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Rapid antibiotic sensitivity testing and trimethoprim-mediated filamentation of clinical isolates of the *Enterobacteriaceae* assayed on a novel porous culture support

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A porous inorganic material (Anopore) was employed as a microbial culture and microcolony imaging support. Rapid Anopore-based antibiotic sensitivity testing (AST) methods were developed to assess the growth of clinical isolates, with the primary focus on testing the response of the *Enterobacteriaceae* to trimethoprim, but with the method supporting a wider applicability in terms of strains and antibiotics. It was possible to detect the growth of *Enterobacter aerogenes* after 25 min culture and to distinguish a trimethoprim-sensitive from a trimethoprim-resistant strain with 40 min incubation. MIC_{90} determinations were made on Anopore; these were in good agreement with the results from the Vitek 2 and E-test methods. The Anopore method correctly identified sensitive (40/40) and resistant (17/17) strains of the *Enterobacteriaceae* and other Gram-negative rods within only 2–3 h culture. Additionally, a trimethoprim-resistant subpopulation (10 % of population) could be detected by microcolony formation within 2 h, and a smaller subpopulation (1 %) after 3·5 h. These results suggest that this is a viable approach for the rapid AST of purified strains, and that it may be able to deal with mixed populations. The microscopic examination of microcolonies during AST is an advantage of this method which revealed additional information. Filamentation triggered by trimethoprim was discovered in many species of the *Enterobacteriaceae* for which this phenomenon has not previously been reported. Filamentation was characterized by heterogeneity in terms of cell length, and also uneven nucleic acid distribution and flattening of damaged cells. The development and application of Anopore-based AST within clinical diagnostics is discussed.

INTRODUCTION

The time taken to culture micro-organisms is generally a rate-limiting step in antibiotic sensitivity testing (AST), taking from days to weeks by conventional methods. An alternative route to rapid AST is prediction of microbial viability after antibiotic treatment using vital dyes combined with a fluorescence-activated cell sorter (Álvarez-Barrientos *et al.*, 2000). Examples of more rapid culture-based assays include the detection of microbial metabolism (Brunello & Fontana, 2000) and the optical imaging of microcolonies (Mejia *et al.*, 1999). Microbial culture is also possible in a variety of non-standard formats, not generally applied to diagnostics, including encapsulation in beads, and growth in capillaries (Shah *et al.*, 1998) or on surfaces other than agar (Nordbring-Hertz *et al.*, 1984; Binnerup *et al.*, 1998). We have shown that microbial culture is possible on strips of the ceramic Anopore (Ingham *et al.*, 2005). Anopore is a rigid and exceptionally porous (2×10^9 pores cm^{-2}; pore diameter 0·2 μm), inorganic, planar material (McKenzie *et al.*, 1992). Anopore is extremely flat, has a low background fluorescence, and is translucent when wet (Jones *et al.*, 1989; Wu *et al.*, 2004), making it appropriate as a cellular imaging substrate. Rapid microbial growth is possible on Anopore, with the nutrients supplied from beneath through the pores. The resulting microcolonies can be visualized in a number of ways, including fluorescence microscopy, allowing early detection of cell growth and division. Additionally, the rigidity, and limited volume changes with wetting and temperature changes, facilitate the micro-engineering of this material. For example, it is possible to print an inert latex barrier on the surface to separate miniaturized microbial culture areas (Ingham *et al.*, 2005).

Abbreviations: AST, antibiotic sensitivity testing; PI, propidium iodide; SEM, scanning electron microscopy.

Trimethoprim is a diaminopyrimidine antibiotic that inhibits the bacterial dihydrofolate reductase which catalyses the conversion of dihydrofolate to tetrahydrofolate (Hitchins, 1989; Huovinen *et al.*, 1995). Trimethoprim has been reported to cause morphological changes in a few bacterial species only, including *Listeria monocytogenes* (Minkowski *et al.*, 2001). Antifolate drugs structurally related to trimethoprim have similar effects on *Escherichia coli* (Nickerson & Webb, 1956). Trimethoprim also affects cell morphology and the synthesis of cell wall precursors in *Enterobacter cloacae* (Richards & Xing, 1994), and cell wall formation in *Enterococcus faecalis* (Richards *et al.*, 1995). It has been suggested that trimethoprim exerts its effect on cell morphology by inhibiting DNA replication (Richards & Xing, 1994). Most aspects of the occurrence, mechanism and clinical significance of trimethoprim-mediated filamentation are unknown. Filamentation has been more extensively investigated for other antibiotics (Cudic & Otvos, 2002; Chadfield & Hinton, 2004), including the β-lactams, for which links to the SOS response and antibiotic survival have been made (Miller *et al.*, 2004).

In this study, Anopore-based growth and *in situ* microcolony imaging were used to perform rapid AST. We have focused on the response of clinical isolates of the *Enterobacteriaceae* to trimethoprim, but have obtained additional data that suggest a wider applicability. An advantage of the method was the direct observation of cells exposed to an antibiotic. The value and potential of Anopore as a rapid diagnostic tool are discussed.

