High-throughput, multiplex microfluidic test strip for the determination of antibiotic susceptibility in uropathogenic *E. coli* with smartphone detection

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Abstract

Antibiotic resistance in urinary tract infections is a major global challenge and improved cost-effective and high throughput antibiotic susceptibility tests (AST) are urgently needed to inform correct antibiotic selection. We evaluated a high throughput microfluidic test strip for AST and minimum inhibitory concentration (MIC) determination in 20 urinary pathogenic *E.*

23   *coli* (UPEC) isolates using six commonly prescribed or therapeutically beneficial antibiotics.

24   The microfluidic MIC performs broth microdilution in 1 microliter volume capillaries, 100 X

25   smaller than standard broth microdilution. Each test strip contains 10 parallel capillaries

26   which are dipped into a single well of a 96 well plate, significantly increasing throughput over

27   a microtitre plate. When tested with clinical UPEC isolates at standardised inoculum density,

28   these devices gave 100% essential agreement (+/- 1 doubling dilution of antibiotic) to the

29   gold standard microplate broth microdilution method described by CLSI. Although for some

30   antibiotic/isolate combinations an earlier endpoint readout reduced accuracy, MIC test strips

31   read at a 6h endpoint still gave 69 – 100 % essential agreement depending on the antibiotic.

32   Growth could be detected significantly earlier than 6h, but with a trade-off between speed vs

33   accuracy. These high-throughput, multiplexed test strips could be used to increase

34   throughput and give faster results than microplates while retaining the core broth

35   microdilution methodology of gold standard techniques for AST and MIC determination.

36   **1 Introduction**

37   Although improved diagnostic technology is widely acknowledged to be essential to combat

38   antimicrobial resistance (AMR) (Trevas et al., 2020), antibiotic susceptibility testing (AST) of

39   clinical samples such as urine remains challenging. Although urinary tract infections (UTI)

40   are often self-limiting, a high prevalence places a significant burden on health systems, and

41   when infections escalate hospital treatment can be required. Current primary care and

42   hospital treatment protocols rely on empirical antibiotic administration at first presentation

43   (Grigoryan et al., 2014, O'Grady et al., 2019), which exacerbates the clinical and economic

44   burden because of a high level of broad and rapidly changing AMR profile necessitating

45   constant updates to guidelines. When urine samples are taken and tested, adjustment of

46   antibiotic is often required when results are returned days later. Resistance ranges up to

47   ~50% of uropathogenic *E. coli* (UPEC) isolates in OECD countries and as high as >80%

48   outside OECD, depending on antibiotic (Bryce et al., 2016) which limit the use of many

49   common antibiotics, and reduce effectiveness of empirical prescribing. In antibiotics with

50 resistance rates > 20% empiric treatment is unsuitable (O'Grady et al., 2019). This leads to

51 consequences for the patient (infection may not be controlled if pathogen is resistant), the

52 clinician (limited treatment choices) and the public (emergence and spread of AMR and

53 subsequent drug-resistant infections). Even if susceptibility testing is requested, urine culture

54 plus AST takes 2-3 days, during which time infections worsen if the empirical antibiotic is

55 ineffective, or if treatment is delayed prior to culture results.

56 Several emerging strain-characterisation technologies to determine AST have been

57 developed to the point of large scale uptake into central microbiology laboratories (Pulido et

58 al., 2013). Many labs are now using mass spectroscopy for identification of clinically

59 important microbes by peptide mass fingerprinting to replace traditional analytical

60 microbiology techniques (Lavigne et al., 2013, Florio et al., 2020). Nucleic acid tests (NAT)

61 such as PCR can detect specific AMR genes, allowing rapid identification of known

62 resistance markers, but do not always reliably predict phenotypic antibiotic susceptibility and

63 can only detect target sequences – as these targets change, tests will need regular updating

64 informed by phenotypic-genotypic surveillance programs (Needs et al., 2020).

65 Phenotypic AST, including the current gold standard methods (Andrews, 2001, Andrews,

66 2005, Jorgensen and Turnidge, 2015) of broth microdilution (BMD) performed in a microtitre

67 plate (MTP) and disc diffusion assays performed on agar plates, demonstrate antibiotic

68 suitability by direct phenotypic identification of which antibiotics achieve bactericidal or

69 bacteriostatic effects (i.e. susceptible), vs which isolates grow in the presence of antibiotic

70 (i.e. resistant). Phenotypic AST currently requires a pure bacterial culture isolated by

71 overnight plating, laborious handling and requiring centralised laboratory equipment.

72 There are several automated phenotypic AST machines currently available. The Vitek® 2

73 (Biomerieux) reports bacterial identification and MIC results within 5-8 h, following bacterial

74 colony isolation after overnight agar plating. In contrast, the Accelerate Pheno[R] system is

75 designed to be used directly with samples (Kaprou et al., 2021). These methods are able to

76   reduce the time to result by hours and potentially days (Kinn et al., 2019) but still require the

77   use of centralised labs for testing.

78   Microfluidic technologies offer several advantages, especially in point-of-care applications,

79   including portability and rapid result times. Recently many examples of microfluidic analytical

80   microbiology devices illustrate the potential for AST miniaturisation (Avesar et al., 2017, Azizi

81   et al., 2018, Wu and Dekker, 2016, Kim et al., 2015, Kang et al., 2019). A "millifluidic" system

82   made from simple FEP tubing illustrated that analysis of AST can be miniaturised using

83   microdroplets allowing parallel analysis of large populations of individual cells (Baraban et

84   al., 2011). Whilst this microdroplet system offers improved resolution in quantitative AST

85   (Jiang et al., 2016) pure cultures from overnight plating and complex instrumentation are still

86   required. Microfluidic devices have also been developed for quantitative susceptibility

87   testing. Multi-chamber devices can distribute bacterial samples into chambers loaded with

88   antibiotics (Cira et al., 2012, Avesar et al., 2017, Azizi et al., 2018, He et al., 2020,

89   Matsumoto et al., 2016), or cells can be observed in microfluidic systems that generate

90   antibiotic gradients (Kim et al., 2019, Li et al., 2014). Microfluidic culture can be combined

91   with digital microscopy and image analysis to allow direct observation of the effects of

92   antibiotics on cell behaviour or growth (Choi et al., 2013, Wistrand-Yuen et al., 2020,

93   Baltekin et al., 2017, Matsumoto et al., 2016). However, microscopic observation of bacterial

94   growth inhibition by antibiotics does not directly map to traditional BMD or disc diffusion

95   results, and extensive equivalency testing is required. Electrochemical detection has been

96   combined with the conventional colorimetric metabolic dye resazurin to detect bacterial

97   growth in microfluidic devices (Besant et al., 2015). Most microfluidic microbiology studies

98   have focused either on exploring microdevice engineering science, or on addressing

99   fundamental biology research questions. One notable exception is a microplate-based

100   microfluidic AST device that has been developed into a fully automated product for testing

101   positive blood cultures to rapidly select antibiotics for the treatment of sepsis (Choi et al.,

102   2014, Choi et al., 2013). However, this laboratory device still requires pre-incubation of blood

103   sample prior to AST, to achieve a threshold level of organisms required for testing.

104   Incubation time and inoculum size are critical parameters in AST and deviation from

105   standardised methods can lead to errors in categorical agreement compared to the gold

106   standard. It is therefore important to understand how both of these may affect any new AST

107   devices or methods, however there are limited data in the literature exploring how these

108   parameters affect microfluidic analytical microbiology.

