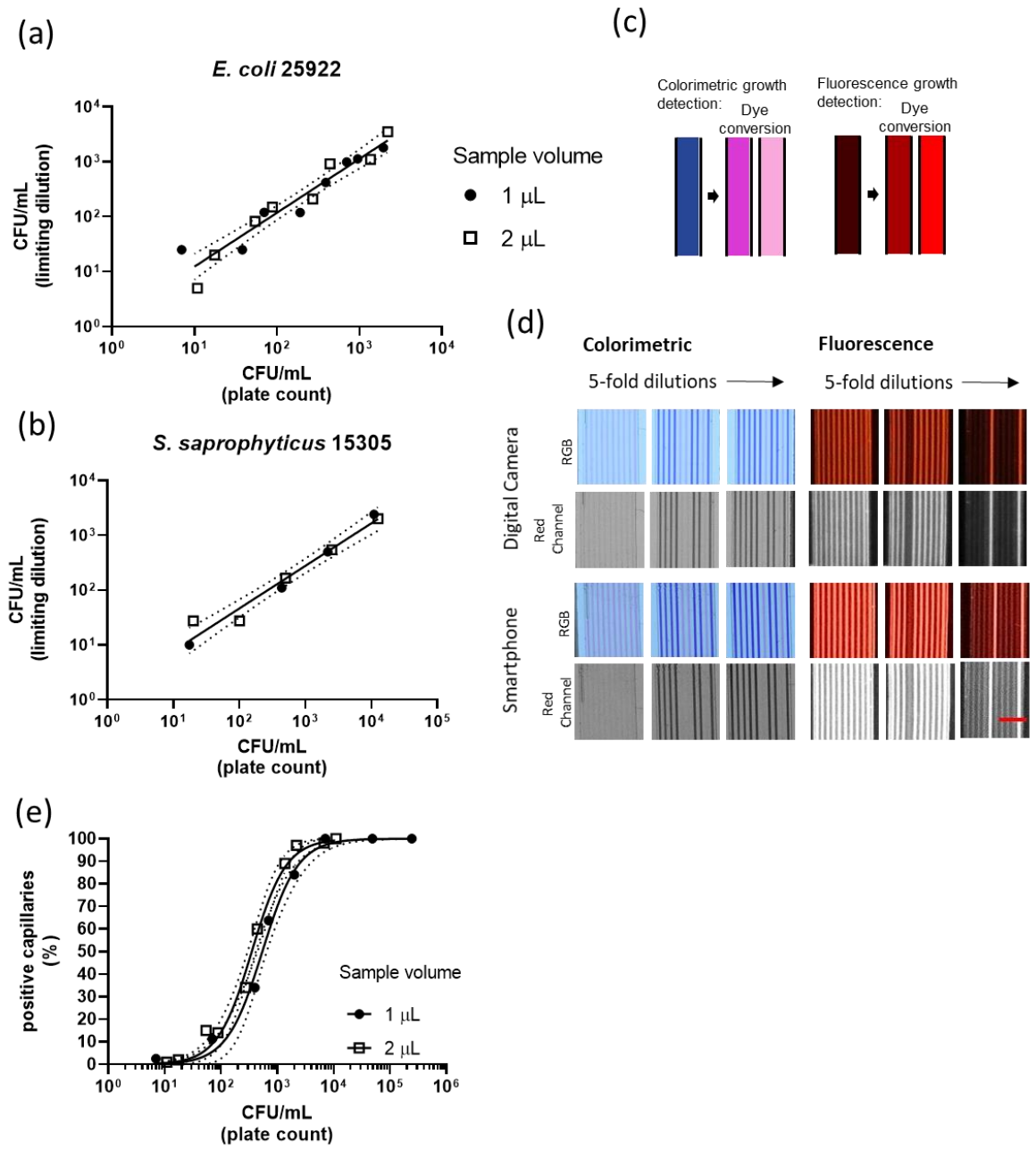
Both *E. coli* and *S. saprophyticus* $\log_{10}$ CFU/mL showed a linear relationship with a slope of 1 and 1.2 respectively indicating close agreement between the microcapillary test strip limiting dilution method vs agar plate counts, confirming that distribution of bacteria in the capillary test strips follows Poisson statistics. There was no significant difference in regression lines between the 1 and 2 µL capillary volumes indicating no difference in distribution or growth quantitation between the two capillary sizes. A small difference in viable cell counts between liquid and solid media might be expected for some samples as not all viable cells form colonies on solid media.