METHODS

Strain characterization and testing. The strains used and the methods for verification of species identity and antibiotic sensitivity testing are summarized in Table 1. Vitek 2, API (both bioMérieux) and E-test (AB Biodisk) assays were performed as recommended by the manufacturers, and interpreted according to National Committee for Clinical Laboratory Standards (NCCLS) criteria and the manufacturers' guidelines.

Manufacture of Anopore chips. Chips were manufactured, washed and sterilized as previously described (Ingham *et al.*, 2005). Briefly, a latex barrier was printed on the upper surface of an 8×36 mm strip of Anopore (60 µm thick, 0·2 µm pore size) to delineate eight identical culture areas. The barrier prevented microbial cross-contamination between compartments on the surface, and also penetrated the Anopore beneath to limit the lateral spread of antibiotics through cross-pores in the Anopore. Printing of trimethoprim (Sigma) onto Anopore was done by dissolving the antibiotic in methanol (5–50 ng ml^{-1}) and placing 2 µl aliquots into a compartment, saturating it evenly. Controls consisted of methanol without trimethoprim. Chips were then air-dried, so that the net result was to coat the pores of the Anopore with the antibiotic within a defined region. The layout was arranged so that the most closely related amounts of antibiotics were placed in adjacent compartments. The quantity of antibiotic printed was expressed as pg trimethoprim mm^{-2}, with the area used in this calculation being the upper (planar) surface of the chip. Chips were stored for up to 2 months, in sealed vials at room temperature in the dark, before use.

Growth, imaging of cells and AST. Anopore chips were placed upon pre-warmed Mueller–Hinton agar plates (Oxoid) for 10 min, then inoculated with 10^4–10^5 c.f.u. (2 µl aliquots) of bacteria per compartment. After waiting a few minutes for the liquid to be drawn into the pores, the plates were incubated inverted in a CO_2 incubator at 37 °C. These chips were used to perform fast AST using trimethoprim. Trimethoprim or other antibiotics either were present in the Mueller–Hinton agar at a known concentration or were printed directly into the compartment of an Anopore strip which was then incubated on Mueller–Hinton agar lacking the antibiotic. After incubation, staining of cells on the chip surface with fluorescent dyes (through the pores of the Anopore from beneath) was performed as previously described (Ingham *et al.*, 2005). Briefly, this was accomplished by the transfer of the chip to a microscope slide covered with a film of solidified low-melting-point agarose (Sigma) containing 10 µM Syto9 (Invitrogen) and in some cases 40 µM propidium iodide (PI; Invitrogen).

Imaging, processing and statistical analysis. Chips were imaged directly (without the use of any immersion oil or a coverslip) using an Olympus BX41 epifluorescence microscope equipped with UmPlan F1 objective lenses and U-MWIBA and U-M41007 filters (Ingham *et al.*, 2005). Image capture used a Kappa charge-coupled device (CCD) camera controlled by Kappa Image Base software. Raw files of 12 bit images or BMP files of 8 bit images were analysed quantitatively using ImageJ software to implement background correction, median filtration, conversion to a binary image and calculation of colony or cell size (Rasband, 2004). Calculations were performed in Microsoft Excel. Images were merged and displayed using Photoshop 8.0 CS (Adobe). Simple paired comparisons were made with Student's *t* test. One-way ANOVA with Tukey post-hoc testing (Turner & Thayer, 2001) was used for multiple comparisons. For AST testing on Anopore, MIC_{90} values were calculated from the mean area of at least 200 microcolonies per data point. Unless stated otherwise, all statistical calculations are the representative result from at least two independent experiments.

Scanning electron microscopy (SEM). Anopore strips cultured with bacteria were glued on a sample holder with conductive carbon cement (Leit-C, Neubauer Chemicalien) and frozen in liquid nitrogen. Samples were transferred under vacuum to the dedicated cryo-preparation chamber (Oxford Cryo-system, CT 1500 HF) onto a sample stage at −90 °C. The samples were freeze-dried for 4 min at −90 °C in a 3×10^{-7} Pa vacuum to remove water vapour contamination. After the sample surface was sputter-coated with 10 nm platinum, it was transferred to the cold sample stage (−190 °C) inside the Cryo-FESEM (JEOL 6300F Field Emission SEM) and subsequently analysed with an accelerating voltage of 5 kV. Images were digitally recorded (Orion, E.L.I.).

Screening for filamentation. A systematic screening was performed using Anopore chips printed with trimethoprim (0–600 pg mm^{-2}). Over 30 strains were tested on two types of medium (Mueller–Hinton and sheep blood agar), using three dilutions of each sample (inoculations of approximately 10^5, 10^4 and 10^3 c.f.u. over a 10 mm^2 area of Anopore) and two incubation times (4–5 and 16 h). After this initial screening, follow-up experiments were performed on strains showing filamentation. These were cultured for 2·5, 5 and 16 h on chips printed with 200 pg trimethoprim mm^{-2}. Filamentation was also tested on Anopore incubated on Mueller–Hinton agar containing 3 µg mitomycin C ml^{-1} (Sigma).