109   Shorter incubation times of 6- 8 h have been explored for disc diffusion assays

110   (Mancini et al., 2020, Hombach et al., 2017, Fröding et al., 2017, Chandrasekaran et al.,

111   2018). While categorical agreement differed depending on antibiotic/bacteria tested, overall

112   agreement was generally >80% and it was concluded this approach was useful as a

113   preliminary result to base urgent clinical decisions on (Fröding et al., 2017). Disc diffusion

114   assays of 11123 Enterobacteroles/antibiotic combinations incubated for 6 h found sharper,

115   more defined areas of inhibition with categorical agreement of 89.8% and major errors and

116   very major errors below 4%; after 8 h the categorical agreement rose to 98.5%. The most

117   common errors came from strains categorised as intermediate (Mancini et al., 2020); for

118   such strains where the inhibitory concentration for antimicrobial is close to the breakpoint for

119   scoring resistance vs susceptibility, there is always greater uncertainty in AST, even with

120   gold standard methods. In a clinical setting it is important to identify resistant strains early,

121   and false susceptibility is more of a risk than false resistance as the former can lead to

122   treatment failure, so for rapid test methods to be clinically useful must be optimised to

123   minimise false susceptibility.

124   The inoculum cell density is likewise an important factor when measuring MIC, and

125   an increased starting inoculum can increase the observed MIC for a number of reasons. This

126   well-known phenomenon is termed the inoculum effect (IE), and varying the starting bacterial

127   cell density can influence inhibition of growth by many antimicrobials; hence guidelines for

128   broth microdilution define acceptable ranges for inoculum density. Even within the CLSI

129   recommended range of inoculum density, categorical errors in antibiotic sensitivity have

130   been observed. An 8-fold difference was found in MIC between $2\text{-}8\text{X}10^5$ CFU/mL for

131   carbapenem-resistant *Enterobacteriaceae* with meropenem (Smith and Kirby, 2018).

132   Estimating and adjusting cell density therefore remains an essential but laborious step for

133   many functional microbiology methods.

134   Healthcare budgets remain a major constraint, with high prevalence of UTI resulting in very

135   large volume of patients presenting with UTI symptoms in primary care. AMR is more

136   prevalent in parts of the world with most limited healthcare budgets (Pokharel et al., 2019).

137   Very low device costs are therefore needed and expensive capital equipment should be

138   avoided, and highly skilled technical sample processing or complex interpretation of results

139   are likewise undesirable. There remains an urgent need for novel AST methods that can

140   reduce workload, provide high quality data and directly test patient urine to without the need

141   for isolation or overnight plating. Ideally new methods would replicate conventional

142   phenotypic assays as closely as possible, to permit ease of interpretation by clinicians

143   accustomed to current gold standard methods. For example, miniaturised BMD utilising

144   growth detection that is similar to current automated lab BMD equipment would accelerate

145   adoption of new technology by permitting simple clinical interpretation of similar results

146   measured and presented in similar ways to current gold standard AST, such as MIC values

147   and scoring resistance/susceptibility at the same breakpoint concentrations. As breakpoint

148   concentrations are constantly monitored, ensuring new methods can make direct use of

149   current guidelines is vital to avoid continuous equivalency testing.

150   Here, we fully evaluate a high throughput system for analytical microbiology that uses a

151   fluoropolymer microcapillary film (MCF) that contains a parallel array of ten microcapillaries.

152   The MCF is manufactured by melt-extrusion and multiplex test strips can be produced in

153   large batches (Barbosa et al., 2015, Castanheira et al., 2015, Barbosa et al., 2014, Edwards

154   et al., 2011), allowing us to make and test hundreds of very low-cost test devices,

155   representing thousands of individual microcapillary tests. These devices incorporate a

156   hydrophilic polymer coating to modify the internal walls of the capillaries allowing sample

157   uptake by capillary action. Using this method, samples can be loaded easily by dipping the

158   test strips into the well of a 96-well plate with the sample drawn up into the 10 capillaries by

159   capillary action. Previously, we showed that these "Lab-on-a-stick" devices can perform a

160   range of classical microbiology tests to identify and count viable cells (Reis et al., 2016,

161   Needs et al., 2019, Dönmez et al., 2020, Needs et al., 2021). Here we present the first full

162   demonstration of MIC measurement and antibiotic susceptibility testing for a range of

163   antibiotics in clinically relevant UPEC isolates. We explore the impact of inoculum cell

164   density on microfluidic broth microdilution and assess the kinetics of antibiotic susceptibility

165   testing in a convenient miniaturised format, to understand better how to use microfluidics to

166   speed up analytical microbiology.

167

2 Material and Methods

2.1 Bacterial Isolates

170   Reference strain *E. coli* 25922 was purchased from ATCC and was used as a quality control.

171   *E. coli* 13352 was purchased from NCTC. The uropathogenic *E. coli* (UPEC) strains were

172   collected at a tertiary care hospital of Pakistan from community acquired UTI patients

173   (Rafaque et al., 2018, Rafaque et al., 2019) under a study that was approved by the Ethical

174   Review Board (ERB) of the Pakistan Institute of Medical Sciences. UPEC were identified

175   using standard microbiological and biochemical tests as described before (Ali et al., 2019).

2.2 Preparation of antibiotic loaded microcapillary test strips

177   MCF of 1 to 5 m lengths were given an internal hydrophilic coating by incubation with a 5

178   mg/mL solution of polyvinyl alcohol (PVOH) in water (MW 146,000-186,000, >99%

179   hydrolysed, Sigma-Aldrich, UK) at room temperature for 2h (Pivetal et al., 2017, Reis et al.,

180   2016). Coated strips were washed with 5 ml of PBS with 0.5 % Tween 20 (Sigma-Aldrich,

181   UK) to remove residual PVOH, and dried on a vacuum manifold for 20 minutes per metre

182   using a SLS Lab Basics Mini Vacuum Pump with PTFE Coated Diaphragm (Scientific

183   Laboratory Supplies, UK).

184   Ciprofloxacin, cefoxitin, trimethoprim and amoxicillin were purchased from Sigma Aldrich.

185   Nitrofurantoin and Cephalexin were from purchased from Fischer Scientific. Serial dilutions

186   of antibiotics in sterilised milliQ water were injected using a 30-gauge needle into individual

187   capillaries in up to 1.5 m long strips of MCF. The MCF strip was cut to 17 mm individual test

188   strips and frozen overnight at – 80 °C. Test strips were freeze-dried for >4 h on an Edwards

189   Modulyo freeze drier. Test strips were vacuum packed and stored at -20 °C until use.

190   **2.3 Microplate Broth Microdilution**

191   Bacterial inocula were prepared according to CLSI standard for growth preparation. Briefly,

192   3-5 individual colonies were inoculated into Mueller-Hinton broth and grown for 4-6 h until

193   visibly turbid. The suspension was diluted to 0.5 McFarland equivalent which corresponds to

194   approximately $1X10^8$ CFU/mL. The culture was diluted 1:150 in Mueller-Hinton to give $1X10^6$

195   CFU/mL suspension and finally $50\mu L$ was added to $50\mu L$ of antibiotic solutions in a

196   microplate to give a 1:2 dilution to give a final suspension of bacteria at $5X10^5$ CFU/mL in

197   two-fold dilutions of antibiotic. For BMD including resazurin, dye was added to a final

198   concentration of 0.25 mg/mL. Plates were incubated at 37 °C overnight. MIC measurements

199   were measured in duplicate (Fig 1a). The MIC was recorded as the lowest concentration of

200   antibiotic that did not show resazurin conversion or turbidity. For tests that varied within

201   duplicate measurements, the highest MIC was recorded. A growth control (no antibiotic) and

202   a sterile control (MH broth only) was included for all isolates.