Endpoint growth determination with resazurin can be followed colorimetrically or fluorescently by either following a blue to pink color change or from low fluorescence to high red fluorescence (figure 2c). Growth versus no growth can be captured at an overnight endpoint using a low-cost smartphone camera or digital camera for both colorimetric and fluorescent detection (figure 2d), permitting microfluidic viable cell counting without laboratory instruments. Endpoint colorimetric growth detection is likely to be the most accessible for laboratories as the change in colour can be observed by eye and imaged without the need for a fluorescence system, further increasing the flexibility of this platform. Fluorescence detection used simple low-cost green LED strips placed in a flight case for excitation and a colored glass emission filter over the smartphone or digital camera lens costing approximately £100 in materials to build.

While the CFU/mL determined by agar plate count or limiting dilution was similar, the relationship between CFU/mL and the number of bacteria positive capillaries is sigmoidal and plateaus as the number of positive capillaries approaches 100 % (figure S3). Increasing the volume of each compartment allows detection of lower cell densities, with a

corresponding reduction in the upper quantifiable concentration. For example, 100 compartments of 1 μL volume gives a total sample volume of 100 μL and has a theoretical range of ~10^1 – 4.6X10^3 CFU/mL. Using a 2 μL compartment volume, with 100 capillaries but doubling the total sampling volume to 200 μL, shifts the measurable range to ~0.5X10^1 – 2.3X10^3 CFU/mL. This is demonstrated by comparing the positive number of capillaries for both 1 and 2 μL (figure 2e), the 2 μL test strip shows a shift to the left and a lower limit of detection than the 1 μL test strip.

Bacterial concentration is determined by the number of positive compartments against total compartments. Increasing the number of compartments increases the range of bacteria that can be detected and quantified using Poisson distribution. Table 1 shows how increasing the number of compartments increases the concentration range of bacteria that can be theoretically quantified. If only a single microcapillary test strip is used (i.e. 10 capillaries) the minimum percentage of positive compartments that can be counted is 10% (with only 1 single positive capillary) and the maximum is 90% (with only 1 single negative capillary) which corresponds to a concentration range of 10^2 – 2.3X10^3 CFU/mL. At the low end of concentrations, for many test strips there will by chance be no bacterial growth detected in any compartment, and the concentration will be unquantifiable in that situation (figure 1b). In contrast, if 10 test strips were used (i.e. 100 capillaries) a minimum of 1 % and a maximum of 99 % (i.e. 1 single empty capillary) can be used to calculate a viable cell concentration, increasing the theoretical range of bacteria that can be counted to 10^1 – 4.6x10^3 CFU/mL.



320

321

322 **Figure 2. CFU/mL calculated by limiting dilution corresponds to agar colony counting and can**
323 **be recorded colorimetrically or fluorescently using a smartphone camera.** The predicted
324 CFU/mL was calculated by the percentage of positive capillaries observed and plotted against the
325 overnight colony counts for (a) *E. coli* and (b) *S. saprophyticus.* CFU/mL was log transformed. r^2 =
326 0.93 and 0.97 for *E. coli* and *S. saprophyticus* respectively. Lines indicate linear regression with
327 dotted lines indicating 95% confidence intervals. Bacterial growth using resazurin can be monitored
328 both colorimetrically and fluorescently (c) and can be monitored using smartphone or digital camera

using low-cost LEDs and glass filters for fluorescent detection (d). Images indicate cropped endpoint images of 1 µL test strips for *E. coli* showing 3 dilutions of bacteria, imaged using Canon Powershot S120 digital camera of iPhone 6s smartphone. Scale bar indicates 2 mm. (e) Starting concentration of *E. coli* was plotted against the number of capillaries with bacterial growth (positive capillaries) from 100 capillaries for 1 and 2 µL capillary volumes. $R^2 = 0.99$ for both 1 µL and 2 µL sample volume