Table 1. Characteristics of strains used

The ID column shows the method of species identification. Filamentation in the presence of trimethoprim was scored as positive if the increase in mean cell length over the inoculum and population cultured without trimethoprim was significant ($P<0{\cdot}01$ by one-way ANOVA with Tukey HSD post-hoc test, comparing all datasets for a given strain; $n=200$ for all samples) after both 4–5 and 16 h for any given strain. Limited filamentation was scored if the increase in cell length at either 4–5 or 16 h was significant, or if the increase was of marginal significance ($P<0{\cdot}05$) at both time points. *Proteus* species were not scored for filamentation due to the potential for confusion with swarmer cells. Otherwise, strains were scored as non-filamenting. [S] indicates sensitivity and [R] resistance to trimethoprim. According to hospital procedures, all strains of *Pseudomonas aeruginosa* were scored as resistant. All data are the representative results of two determinations. ND, Not determined.

Species	Strain	ID	Filamentation	Standard methods	Anopore method
				MIC (μg trimethoprim ml^{-1})	MIC$_{90}$ (μg trimethoprim ml^{-1})
Aeromonas hydrophila	2324	Vitek 2	No	>16 Vitek 2 [R], 12 E-test [R]	>10 [R]
Brevundimonas versicularis	2412	API 20E	No	>16 E-test [R]	>10 [R]
Citrobacter diversus (*koseri*)	711	Vitek 2	Yes	2 Vitek 2 [S], 0·25 E-test [S]	1 [S]
Citrobacter freundii	2452	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 7	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
Citrobacter koseri	JBZA 20	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
	JBZA 21	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
Enterobacter aerogenes	1468	Vitek 2	No	>16 Vitek 2 [R], >32 E-test [R]	>10 [R]
	1499	Vitek 2	Yes	<0·5 Vitek 2 [S], 0·25 E-test [S]	0·5 [S]
Enterobacter cloacae	2364	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
	1170	Vitek 2	Yes	0·25 E-test [S]	0·1 [S]
	JBZA 33	Vitek 2	Yes	<0·5 Vitek 2 [S]	1 [S]
	JBZA 22	Vitek 2	Yes	<0·5 Vitek 2 [S]	1 [S]
Escherichia coli	2613	Vitek 2	Yes	<0·5 Vitek 2 [S], 0·12 E-test [S]	0·1 [S]
	2657	Vitek 2	Limited	<0·5 Vitek 2 [S], 0·25 E-test [S]	0·2 [S]
	JBZA 18	Vitek 2	Yes	<0·5 Vitek 2 [S]	2 [S]
	JBZA 24	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 26	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 28	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 29	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
	JBZA 30	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 31	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 32	Vitek 2	Yes	<0·5 Vitek 2 [S]	1 [S]
	JBZA 35	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
	JBZA 10	Vitek 2	No	>16 Vitek 2 [R]	>10 [R]
	JBZA 15	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
	JBZA 25	Vitek 2	Yes	<0·5 Vitek 2 [S]	1 [S]
Hafnia alvei	981	Vitek 2	Yes	<0·5 Vitek 2 [S], 0·5 E-test [S]	0·2 [S]
	5636	Vitek 2	No	<0·5 Vitek 2 [S], 0·5 E-test [S]	0·5 [S]
Klebsiella ornithinolytica	JBZA 19	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·5 [S]
Klebsiella pneumoniae	2600	Vitek 2	No	<0·5 Vitek 2 [S]	0·5 [S]
	JBZA 5	Vitek 2	Yes	<0·5 Vitek 2 [S]	2 [S]
	JBZA 13	Vitek 2	Yes	<0·5 Vitek 2 [S]	1 [S]
Listeria monocytogenes	620	API Rapid	No	0·12 E-test [S]	ND
	1444	API Rapid	Yes	0·12 E-test [S]	ND
Morganella morganii	JBZA 2	Vitek 2	No	<0·5 Vitek 2 [S]	0·5 [S]
Pantoae sp.	28	API 20E	Yes	<0·5 Vitek 2 [S], 0·06 E-test [S]	0·05 [S]
Proteus mirabilis	2612	Vitek 2	No	1 Vitek 2 [S]	1 [S]
	JBZA 1	Vitek 2	ND	>16 Vitek 2 [R]	>10 [R]
	JBZA 8	Vitek 2	ND	>16 Vitek 2 [R]	>10 [R]
	JBZA 12	Vitek 2	ND	2 Vitek 2 [S]	2 [S]
	JBZA 14	Vitek 2	ND	>16 Vitek 2 [R]	>10 [R]
	JBZA 16	Vitek 2	ND	1 Vitek 2 [S]	1 [S]
	JBZA 23	Vitek 2	ND	>16 Vitek 2 [R]	>10 [R]

Table 1. cont.