203   **2.4 Microcapillary Broth Microdilution**

204   Individual 17 mm test strips were clipped into 3D printed holders, able to hold up to 12 test

205   strips. For MIC tests in MCF strips, bacteria inoculum was prepared as per the CLSI

206   standard for growth preparation as before at a final concentration of $5X10^5$ CFU/mL and

207   mixed in a 96 well plate with a final concentration of 0.25 mg/mL resazurin in a total volume

208   of 200 µL. The test strips were dipped into each well. Once all capillaries were filled

209   (approximately 3 seconds) end covers filled with silicone grease were placed on each end to

8

210   stop sample evaporation. Samples were incubated overnight at 37 °C and color change

211   monitored using an in-house automated raspberry pi camera imaging system (Needs et al.,

212   2019). MIC measurements were taken in duplicate with two wells used for each

213   measurement (Fig 1a). The MIC was recorded as the lowest concentration of antibiotic that

214   did not show resazurin conversion or turbidity. For tests that varied in duplicate

215   measurements, the highest MIC was recorded. A growth control (no antibiotic) capillary was

216   included for all test strips.

217   For microcapillary BMD experiments using a higher inoculum density, CFU/mL was

218   calculated using a spot plating protocol. Briefly, 10 µL of serially diluted culture were taken

219   from the microtitre wells and plated on both LB agar and the Gram-negative selective media,

220   MacConkey agar to determine cell count and ensure no contamination with other organisms.

221   Plates were incubated overnight at 37 °C.

222   **2.5 Data Analysis**

223   Two recordings of the test strips were made. A single endpoint was recorded by colorimetric

224   measurement after overnight incubation at 37 °C using a smartphone camera (iPhone 6s) or

225   digital camera (Canon Powershot S120). The strips were placed on an even white light

226   illumination screen. Isolates were scored suspectable or resistant based on EUCAST v11.0

227   breakpoint values in Table 1.

228   For time-lapse imaging, resazurin conversion was recorded every 15 minutes using the

229   POLIR robot (Needs et al., 2019), with 3280 x 2464 resolution images taken with a

230   Raspberry Pi v2 camera. MatLab scripts were used to analyse time-lapse image series of

231   bacterial growth in MCF, and the code can be accessed here:

232   https://gitlab.com/sneeds/code-repository. Briefly, color images were split into red, blue and

233   green (RGB) channels and the red channel analysed for absorbance using:

234    $$A = log_{10}\left(\frac{I_o}{I}\right)$$

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235 Time to resazurin conversion was calculated at which half the starting absorbance in the red

236 channel was reached.

3 Results

3.1 Broth microdilution in microcapillary test strips accurately determines MIC for uropathogenic *E. coli*

240 The novel microcapillary BMD method conducts AST in dip-and-test microcapillary film

241 (MCF) strips, with each test strip containing 10 different conditions (9 antibiotic

242 concentrations plus no antibiotic control) in 1 μL samples, thereby reducing the test volume

243 over 100-fold from microplates. To understand if miniaturisation affects BMD performance,

244 we validated this microcapillary BMD AST, by comparing to a standard microplate BMD

245 following CLSI guidelines. Growth was quantified using the colorimetric growth indicator,

246 resazurin, and although full growth kinetics were recorded, we initially focussed on an

247 overnight endpoint readout. Microplate BMD and microcapillary BMD were performed in

248 parallel, testing a panel of 6 antibiotics commonly used in the treatment of UTIs, against an

249 *E. coli* quality control (QC) strain, an *E. coli* reference strain, and 20 UPEC isolates.

250 *E. coli* cultured in Mueller-Hinton broth were diluted to a standard inoculum cell density of

251 5×10^5 CFU/mL in Mueller-Hinton broth with a final resazurin concentration of 0.25 mg/mL in

252 both microplates and MCF test strips in parallel, and overnight colorimetric endpoint images

253 taken to score growth/no growth, and the same colour changes were observed in both

254 microplate and microcapillaries (Fig 2). The capillaries in MCF test strips have a 20-fold

255 shorter light pathlength than a microtitre plate, therefore the concentration of resazurin had

256 to be high enough to be clearly visible in the capillaries. Endpoint colour change was scored

257 after overnight growth from digital images, with pink/white turbid capillaries indicating

258 bacterial growth whereas samples with a susceptible bacterial population remained blue (Fig

259 1b and 2).

260   BMD in MCF and microplates was performed in duplicate and the highest MIC value for

261   each isolate/antibiotic combination was recorded in Table S1. A total of 2640 data points

262   were collected from the combination of 6 antibiotics and 22 samples (20 UPEC isolates plus

263   1 QC strain and 1 reference strain, totalling 132 antibiotic/isolate combinations). The *E. coli*

264   25922 strain was used as the QC strain. All MIC measured by microplate BMD and

265   microcapillary BMD were within the acceptable MIC range according to EUCAST QC Table v

266   11.0 for this QC strain. Out of all combinations of *E. coli* strains tested against the 6

267   antibiotics, there were 56 combinations where MIC was measurable and for these there was

268   100% (56/56) essential agreement between microcapillary and microplate, with MIC values

269   for each method within ±1 doubling dilution of antibiotic. No MIC value was attributed to 74

270   microplate BMD tests and 71 microcapillary BMD test strips. Of these, no MIC value was

271   attributed for 3 microplate BMD conditions in which growth was only detected in the control

272   without antibiotic (i.e. MIC was below the lowest concentration; scored as susceptible), and

273   for 71 microplate BMD and 71 microcapillary BMD test strips where all antibiotic containing

274   capillaries showed growth thus MIC was greater than the highest included concentration

275   (scored as resistant; Table 2). Categorical agreement was achieved when isolates were

276   scored as susceptible or resistant at the EUCAST breakpoint values shown in Table 1 by

277   both methods. There was a 96% (127/132 isolate/antibiotic combinations) categorical

278   agreement across all antibiotics tested and 100% categorical agreement for all isolates

279   tested against trimethoprim, nitrofurantoin, cephalexin, and amoxicillin (Table 2). The

280   microcapillary BMD had 2.3% (3/132) very major errors where 3 isolates were categorised

281   as susceptible in contrast to microplate BMD that categorised them as resistant. All three

282   major errors occurred with cefoxitin for which the MIC determined by microplate BMD was

283   within ±1 $\log_2$ fold change of the breakpoint, indicating the breakpoint value is close to the

284   threshold for inhibition. In this situation the variability in resistance/susceptibility scoring is

285   known to increase significantly, leading to higher uncertainty in AST results even with gold

286   standard methods. Microcapillary BMD showed a further 1.5% (2/132) major errors in which

287   isolates tested for cefoxitin and ciprofloxacin were identified as resistant but categorised as

288   susceptible by microplate BMD.

289   Overall, we conclude that microcapillary BMD performs very well and that the miniaturisation

290   of broth microdilution from microplate wells down to microcapillaries has little if any impact

291   on MIC determination and scoring for antibiotic resistance.