The sample volume for this array of MCF strips sits between microwell plates and smaller microfluidic bacterial devices, and therefore can be used to measure a distinct range of cell concentrations. In the case of RAPiD (resazurin amplified picoarray detection), a total of 600 chambers each holding 250 pL of sample (Hsieh et al., 2018), requires a minimum of $6.4X10^3$ CFU/mL to theoretically detect a single positive CFU (although this device also has a higher maximum concentration that can be quantified). In contrast, the higher sample volume tested in microtitre plates can be used to quantify much lower cell densities, but significant dilution is essential for sample concentrations above ~10 CFU/mL although we do not propose cell segregation in a microtitre plate.

The low LOD of the microcapillary dip and test strips allows for analysis of samples with extremely low CFU counts, hence with the addition of several serial dilutions even samples with high CFU load can be quantified using this method.

Table 1. Theoretical range of bacteria that can be calculated based on Poisson distribution depending on chamber number and volume.

Microcapillary 'dip and test' device					96-well microtitre plate					RAPiD (Hsieh et al., 2018)				
Test strips (Chamber) (n)	Total sample volume	Positive chamber range (%)	Lower CFU/mL limit	Upper CFU/mL limit	Chamber (n)	Total sample volume	Positive chamber range (%)	Lower CFU/mL limit	Upper CFU/mL limit	Chamber (n)	Total sample volume	Positive chamber range (%)	Lower CFU/mL limit	Upper CFU/mL limit
Chamber volume	1 µL				200 µL					250 pL				
1 (10)	10 µL	10-90%	100	$2.3X10^3$	12	2.4 mL	8.3 - 91.6 %	0.44	12	600	150 nL	0.1 – 99.8 %	$6.6X10^3$	$2.6X10^7$
2 (20)	20 µL	5-95%	51	$3X10^3$	24	4.8 mL	2.5-97.5%	0.2	15					
4 (40)	40 µL	2.5-97.5%	25	$3.7X10^3$	46	9.2 mL	2.1-97.8%	0.11	19					
8 (80)	80 µL	1.2-98.7%	13	$4.4X10^3$	58	11.6 mL	1.7-98.2%	0.09	20					
10 (100)	100 uL	1-99%	10	$4.6X10^3$	96	19.2 mL	1-98.9%	0.05	22					

We assessed our experimental data to see if this theoretical concentration range matched experimental observations. The theoretical concentration predicted by Poisson distribution was compared to that observed for both 10 replicate test strips (100 compartments) and for just 1 test strip (i.e. only 10 compartments) interpolated from plots in figure 2e (table 2). Using only 10 replicate test strips (at 1 µL capillary volume) the theoretical limit of detection would be 10 CFU/mL. Interpolating for 1% positive capillaries from figure 2e we observed 19 CFU/mL and a 95% confidence interval of 9 – 74 CFU/mL. As the theoretical limit fits within these interpolated confidence interval we believe these estimated measurement ranges are credible.

Table 2. Observed quantifiable range of bacteria in 'dip and test' microfluidic strips.

The observed CFU/mL for *E. coli* was interpolated for 1 and 10 % bacteria positive capillaries for each sample volume from figure 1e.

Capillary volume	Test strip (n)	Total sample volume	Theoretical LOD (CFU/mL)	Observed LOD (CFU/mL)
1 µL	10 (100 capillaries)	100 µL	10	19 (95% CI = 9-74)
	1 (10 capillaries)	10 µL	100	109 (95% CI = 66-181)
2 µL	10 (100 capillaries)	200 µL	5	13 (95% CI = 7-35)
	1 (10 capillaries)	20 µL	50	75 (95% CI = 52-108)