Species	Strain	ID	Filamentation	Standard methods	Anopore method
				MIC (μg trimethoprim ml^{-1})	MIC_{90} (μg trimethoprim ml^{-1})
	JBZA 27	Vitek 2	ND	<0·5 Vitek 2 [S]	1 [S]
	JBZA 34	Vitek 2	ND	<0·5 Vitek 2 [S]	2 [S]
Proteus vulgaris	1790	Vitek 2	No	0·25 E-test [S]	0·5 [S]
Providencia rettgeri	2699	Vitek 2	No	1 Vitek 2 [S]	1 [S]
Pseudomonas aeruginosa	37	Vitek 2	No	[R]	>10 [R]
	JBZA 6	Vitek 2	No	[R]	>10 [R]
	JBZA 9	Vitek 2	No	[R]	>10 [R]
Pseudomonas stutzeri	JBZA 4	Vitek 2	No	<0·5 Vitek 2 [S]	1 [S]
Salmonella enteritidis	2627-2	Vitek 2	No	<0·5 Vitek 2 [S]	0·2 [S]
Serratia marcescens	2553	Vitek 2	No	<0·5 Vitek 2 [S]	0·5 [S]
Shigella flexneri	M1	API 20E	Limited	<0·5 Vitek 2 [S]	0·2 [S]
Shigella sonnei	2627-1	Vitek 2	Yes	<0·5 Vitek 2 [S]	0·2 [S]
Shigella sp.	5653	Vitek 2	No	<0·5 Vitek 2 [S]	0·5 [S]
Stenotrophomonas maltophilia	JBZA3	Vitek 2	Limited	<0·5 Vitek 2 [S]	1 [S]
Yersinia enterocolitica	2636	Vitek 2	No	4 Vitek 2 [S]	1 [S]

RESULTS AND DISCUSSION

Growth and rapid AST of *Enterobacteriaceae* on Anopore

Ent. aerogenes strains 1499 (trimethoprim-sensitive; Table 1) and 1468 (resistant) both grew rapidly on Anopore incubated on Mueller–Hinton agar. The doubling times of these strains at 37 °C was calculated from the mean number of cells per microcolony ($n>200$ at each data point) during the first 2·5 h growth (Ingham *et al.*, 2005). The doubling time during exponential growth on Anopore was 27 min for strain 1499 and 29 min for strain 1468, with lag times of 25–30 min for both strains (all times are the average of two values that differed by less than 20 %). This compared to doubling times of 23 and 24 min, respectively, for strains 1499 and 1468 in shaken Mueller–Hinton broth at the same temperature. With only 25 min culture it was possible to detect growth of strain 1499 by comparing the mean microcolony area of the inoculum (generally 1–2 cells) with that of the cultured population (one-way ANOVA, $n=924$ for the inoculum, $n=774$ cultured without trimethoprim, $n=567$ for culture with trimethoprim; $P<0·01$ by Tukey HSD). No significant inhibitory effect on growth was found with trimethoprim ($P>0·05$ by Tukey HSD) within 25 min. However, with 40 min incubation (Fig. 1), trimethoprim reduced growth ($n=594$ for the inoculum, $n=602$ cultured without trimethoprim, $n=642$ cultured with trimethoprim; $P<0·01$ comparing treated and untreated populations by Tukey HSD). Growth occurred in both the treated and untreated population, as indicated by significant increases in the microcolony areas (mean microcolony area of both cultured populations compared to that of the inoculum; $P<0·01$ in both cases by Tukey HSD). For the resistant strain 1468 (Fig. 1b), trimethoprim had no significant effect on growth at 40 min ($n=604$ for the inoculum, $n=615$ cultured without trimethoprim, $n=628$ cultured with trimethoprim; one-way ANOVA with Tukey HSD, $P>0·05$ comparing treated and untreated populations). Taken together, these data suggest that 40 min was close to the minimal time in which it was possible to distinguish a sensitive from a resistant strain by this method.

Longer periods of growth increased the difference in microcolony area between the trimethoprim-treated and untreated populations of strain 1499. Culture of *Ent. aerogenes* for 2 h on Anopore on Mueller–Hinton agar containing defined concentrations of trimethoprim was sufficient to allow calculation of MIC_{90} values. Strain 1499 had a MIC_{90} of 0·5 μg trimethoprim ml^{-1} (Fig. 2a, Table 1). This compared to MICs of 0·25 μg ml^{-1} by E-test, and <0·5 μg ml^{-1} by Vitek 2 (Table 1). The resistant strain (1468) had a MIC_{90} of >10 μg ml^{-1} by the Anopore method and MICs of >32 μg ml^{-1} by E-test and >16 μg ml^{-1} by Vitek 2 (Fig. 2a, Table 1). MIC_{90} determinations were then extended to other members of the family *Enterobacteriaceae* and some other Gram-negative rods, such as pseudomonads (Table 1). During the testing period, swarming behaviour by *Proteus mirabilis* or *Proteus vulgaris* on Anopore was not observed. There was excellent agreement between established techniques and the Anopore method of AST: resistance (17 strains) and sensitivity (40 strains) to trimethoprim were assigned identically on Anopore when compared to Vitek 2 and/or E-test determinations.