292   **3.2 Time-resolved growth analysis in microcapillary test strips allows earlier endpoint**

293   **MIC giving accurate antibiotic susceptibility within 6h**

294   Previously we established that even at low cell densities, *E. coli* growth can be detected

295   within 7 h and at the inoculum recommended for AST within approximately 3-5 h (Needs et

296   al., 2021) (Fig S1). MIC were measured and susceptibility/resistance scored for

297   microcapillary BMD after 6 h incubation (Fig S2-7), and results compared to overnight

298   microplate BMD results (Table 3). In only two cases, MIC was not determinable due to no

299   growth detected at 6h in the no antibiotic control, for *E. coli* 13352 tested against

300   trimethoprim and cefoxitin. No MIC value was attributed for 9 microcapillary nitrofurantoin

301   BMD test strips, in which growth was only detected in the growth control without antibiotic

302   (i.e. MIC below lowest concentration; scored as susceptible), or for a further 72

303   microcapillary BMD test strips where all antibiotic containing capillaries showed growth and

304   thus the MIC was above the highest concentration (scored as resistant; Table 3).

305   For those conditions where MIC was within the measurable range, overall essential

306   agreement in MIC level across the antibiotics tested dropped to 78% (38/49 isolate/antibiotic

307   combinations). However, overall categorical agreement remained high at 91% (120/132

308   isolate/antibiotic combinations). Very major errors were identified in 7.7% (10/130) of cases.

309   The accuracy was reduced in a number of ways. Where growth was not clearly detected at

310   the 6h timepoint, because the antibiotic slows growth, false susceptibility could be scored,

311   but at an overnight timepoint even the slower growth still leads to a positive score. On the

312   other hand, slower growing isolates growth may not be strongly detected at 6h even in

313   control without antibiotic; for these strips susceptibility and MIC cannot be scored.

314   Kinetics of growth was clearly influenced more by some but not other antibiotics, depending

315   on susceptibility/resistant status. Cefalexin and ciprofloxacin show very similar MIC results at

316   6 and 16 h varying by only one doubling dilution of antibiotic (Table S3, Fig 3a-b). Isolates

317   categorised as resistant show indistinguishable growth kinetics in the presence of antibiotics

318   (Figure 3c). Nitrofurantoin, in which all isolates were categorised as susceptible, showed the

319   most distinct delay in growth kinetics in the presence of antibiotic (Fig 3d). Shorter incubation

320   times may be less suitable for high resolution quantitation of MIC, and may be more

321   accurate for some antibiotics than others, however overall accuracy of microcapillary BMD

322   for scoring resistance vs sensitivity remained high even with a 6h endpoint readout. As with

323   overnight endpoint, the growth could readily be recorded using a smartphone camera or if

324   necessary, scored by eye. Overall, this indicates that early endpoints can be reliable for

325   determining resistance and may aid in decision making for resistant isolates.

326   **3.3 Effect of inoculum density on microfluidic MIC determination**

327   Detecting antibiotic susceptibility and measuring MIC is known to be significantly

328   affected by the initial inoculum density, and in the development of new methodologies it is

329   vital to identify how sensitive results are to variables such as inoculum cell density that are

330   not easy to control. Here, we focussed on understanding how starting cell density affects

331   microcapillary BMD performance, and seeing if IE reduces accuracy for both MIC

332   quantitation and AST scoring, as it does for conventional microplate methods. Microcapillary

333   BMD test strips were dipped into serial 10-fold dilutions of each bacterial isolate, starting with

334   10^7 CFU/mL (Fig 4), 100X higher than the recommended density. Inoculum densities 100X

335   above the CLSI recommended resulted in growth at antibiotic concentrations significantly

336   above the MIC, giving false resistance scores for *E. coli* 25922 and UPEC 2165 for

337   ciprofloxacin and cefalexin and in UPEC 2165 for nitrofurantoin (Fig 4b and Table 4). This

338   confirms that as for microplate BMD, the starting inoculum should be standardised to avoid

339   false resistance from high starting cell density.

340   Inoculum densities below the CLSI recommended density gave MIC measurements within

341   the ± 1 $\log_2$ fold acceptable range, suggesting the microcapillary BMD is relatively robust as

342   long as inoculum density remains low enough to avoid false resistance or raised MIC levels.

343   This indicates that the microcapillary BMD test strips are compatible with lower bacterial

344   inoculum, suggesting this method may be robust enough to be used in environments where

345   precise inoculum density may be harder to achieve (e.g. less well-resourced laboratories).

3.4 Detecting delay in growth from kinetic analysis allows fast and accurate

categorical determination at higher cell densities

348   Rapid AST microdevices have been developed that use time-lapse digital microscopy

349   imaging to determine delayed growth in the presence of antibiotics compared to a growth

350   control (Avesar et al., 2017, Kang et al., 2019, Busche et al., 2019, Sun et al., 2019, Osaid et

351   al., 2021). Higher inoculum densities convert resazurin faster. The use of miniaturised BMD

352   tests combined with kinetic data collection offers further opportunity to gain more detailed

353   insight into bacterial response to antimicrobial agents, for example through detection of

354   differential growth kinetics indicating susceptibility. MIC was calculated based on differential

355   growth kinetics for different inoculum densities (Table 5, Fig S8-9). MIC was calculated by

356   scoring susceptibility when growth was delayed, but resistant when at a particular antibiotic

357   concentration, growth was detected within 1 h of the no antibiotic control. It was evident that

358   isolates that were categorised as susceptible, and with MIC well below the breakpoint

359   concentration, were consistently scored as susceptible by this method, even at inoculum

360   densities 100X higher than the recommended range. Nitrofurantoin showed significantly

361   delayed growth compared to the growth control at all inoculum densities (Fig 4c). False

362   resistance at higher inocula was still observed for some isolate/antibiotic combinations but

363   with an MIC still within one doubling dilution of the breakpoint antibiotic concentration. For

364   example, *E. coli* 25922 has an MIC for ciprofloxacin of 0.015 µg/mL. Based on growth

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365 kinetics, inocula as high as 10^7 CFU/mL would be scored as susceptible. The UPEC isolate

366 2165 with an MIC of 0.25 μg/mL – the same value as the breakpoint – exhibited false

367 resistance at inoculum densities greater than 10^5 CFU/mL. It is clear therefore that the

368 fastest way to score resistance or susceptibility, or to estimate MIC, is through detecting

369 delayed growth with a high inoculum density that allows early growth detection. The

370 accuracy of this method varies depending on the antibiotic, and as with even gold standard

371 methods, if the MIC of isolates is close to breakpoint concentrations, AST is less reliable.

4. Discussion

373 Overall, by measuring growth with large panels of antibiotic combined with a set of *E. coli*

374 strains with a range of sensitivities, we observed that microfluidic broth microdilution

375 performs very similarly to microplate broth microdilution. While agreement between the

376 methods was very close (Table 2), the conditions where different results were obtained

377 represented samples with marginal MIC where variability in AST is known to be highest. We

378 observed the IE, that is well characterised for microplate measurements, also affects

379 microfluidic broth microdilution (Fig 4), with implications for direct testing methods where

380 bacterial inoculum density may be unknown or difficult to control. As might be expected,

381 faster results can be obtained with earlier growth readouts, yet some trade-off is apparent

382 between speed and accuracy. Nevertheless, even with 6h endpoint (Table 3) or when

383 differential growth is used to score susceptibility (Table 5), accuracy remained excellent for

384 some antibiotics and overall was 91%.