Kinetics of resazurin conversion is dependent on bacterial concentration

Counting bacteria in microcapillary chambers by limiting dilution permits accurate viable cell quantitation, however, the quantifiable range of CFU/mL is dependent on chamber number and volume. It is also important to know the time taken for a single CFU to grow to a detectable resazurin conversion, to set the earliest endpoint measurement. Others have shown that the reproducible rate of bacterial growth can be used to quantify starting viable cell concentration in a sample, with the time taken to reach a threshold of resazurin conversion being inversely proportional to a log of bacterial density (Travnickova et al., 2019, Borra et al., 2009). We explored whether this approach could be used for counting higher cell concentrations, where more than 1 CFU is present per microcapillary of an MCF test strip. Growth curves were recorded by time-lapse imaging of resazurin conversion for *E. coli* and *S. saprophyticus* over a range of starting cell densities using serially-diluted samples from a top concentration of 10^7 CFU/mL in 1 and 2 µL sample microcapillary test strips, and growth kinetics compared to microtitre plates.

Images of the MCF test strips and microtitre plates were taken every 15 minutes over 20h incubation to monitor the conversion of resazurin to the fluorescent form resorufin using an open source imaging robot (Needs et al., 2019). The time taken to reach a threshold of resazurin conversion indicating the earliest detection of growth was interpolated from the growth curves (figure S2) and plotted against CFU/mL calculated from agar plate colony counts. The time to resazurin conversion for five-fold serial dilutions was compared to calculate the generation time for each bacterial species. The generation time for *E. coli* and *S. saprophyticus* was 24 and 59 minutes respectively, in line with expected log phase growth kinetics for these species.

The $\log_{10}$ CFU/mL and time to resazurin conversion follow a linear regression described as Y = -1.3x+10 and Y = -3.6x+25 for all *E. coli* and *S. saprophyticus* sample volumes with a goodness of fit of $r^2 = 0.97$ and 0.93 respectively (figure 3). Studying matched experiments, bacterial concentration in different sample volumes, and between microtitre plate wells vs microcapillaries, there was no significant difference between the slope or intercept for any of the sample volumes for either *E. coli* or *S. saprophyticus* indicating growth kinetics were independent of device and volume.

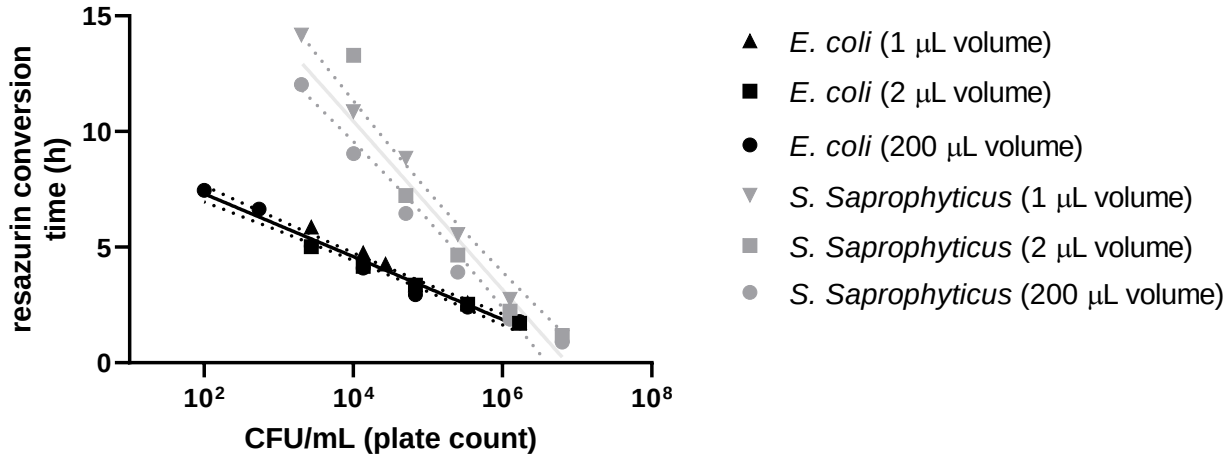


Figure 3. Resazurin conversion kinetics is dependent on bacterial concentration but independent of compartment volume. Analysis of resazurin conversion kinetics by *E. coli*, *S. saprophyticus* in 1 µL and 2 µL volume microcapillary film vs 200 µL in microtitre plates. CFU/mL log transformed. Time to resazurin conversion was set at a threshold fluorescence intensity calculated by the mean intensity of a no bacteria control + 3x(standard deviation of control). Line indicates linear regression for each bacterial species for all sample volumes combined, for $\log_{10}$ CFU/mL against resazurin conversion time. $r^2 = 0.97$ and 0.93 for *E. coli* and *S. saprophyticus* respectively.