The method was not specific to the mode of action of trimethoprim: the effect of other antibiotics could be detected rapidly by microcolony analysis on Anopore. The inhibitory effect on the growth of *Ent. aerogenes* 1468 (Rif^{S}) of 10 μg rifampicin ml^{-1} in Mueller–Hinton plates could be

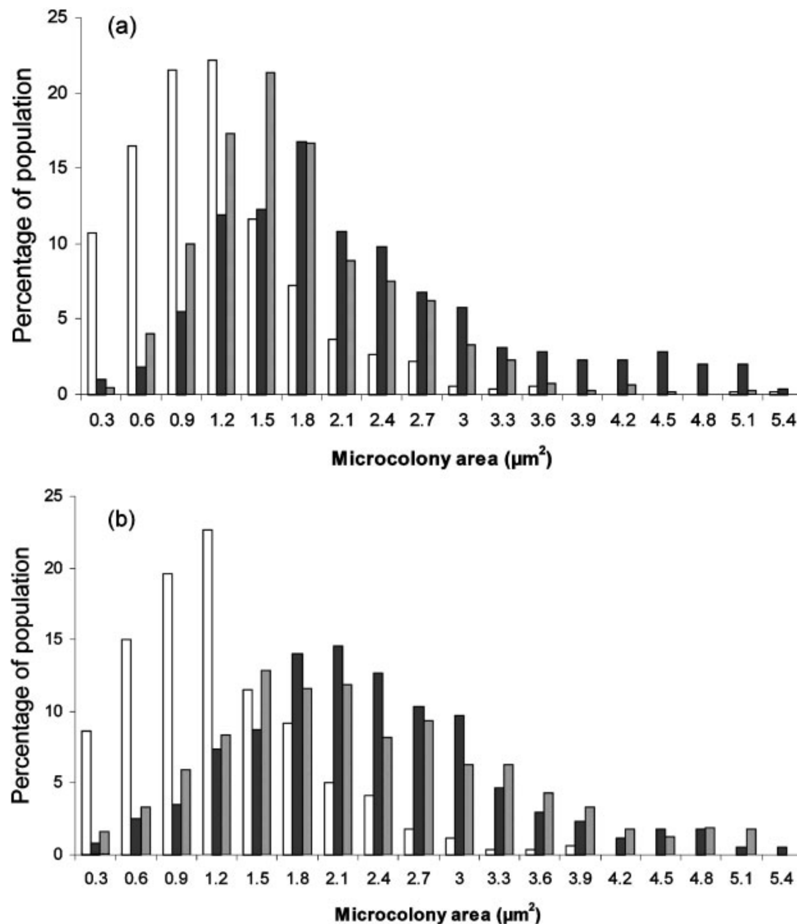

Fig. 1. Distribution of the microcolony area of populations of *Ent. aerogenes* cultured on Anopore for 40 min, in the presence or absence of trimethoprim. (a) Trimethoprim-sensitive strain 1499; (b) trimethoprim-resistant strain 1468. White bars, inoculum; black bars, incubation on untreated Anopore; hatched bars, incubation on Anopore printed with 200 pg trimethoprim mm^{-2}.

detected within 1 h incubation at 37 °C by comparing microcolony areas ($n=200$ for all samples; $P<0{\cdot}001$), but there was no inhibitory effect on a spontaneous rifampicin-resistant mutant (MIC >25 μg ml^{-1}) derived from this strain ($P>0{\cdot}05$). The results were similar using neomycin, ampicillin and tetracycline for *E. coli* 2613, which was sensitive to all three antibiotics. In all cases the results were obtained by comparing the microcolony areas of treated populations cultured on Anopore with those of untreated populations, employing an antibiotic concentration above the MIC ($n=200$–400; $P<0{\cdot}01$ in all comparisons, Student's *t* test). Additionally, *Candida albicans* could be shown to be sensitive to amphotericin B within 2 h by a similar method on Sabouraud agar at 37 °C, with the strain sensitivity verified by E-test in 36 h (C. J. Ingham and P. M. Schneeberger, unpublished results). Taken together, these data suggest that the action of antibiotics with very different chemistries and modes of action could be assayed rapidly, and that a broadening of the range of antimicrobials tested by this method is possible.

The MIC_{90} determinations described above were made using a defined concentration of the antibiotic in agar plates. Trimethoprim-printed Anopore chips, when placed on Mueller–Hinton agar lacking trimethoprim, could also be used to rapidly distinguish a resistant from a sensitive strain of *Ent. aerogenes* (Fig. 2b). A dose-dependent response to trimethoprim was observed after 2 h culture. Minimal inhibitory amounts of trimethoprim were 25 pg mm^{-2} for the sensitive strain (1499) and >600 pg mm^{-2} for the resistant strain (1468). Anopore chips printed with 200 pg trimethoprim mm^{-2} were also used to assess the minimal culture time required to distinguish the sensitive from the resistant strain of *Ent. aerogenes* by calculating the mean area of the microcolonies.