385 Antibiotics were chosen based on their use in UTI treatment but also to cover a range of

386 antibiotic classes. UTIs are the most common outpatient infection (Medina and Castillo-Pino,

387 2019). In the UK, the 2018 NICE guidelines recommend a first-choice treatment for

388 uncomplicated UTI in women over 16 years old of Nitrofurantoin or Trimethoprim, despite

389 resistance rates to trimethoprim around 30% (Watts et al., 2020). During pregnancy, the

390 need for safe and effective drugs is increased, while nitrofurantoin remains first choice,

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391 amoxicillin is only recommended if the susceptibility is known due to resistance rates above

392 50% in some demographics (Watts et al., 2020). Cefoxitin, while not currently used for

393 clinical prescription, has been cited as an effective *in vitro* against UTI pathogens including

394 extended-spectrum beta-lactamase producing Enterobacteriaceae (ESBL-E) (Mambie et al.,

395 2016, Senard et al., 2018, Guet-Revillet et al., 2014). Since antibiotic loading of the strips is

396 by freeze-drying, it is expected that the test can be adapted to most antibiotics for different

397 applications.

398   Bacterial growth was monitored using the metabolic indicator resazurin. Resazurin

399 has been used in many different plate assays studying bacterial and cellular growth,

400 mitochondrial function, and MIC of antimicrobial agents. However, with many cell viability

401 test methods the resazurin is added towards the end of the experiment and incubated for

402 several hours to measure metabolic activity after a primary incubation period with

403 antimicrobial agent but no dye, to reduce the risk of the dye interacting with the antimicrobial

404 (Kim and Jang, 2018, Teh et al., 2017, Rakhmawatie et al., 2019, Elshikh et al., 2016,

405 Sarker et al., 2007). In contrast, for the novel microcapillary BMD method, the resazurin is

406 present from the start. Microplate BMD with the addition of resazurin at the start was

407 therefore compared to microplate BMD without dye to evaluate if the presence of resazurin

408 dye affected antimicrobial susceptibility and MIC results. There was 100% essential

409 agreement when microplate BMD was compared with and without resazurin dye for the MIC

410 values of nitrofurantoin and cephalexin, and 100% essential agreement between

411 microcapillary BMD (that includes resazurin) vs microplate BMD conducted without resazurin

412 (Table S2). This confirms that the inclusion of resazurin does not interfere with MIC

413 determination for *E. coli* when mixed with the sample at 0.25 mg/mL and incubated

414 overnight. Resazurin can be used with fluorescence detection (Needs et al., 2021) with the

415 potential for earlier growth detection compared to colorimetric detection, however, in

416 previous studies, some isolates showed a decay in fluorescence after the initial increase

417 (Needs et al., 2019). This has been observed previously in assays in which fluorescent

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418   resorufin is further reduced to the colorless, non -fluorescence hydroresorufin. This reduces

419   the reliability of an endpoint measurement as loss of fluorescent signal may be mistakenly

420   interpreted as negative growth i.e. scored as false susceptible (Uzarski et al., 2017). For this

421   reason, we measured growth colorimetrically. Although small, the individual capillaries are

422   large enough to be scored by the naked eye if digital camera is not available to record result.

423   A smartphone camera is ideal for digitally recording these test results, and wide range of

424   different quality digital cameras have been shown to be equally effective for recording

425   microcapillary bioassay results (Needs et al., 2021, Jégouic et al., 2021).

426   The ability to detect growth of very low viable cell numbers – ideally down to a single colony

427   forming unit – may be critical for some analytical applications. We previously identified the

428   limit of detection of a 1 μL microcapillary test strip to be in the order of 5×10^3 CFU/mL; at this

429   concentration 99% of capillaries are predicted to contain at least 1 bacteria every test,

430   allowing MIC measurement (Needs et al., 2021). The initial inoculum density correlates to

431   the speed of detecting resazurin conversion, as early growth at low density does not convert

432   enough dye to influence the capillary color, thus lower densities take longer before growth

433   can be detected (Needs et al., 2021). Thus, although reduced inoculum density still gave

434   categorical agreement with microplate BMD, early timepoint endpoint readings at 6h are not

435   possible as growth is not always detected without antibiotic at this timepoint (Fig S1).

436   Whilst overnight plating to obtain individual colony isolates, plus cell density adjustment prior

437   to testing is still required, these results confirm that microcapillary BMD has equivalent

438   performance to standard microplate BMD. The MCF test strips significantly increase

439   throughput compared to microplates alone, whilst remaining compatible with conventional

440   microtitre plate format found in most laboratories for sample preparation. While one standard

441   microplate BMD can test 6 isolates in duplicate with 6 concentrations of a single antibiotic,

442   the microcapillary BMD can test 96 isolates, with a single isolate per well. Each

443   microcapillary BMD test strip provides 9 antibiotic concentrations plus control, and each well

444   can be dipped with multiple test strips for replicates. Assuming picking and adjusting isolate

445 concentration, and plating samples out, is rate limiting, microcapillary BMD is therefore 16

446 times higher throughput than microplates.

4 Conclusions

448 We present a high throughput method for determining antibiotic resistance or susceptibility

449 and for measuring MIC, using a simple to use microfluidic dipstick device, and validate this

450 method with uropathogenic *E. coli* and antibiotics commonly used to treat UTI. The test is

451 fully compatible with existing lab equipment such as multichannel pipettes and 96-well

452 format microplates for sample preparation and does not require additional equipment such

453 as pumps or controllers for microfluidic liquid handling. The colorimetric detection of bacterial

454 growth allows easy readout and simple analysis, reducing the need for equipment

455 investment – most digital cameras including smartphone cameras are capable of recording

456 color change, or growth can be scored by eye. The microcapillary BMD can provide rapid

457 categorical susceptibility/resistance scoring at time points within 6 h based on differential

458 growth. Kinetic analysis can provide robust S/R categorisation above the recommended

459 inoculum density, however, isolates with MIC within two-fold dilution of the breakpoint may

460 show false resistance.

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469 **Tables**

470 **Table 1. EUCAST v 11.0 antibiotic MIC breakpoints used for categorical analysis**

Antibiotic	EUCAST MIC breakpoints (mg/L) ≤S/>R
Cefoxitin	≤8
Ciprofloxacin	≤0.25
Trimethoprim	≤4
Nitrofurantoin	≤64
Cephalexin	≤16
Amoxicillin	≤8

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472 **Table 2. AST agreement between microplate BMD and microcapillary BMD.**

Antibiotic	Microplate BMD		Microcapillary BMD		% Essential Agreement	% Categorical Agreement
	Susceptible	Resistant	Susceptible	Resistant		
Trimethoprim	18% (4/22)	82% (18/22)	18% (4/22)	82% (18/22)		**100%** (22/22)
Nitrofurantoin	100% (22/22)	0% (0/22)	100% (22/22)	0% (0/22)		**100%** (22/22)
Cephalexin	14% (3/22)	86% (19/22)	14% (3/22)	86% (19/22)	**100%** (55/55)	**100%** (22/22)
Amoxicillin	5% (1/22)	95% (21/22)	5% (1/22)	95% (21/22)		**100%** (22/22)
Ciprofloxacin	18% (4/22)	82% (18/22)	17% (3/22)	86% (19/22)		**95%** (21/22)
Cefoxitin	27% (6/22)	73% (16/22)	36% (8/22)	64% (14/22)		**82% (**18/22)

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Table 3. Agreement between microcapillary test strips read at 6h compared to overnight

Antibiotic	BMD (16 – 18 h)		Microcapillary BMD (6h)		% Essential Agreement	% Categorical Agreement
	Susceptible	Resistant	Susceptible	Resistant		
Trimethoprim[1]	18% (4/22)	82% (18/22)	14% (3/21)	86% (18/21)	**100%** (3/3)	**100%** (21/21)
Nitrofurantoin	100% (22/22)	0% (0/22)	100% (22/22)	0% (0/22)	**69%** (9/13)	**100%** (22/22)
Cephalexin	14% (3/22)	86% (19/22)	14% (3/22)	86% (19/22)	**83%** (5/6)	**100%** (22/22)
Amoxicillin	5% (1/22)	95% (21/22)	5% (1/22)	95% (21/22)	**100%** (1/1)	**100%** (22/22)
Ciprofloxacin	18% (4/22)	82% (18/22)	27% (6/22)	73% (16/22)	**100%** (6/6)	**91%** (20/22)
Cefoxitin[2]	27% (6/22)	73% (16/22)	62% (13/21)	38% (8/21)	**71%** (15/21)	**62%** (13/21)

[1] *E. coli* 13352 unable to determine MIC at 6h in microcapillary BMD.
[2] *E. coli* 13352 unable to determine MIC at 6h in microcapillary BMD.