When approaching the concentration at which not all capillaries have any observed bacterial growth, the time to conversion becomes non-linear. At concentrations falling below 1 CFU/capillary where a single CFU is present per compartment, the maximum observed time for *E. coli* to convert resazurin was 7.5 h, indicating that even at very low concentrations of cells the resazurin signal for growth of one CFU can be detected on the same day. The slower-growing *S. saprophyticus* required a maximum of 16 h at these limiting dilutions (figure S4), representing the maximum time needed for endpoint cell counting to detect a single bacterial colony growth and count these two organisms by limiting dilution. This is similar to growth kinetics in other microfluidic devices. The growth of $5X10^5$ CFU/mL *E. coli* grown in nanolitre volumes can be detected using resazurin within 4 h (Avesar et al., 2017), while growth of *E. coli* in our dip and test devices at $5X10^5$ CFU/mL is interpolated at 3.5 h indicating a robust measurement of *E. coli* growth using resazurin between microfluidic systems.

Bacterial counting by limiting dilution and single cell segregation is effective in urine samples

The rapid detection of bacteria in clinical samples is important for diagnosis and to inform correct treatment (such as antibiotic selection). One of the most sought after ways to reduce a time to result from a clinical sample such as urine is by direct sampling, minimising both sample processing time and reducing time-to-result below the duration of two cycles of

bacterial growth required for broth microdilution or disc diffusion testing of isolates taken from agar plates (Needs et al., 2020, Chandrasekaran et al., 2018, Davenport et al., 2017). Urine is a complex sample matrix with variable composition containing a number of components that can affect bacterial growth and resazurin fluorescence including pH, nitrite levels and ascorbic acid (Carlsson et al., 2001).

To test whether counting remains accurate with direct sampling methods in the presence of urine sample matrix, *E. coli* and *S. saprophyticus* cells were spiked into four individual urine samples donated by healthy volunteers (table S1) and the kinetics of resazurin conversion monitored. Comparison of multiple individual urine samples is important as pooled urine may have a more uniform pH than individual samples, missing potential for interference. The urine was diluted into concentrated Mueller-Hinton broth with resazurin such that each simulated patient sample was made up of 90% urine, but with broth and dye present at the same final concentration as prior experiments with medium alone. While all samples showed bacterial growth, the generation times differed between samples indicating the urine matrix can affect detection kinetics, with significantly slower growth noted for sample 012 (figure 4a-b). The generation times were 34 minutes, 26 minutes and 22 minutes in urine samples 009, 012 and 001 respectively. Further dilution of urine matrix to 20% urine reduced the delayed growth in all samples and brought the detection times of all the urine samples to within the 95% confidence limit of *E. coli* grown in Mueller-Hinton alone (figure 4b). We conclude that to study bacterial growth kinetics with direct urine sampling, a sample processed simply by diluting only into unmodified Mueller-Hinton broth (i.e. no centrifugation and cell recovery from urine), it may be necessary to dilute the sample 1:5 with broth to keep final urine concentration at 20% or lower, in order to reduce the impact of urine matrix on bacterial growth rate.