In these assays, trimethoprim was present in the growth medium at a defined concentration or was printed onto Anopore, dried and then allowed to rehydrate. The former method allowed known concentrations of antibiotics to be used, and the agar base may potentially be replaced with a microfluidic underlay or growth matrix in the future, permitting highly multiplexed AST with a wide range of antibiotics, both singly and in combination. Printing the antibiotic directly onto the Anopore means that the breakpoints must be experimentally determined with calibration strains. An inhibition by subnanogram quantities of trimethoprim was observed, possibly because of a localization of antibiotics at a high concentration within the limited volume of the pores in very close proximity to the

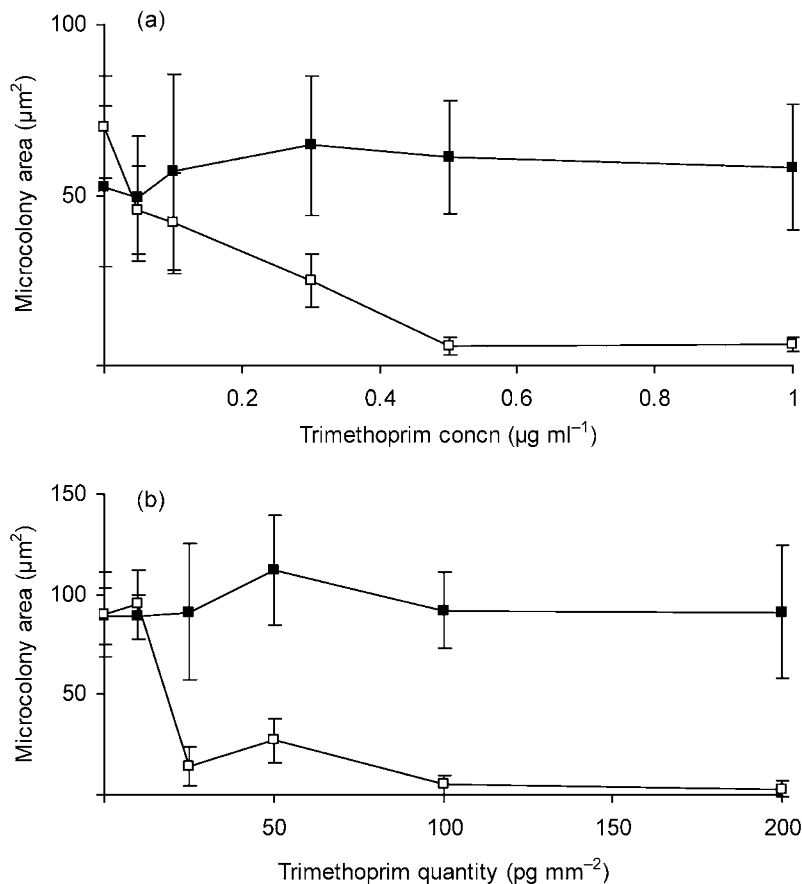

Fig. 2. Dose–response curves derived for the trimethoprim-sensitive (1499, □) and -resistant (1468, ■) strains of *Ent. aerogenes* cultured on Anopore for 2 h. Error bars show SD of the mean. (a) Trimethoprim present in the Mueller–Hinton agar. Additionally, strain 1468 was tested up to 10 μg trimethoprim ml^{-1} with no inhibition of growth. (b) Trimethoprim printed on Anopore. Strain 1468 was tested up to 600 pg trimethoprim mm^{-2} with no growth inhibition.

test bacteria. That small amounts of trimethoprim were bioactive suggests that this approach also has potential for high-throughput screening of compounds from antimicrobial drug libraries which are often limited in quantity. Additionally, antibiotic-printed Anopore was very convenient to store and deploy, and this may facilitate the storage of compound libraries 'ready to use'.

Detection of a resistant subpopulation

In order to simulate a simple mixed population, a culture of strain 1499 was spiked with strain 1468, so that the latter was in a minority (10 % of the population). After culture for 2 h, this mixed population could be distinguished from the 1499 monoculture by the appearance of microcolonies comprising 12–32 cells, but at a lower frequency than the 1468 monoculture. Comparison of microcolony areas indicated that the spiked population had a mean significantly larger than that of the sensitive population ($n=650$ for all populations; one-way ANOVA with $P<0{\cdot}01$ by Tukey HSD), but smaller than that of the resistant population ($P<0{\cdot}01$). After 2 h culture, the resistant subpopulation was still calculated to be in the minority (~30 %). A resistant strain at 1 % of the population could not be reliably detected with 2 h culture. After 3·5 h, a small number of obviously resistant microcolonies (>100 cells) were observed in the spiked population, but not in the 100 % sensitive population. These data suggest that it is possible to resolve mixtures of micro-organisms of clinical significance on Anopore in an analogous way to that possible by the spread-plating of microbes on nutrient agar, but on a far smaller scale. This is an advantage compared to most other methods that rely on culture in liquid media, since in these methods, an antibiotic-resistant subpopulation tends to be detected reliably only when it grows to outnumber antibiotic-sensitive cells. Therefore, the method may be applicable to heterogeneous or mixed populations. In this context, removing the requirement for purification to a single colony has obvious advantages in terms of speed.