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481 **Table 4. Determination of endpoint MIC using varying inoculum density.**

	UPEC 2165					
	Nitrofurantoin		Cefalexin		Ciprofloxacin	
CFU/mL	Endpoint Microplate BMD (5×10^5 CFU/mL)	Microcapillary BMD	Endpoint Microplate BMD (5×10^5 CFU/mL)	Microcapillary BMD	Endpoint Microplate BMD (5×10^5 CFU/mL)	Microcapillary BMD
4×10^7		>512 (R)		> 64 (R)		>1 (R)
4×10^6		8 (S)		16 (S)		1 (R)
4×10^5	4 (S)	4 (S)	8 (S)	16 (S)	0.25 (S)	0.25 (S)
4×10^4		≤2 (S)		16 (S)		0.25 (S)
4×10^3		≤2 (S)		16 (S)		0.25 (S)
	E. coli 25922					
2×10^7		64 (S)		>64 (R)		1 (R)
2×10^6		4 (S)		>64 (R)		0.06 (S)
2×10^5	4 (S)	4 (S)	16 (S)	32 (R)	0.015 (S)	0.015 (S)
2×10^4		4 (S)		16 (S)		0.03 (S)
2×10^3		4 (S)		16 (S)		0.015 (S)

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493 **Table 5. MIC determination based on delayed growth in the presence of antibiotics**

		UPEC 2165					
		Nitrofurantoin		Cefalexin		Ciprofloxacin	
CFU/mL	Growth Control - Time to resazurin conversion (h)	Endpoint Microplate BMD ($5X10^5$CFU/mL)	Kinetic MIC	Endpoint Microplate BMD ($5X10^5$CFU/mL)	Kinetic MIC	Endpoint Microplate BMD ($5X10^5$CFU/mL)	Kinetic MIC
$4X10^7$	1		8 (S)		> 64 (R)		>1 (R)
$4X10^6$	2.8		≤2 (S)		16 (S)		1 (R)
$4X10^5$	4.4	4 (S)	≤2 (S)	8 (S)	16 (S)	0.25 (S)	0.25 (S)
$4X10^4$	5.8		≤2 (S)		16 (S)		0.25 (S)
$4X10^3$	7.7		≤2 (S)		8 (S)		0.125 (S)
		E. coli 25922					
$2X10^7$	1.5		≤2 (S)		>64 (R)		0.25 (S)
$2X10^6$	3.1		≤2 (S)		>64 (R)		0.015 (S)
$2X10^5$	4.9	4 (S)	≤2 (S)	16 (S)	8 (S)	0.015 (S)	0.015 (S)
$2X10^4$	5.6		≤2 (S)		8 (S)		0.007 (S)
$2X10^3$	7.7		≤2 (S)		4 (S)		0.007 (S)