Viable cell counting by limiting dilution for bacterial concentration determination was also evaluated in the presence urine. For counting of bacterial cells, *E. coli* and *S. saprophyticus* cultures were spiked into urine samples. All starting concentrations were diluted only into

90% urine, as diluting further in media would restore bacterial growth and the impact of urine matrix on growth rate was strongest at this high sample matrix concentration. Endpoint analysis after overnight incubation was recorded using a smartphone camera and digital camera. For both bacterial species, accurate counting of bacterial cells was observed even in samples containing 90% urine and with no significant difference in counts obtained by limiting dilution in the presence of 90% urine vs Mueller-Hinton broth alone (figure 4c-d). Furthermore, the urine did not affect the imaging of resazurin conversion, and the fluorescent signal was clearly visible using a smartphone camera even in the presence of urine matrix (figure 4e).

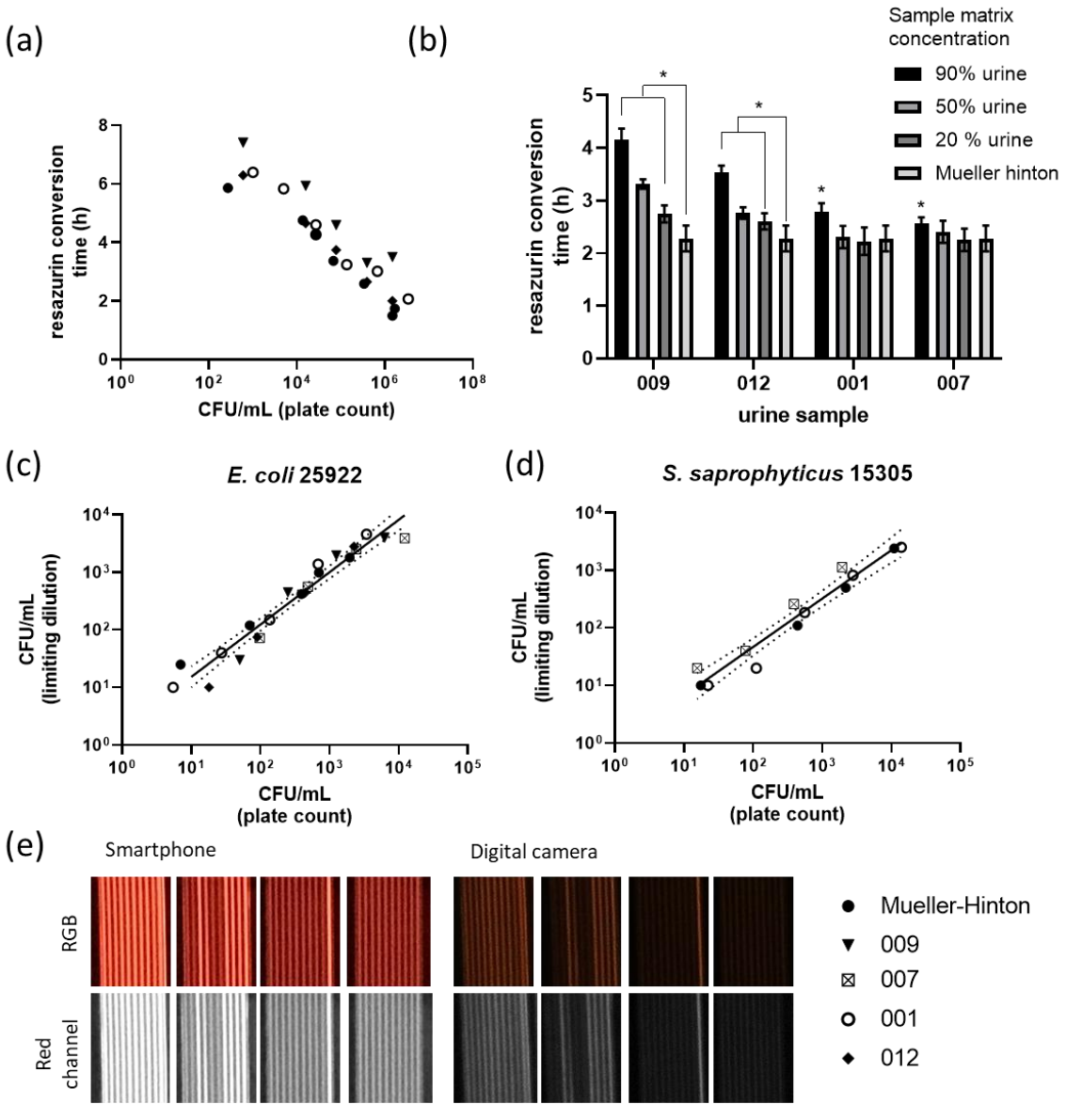


Figure 4. Bacterial counting by limiting dilution is still accurate in urine samples, even when growth kinetics is delayed by sample matrix. *E. coli* 25922 was grown in urine samples diluted with concentrated Mueller-Hinton media and resazurin such that the final concentration of urine was 90% from two healthy volunteers. (a) The time to resazurin conversion was calculated for each sample. (b) Time to resazurin conversion of $1.5x10^6$ CFU/mL *E. coli* grown in decreasing concentrations of urine. Data indicates the average of 10 replicate capillaries ± SD. Two-way ANOVA, Dunnetts post hoc to a Mueller-Hinton control. * indicates $p<0.05$ (c) The CFU/mL for *E. coli* (d) and *S. saprophyticus* in 90 % urine was calculated from 100 capillaries for each urine sample, $r^2 = 0.94$ and 0.95 for *E. coli* and *S. saprophyticus* respectively. The solid line indicates linear regression, dotted lines indicate 95% confidence intervals. (e) Representative fluorescent images of overnight incubation of *E. coli* in 90% urine sample 001 taken with smartphone iPhone 6S and Canon Powershot S120 digital camera.

When using these methods of bacterial quantitation, it is important to be aware that quantitation of bacteria by resazurin conversion rates is impacted significantly by differences in growth conditions (i.e. sample matrix). However, quantitation by limiting dilution proved robust in multiple different samples across gram-negative and gram-positive bacteria. One significant advantage we envisage for this device is the loading of samples in a field location, when a sample is unable to be tested in a laboratory in time. The simple nature of the sample loading (capillary action) means the sample can be loaded into the test strips before overgrowth of bacteria can occur (i.e. during transport of samples), with the inclusion of a small series of dilutions that can be performed with disposable pipettes.