Morphological effects of trimethoprim

Direct microscopic observation of AST assays on Anopore resulted in the finding that elongated cells with irregular nucleic acid distributions were a common effect of trimethoprim treatment of sensitive strains. Other antibiotics, including β-lactams, had a more limited or no obvious effect. Trimethoprim often triggered morphological changes that were quite extreme. The AST assays, backed up by additional screening on Anopore, indicated that filamentation is a common but underreported effect of trimethoprim (Table 1). Filamentation was observed for many trimethoprim-sensitive isolates of the *Enterobacteriaceae*, including *Ent. aerogenes* 1499, *Pantoea*

sp. X28, *Hafnia alvei* 981, *Shigella sonnei* 2627-1, *Shigella flexneri* M1, *Klebsiella pneumoniae* JBZA5, *Citrobacter freundii* JBZA7 and *Citrobacter diversus* 711 (Table 1). These are all representatives of species for which trimethoprim has not been previously been reported to induce filamentation. Quantitatively, the results for these strains were generally similar to those of *Ent. aerogenes* (Fig. 3) in terms of the distribution of cell lengths.

Previous reports that *Ent. cloacae* (Richards *et al.*, 1993) and *L. monocytogenes* (Minkowski *et al.*, 2001) show filamentation when treated with trimethoprim were confirmed (Table 1, strains 2364 and 1170 of *Ent. cloacae* and *L. monocytogenes* 1444). Not all isolates of a species showed the same response. *E. coli* 2657, *H. alvei* 5636 and *L. monocytogenes* 620 were less prone to trimethoprim-mediated elongation than other clinical isolates of the same species (Table 1), but were not trimethoprim resistant. Nor was trimethoprim-induced filamentation found in all species tested (Table 1). Given the variation in trimethoprim-mediated filamentation between isolates we cannot conclude that *Serratia marcescens* or *Salmonella typhimurium* do not filament (Table 1), merely that this response is a common but not universal one for trimethoprim-sensitive *Enterobacteriaceae*.

The distribution of filamented cells over the surface of the Anopore was most often in discrete microcolonies, generally clusters of <50 cells. There was great variation in both the length and in the permeability to PI within a microcolony (data not shown). A moderate degree of broadening and flattening of the more highly damaged cells was, however, observed (Fig. 4). This is consistent with reports that trimethoprim interferes with the supply of cell-wall precursors in *Ent. cloacae* (Richards & Xing, 1994; Richards *et al.*, 1995). A wide range of responses was observed in what must (in most cases) be the product of a single cell, or pair of cells, generating diversity during the course of the assay in the presence of trimethoprim above the minimal inhibitory amount. Such microcolonies did not develop into visible colonies on Anopore with extended incubation for 24 h, and only trimethoprim-sensitive isolates could be recovered from these experiments. This suggested that trimethoprim-resistant mutants were not being generated. Antibiotic-refractory subpopulations (e.g. 'persister' cells) have been shown to exist within populations of bacteria (Balaban *et al.*, 2001). The phenotypes of such subpopulations do not appear to be determined by any stable genetic change. Assessing heterogeneity within microcolonies may be a relatively simple way of looking at this phenomenon. Extreme variation in the response to trimethoprim was also seen by SEM for *Ent. aerogenes* 1499 (Fig. 4). In addition, variation in cell width and in the intactness of the cell envelope was observed. In extreme cases, cells seemed to be collapsing, showing both flattening and visible lesions in the cell wall (Fig. 4c). It was possible to see the remains of these lysed and fragmented cells in close proximity to apparently intact ones, with fragments preserved on the Anopore support in a way that would not have been possible with liquid culture. Given the possibility of withdrawing antibiotics by transfer of an Anopore chip to a new medium, this form of *in situ* imaging may have further potential in the observation of highly damaged and also of recovering cells in the study of antibiotic effects.

Mitomycin C and trimethoprim are both well-known inducers of the SOS response (Lewin & Amyes, 1991; Ahmad *et al.*, 1998). Treatment of *Ent. aerogenes* with mitomycin C also caused filamentation (Fig. 3). The resulting population was similar to the trimethoprim-treated population in terms of the length and heterogeneity of DNA distribution and PI permeability. A more extensive screening suggested that the induction of the SOS response leading to filamentation was general, and included strains that did not show obvious filamentation with trimethoprim (data not shown). The SOS response can result in the inhibition of cell division, but still allows growth leading to filamentation (Neidhardt *et al.*, 1990). Given the widespread presence of the SOS pathway in Eubacteria (Friedberg *et al.*, 1995), this is an obvious explanation of why trimethoprim-mediated filamentation was so frequently observed in this study. The SOS response has also been implicated in mediating the