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503 **Figures**

504 **Figure 1.**

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507 **Figure 2.**

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510   **Figure 3.**

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519 **Figure 4.**

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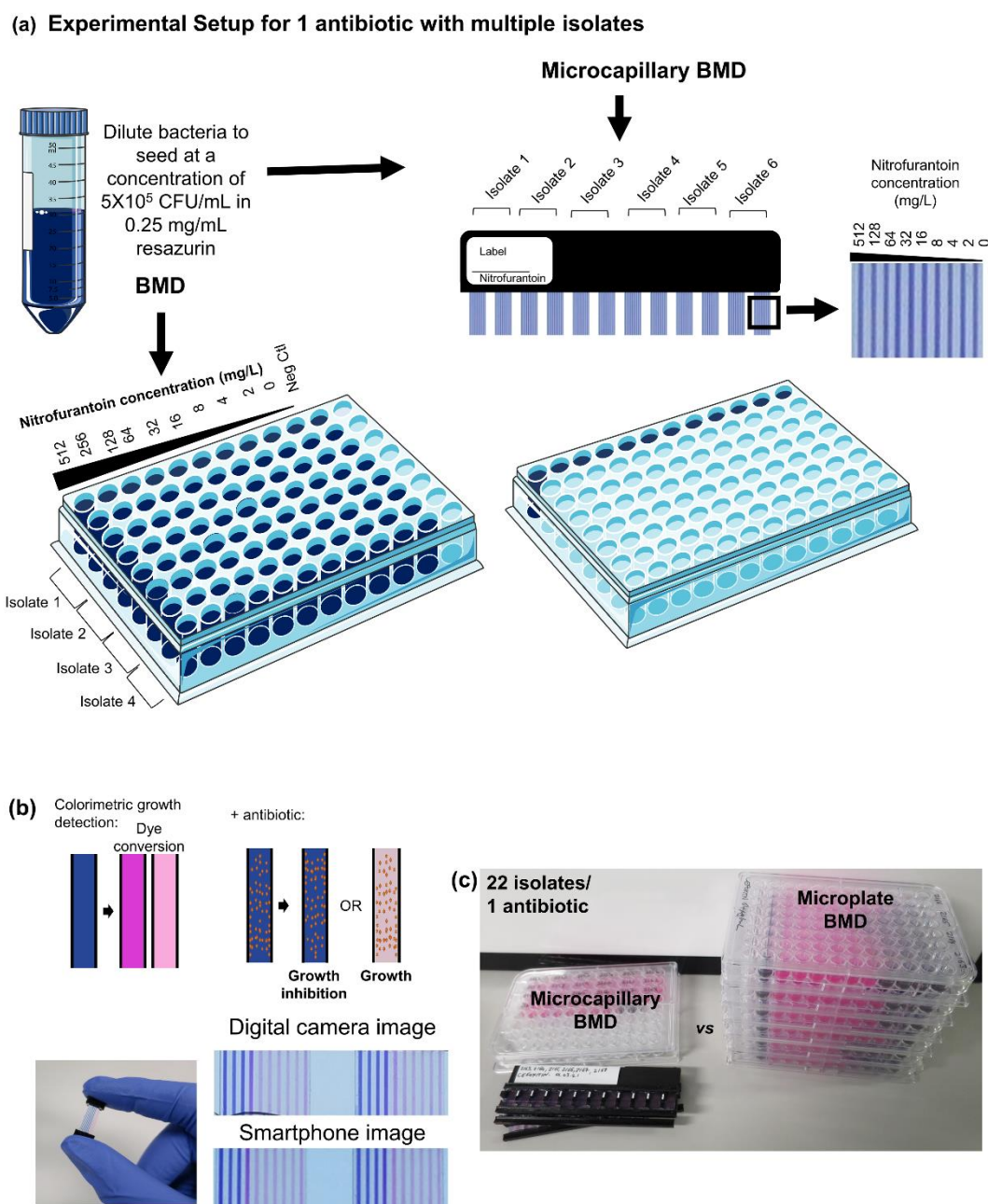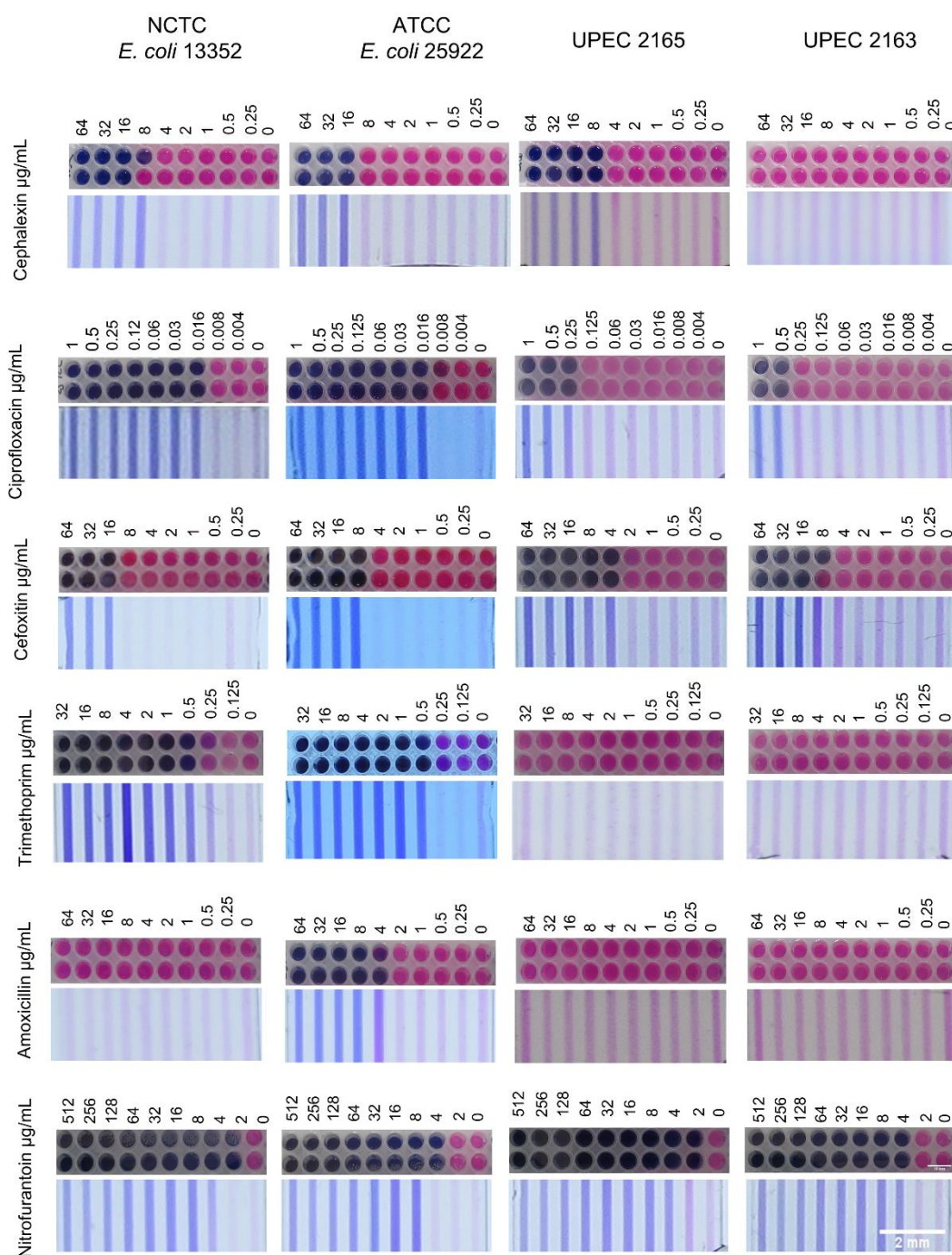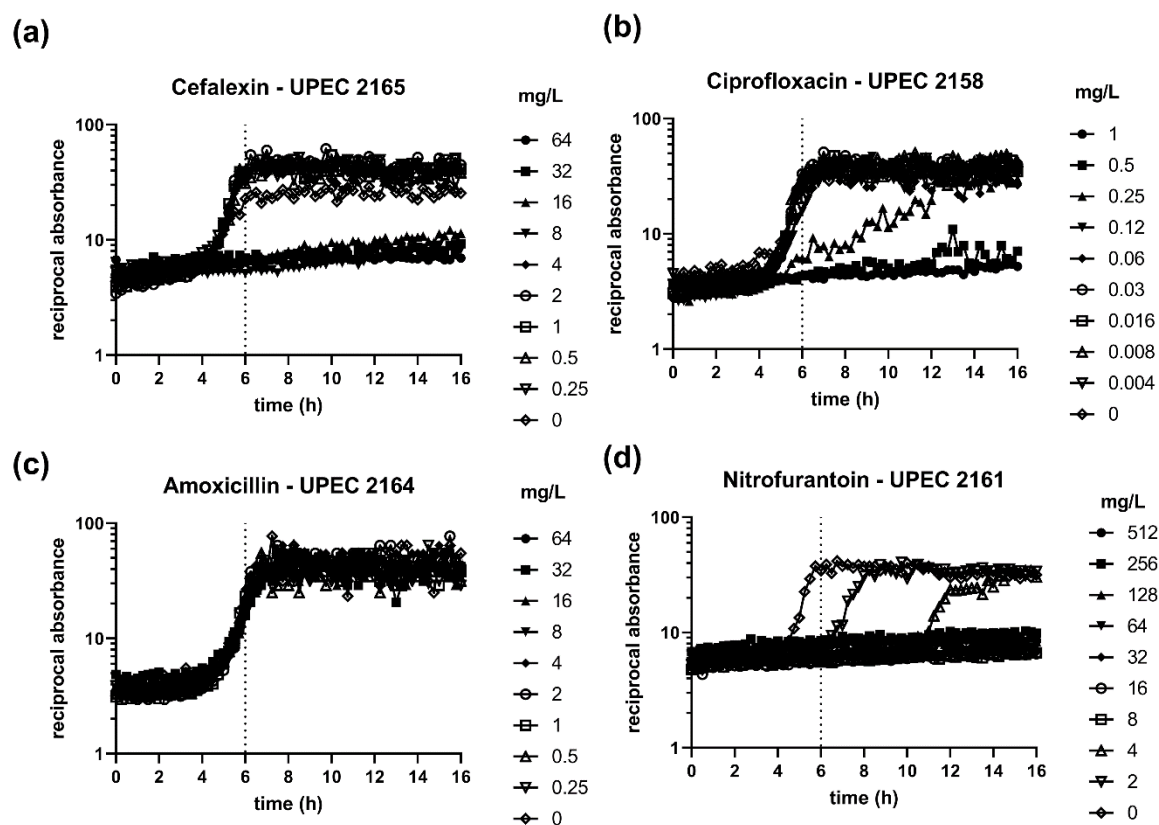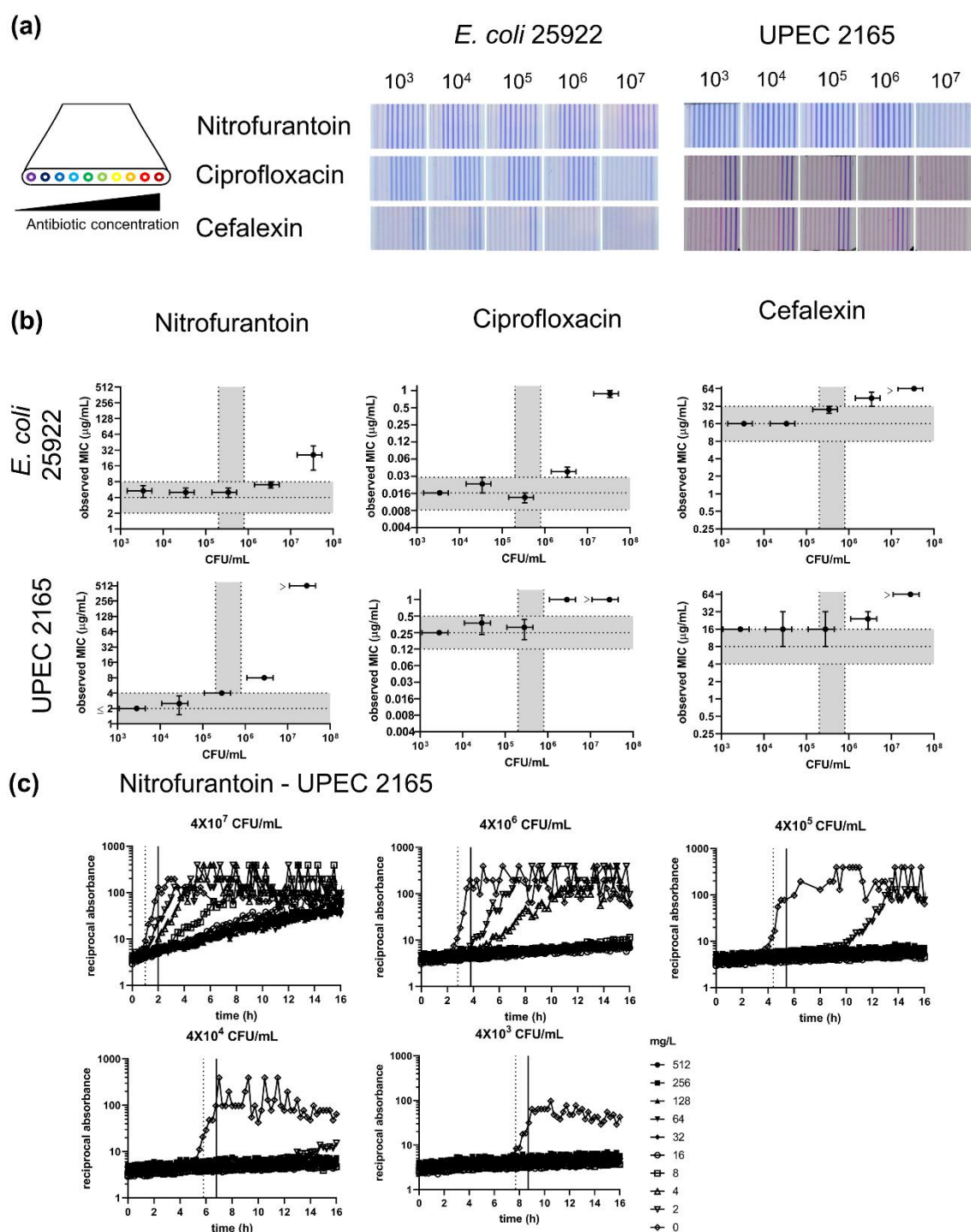
521 **Figure Legends**