Conclusions

This simple ‘dip and test’ microfluidic device can be used to accurately determine bacterial viable cell concentrations by limiting dilution or by growth kinetics. This simple test can be monitored either colorimetriclly or fluorescently and results recorded using low-cost smartphone cameras. The principles presented in this study can be applied to any device using compartmentalisation to separate bacterial cultures. Using a simple device operation that does not require complex liquid handling allows existing microbiology methods to be combined with high throughput microfluidic devices. Both the 1 µL and 2 µL test strips are compatible with standard microtitre plates and 3D printed ‘ladder’ combs pitched 9 mm allows easy use of multiple strips for screening purposes, expanding a 96 well plate into 960 individual measurements (or more, if multiple strips are dipped). The minimal requirement for equipment or instrumentation allows untrained users from non-microfluidic labs to easily adopt this device. We demonstrated that quantitation by limiting dilution in microfluidics remains accurate for gram-negative and gram-positive species in the presence of different urine sample matrix, using urine samples from healthy volunteers. This device can be ‘tuned’ to detect a specific clinical threshold of bacteria by varying the capillary diameter or length to change the sample volume of each chamber. Using this method, the bacterial load of an infection such as urinary tract infections could be quantified without the need for sub-culturing.

Competing Interests

ADE is one of the inventors of patent application protecting aspects of the novel microfluidic devices tested in this study, and is a director and shareholder in Capillary Film Technology Ltd, a company holding a commercial license to this patent application: WO2016012778 "Capillary assay device with internal hydrophilic coating" AD Edwards, NM Reis.

Author Contribution

SHN: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project administration, Software, Visualization, Writing – original draft, Writing – review & editing

ADE: Conceptualisation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing

HMIO – Writing – original draft, Writing – review and editing

Role of the funding source

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Ethical Considerations

Ethical consent for the collection of urine from healthy donors was received from the University of Reading, reference code 19/59. Informed written consent was obtained from all participants.

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