522 **Figure 1. Microcapillary test use. (a)** An array of up to 12 test strips were clipped into a

523 'ladder' holder with a 9mm pitch. Each test strip contains 10 capillaries each loaded to

524 release doubling dilutions of antibiotic. Test strip arrays were dipped directly into 96-well

525 microtitre plate wells allowing 1 microlitre of the sample to be drawn up by capillary action

526 into all 10 microcapillaries. Plastic end covers filled with silicone grease were slid over the

527 ends of the test strips, sealing the capillary ends to prevent evaporation. Image created

528 using servier medical art **(b)** Bacteria growth and antibiotic inhibition is determined by

529 resazurin color change. Blue to pink/white indicates growth and if antibiotic is present,

530 indicates that concentration is below the minimum inhibitory concentration (MIC). At or

531 above MIC, the capillary remains blue. **(c)** Each ladder of microcapillary BMD test strips

532 provides up to 16X higher throughput than microtitre plates. Image illustrates the number of

533 plates vs microcapillaries required to test 22 isolates in duplicate for a single antibiotic at 9

534 concentrations plus no antibiotic.

535 **Figure 2. Agreement between microwell broth microdilution and microcapillary film**

536 **MIC.** Representative images showing MIC determination of two reference strains, *E. coli*

537 25922 and *E. coli* 13352 and urinary pathogen *E. coli* isolate 2165 MIC in microplate and

538 microcapillary film. Error bar on microplates indicate 10 mm. Error bar on microcapillary film

539 indicates 2 mm. Similar agreement was seen with all isolates tested in duplicate.

540 **Figure 3. Microcapillary BMD with a fixed inoculum density growth kinetics in the**

541 **presence of antibiotics. (a)** UPEC 2165 isolate gives the same MIC result after 6h

542 incubation compared to overnight. **(b)** UPEC 2158 recorded MIC is within 1 doubling dilution

543 of antibiotic for ciprofloxacin. **(c)** Growth of UPEC 2164 is identical at all antibiotic

544 concentrations of amoxicillin. **(d)** Nitrofurantoin shows the greatest amount of delayed

545 growth. UPEC 2161 only observes a positive growth control at 6h, indicated an MIC ≤2 mg/L

546 compared to an endpoint MIC of 8 mg/L.

547 **Figure 4. Endpoint MIC measurement of isolates using range of inoculum density. (a)**

548 Schematic showing antibiotic cocnetration is highest in capilary 10. Capilary 1 contains no

549 antibtioc and acts as a growth control. Representative images of capillary test strips at

550 various incoculum density ranging from $10^3 – 10^7$ CFU/mL. **(b)** MIC observed at different

551 inoculum density. Data indicates average of duplicate capillaries for two independent

552 experiments. Vertical error bars indicate ± SEM and horizontal error bars indicate ± SD for

553 inoclum density determiend by overnight agar plating. **(c)** Growth kinetics of UPEC 2165

554 against nitrofurantoin. Dotted line indicates detection time for the growth control, solid line

555 indicates 1 h cuttoff from growth control. Data points indicate average of duplicate

556 capillaries.

557 **Author Contributions**

558 SHN: Conceptualisation, Data curation, Formal analysis, Investigation, Methodology, Project

559 administration, Software, Visualization, Writing – original draft, Writing – review & editing.

560 WI: Resources, Writing – review & editing. ZR: Resources, Writing – review & editing .SA:

561 Resources, Writing – review & editing. PR: Writing – review & editing. ADE:

562 Conceptualisation, Funding acquisition, Methodology, Project administration, Supervision,

563 Writing – original draft, Writing – review & editing

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565 Figure 1a was adapted from elements from Servier medical art under CC BY 3.0 licence.

566 **Declaration of Competing Interests**

567 ADE is one of the inventors of patent application protecting aspects of the novel microfluidic

568 devices tested in this study and is a director and shareholder in Capillary Film Technology

569 Ltd, a company holding a commercial license to this patent application: WO2016012778

570 "Capillary assay device with internal hydrophilic coating" AD Edwards, NM Reis.

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575 **Ethical Approval**

576 The collection of urinary pathogenic *E. coli* from a tertiary care hospital of Pakistan from

577 community acquired UTI patients and was approved by Ethical Review Board (ERB) of

578 Pakistan Institute of Medical Sciences.

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