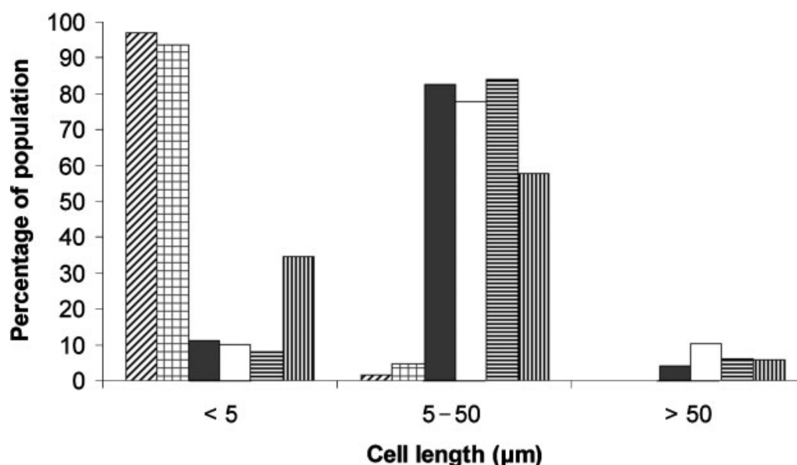

Fig. 3. Quantification of trimethoprim-mediated filamentation of *Ent. aerogenes* 1499. Diagonal hatching, distribution of cell lengths immediately after inoculation; square hatching, distribution after 2·5 h; black, distribution after 4–5 h; white, distribution after 16 h. Culture for 5 h on Anopore on Mueller–Hinton plates containing 5 μg trimethoprim ml^{-1} is indicated by horizontal hatching, and for 5 h with 3 μg mitomycin C ml^{-1} by vertical hatching.

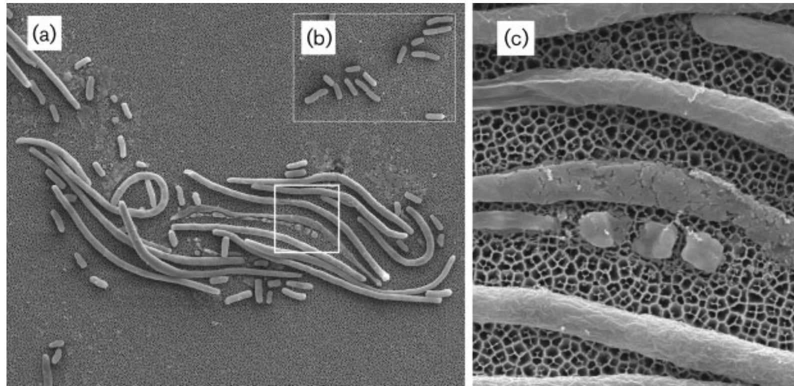

Fig. 4. SEM of *Ent. aerogenes* 1499 cultured on Anopore, showing filamentation and damage due to the action of trimethoprim. (a) Incubation for 5 h with 200 pg trimethoprim mm^{-2}; (b) culture without trimethoprim. (c) Detail of image to the left, showing cell envelope damage on cells and the fine structure of the Anopore support.

filamentation of *E. coli* in response to β-lactam antibiotics, via a periplasmic binding protein and a two-component regulatory system (Miller *et al.*, 2004). The SOS response is also known to facilitate the horizontal dissemination of resistance genes for a range of antibiotics, including trimethoprim (Beaber *et al.*, 2003). The induction of filamentation by mitomycin C suggests that activation of the SOS response can indeed trigger filamentation in the strains we have studied, and the effect on microcolony heterogeneity was similar to that of trimethoprim. However, this does not explain why filamentation was not a universal response in trimethoprim-sensitive strains. If the SOS response is involved, the relationship may not be a simple one.

Conclusions and clinical potential

This is a new application of an unusual but appropriate material in culture-based diagnostics. The data presented demonstrate rapid and accurate AST testing in a few hours, rather than overnight. This speed was made possible by the ability to stain and image microcolonies *in situ* with little redistribution of organisms on the Anopore surface. Images of fields of microcolonies could be rapidly captured, digitized and compared. This was done using a type of microscope available in most microbial diagnostics laboratories, with data analysis performed using publicly available software (Rasband, 2004). This study has concentrated on a single antibiotic, trimethoprim, and strains within the family *Enterobacteriaceae*, but we have demonstrated examples of AST with other Gram-negative bacteria and antibiotics, suggesting that this is a generally applicable technique. Clearly, this approach needs to be tested more widely, focusing on the important issues of intermediate resistance, the interactions between subpopulations, and inducible resistance mechanisms. However, there are obvious advantages in the speed and imaging simplicity of the method that should make this effort worthwhile. The early detection of growth may prove even more advantageous when applied to slower growing organisms. Preliminary results for drug testing of *Mycobacterium tuberculosis* on Anopore (A. Ben Ayad, and others, unpublished results) suggest that this difficult bacterium can be tested for rifampicin and isoniazid resistance with only a few days culture. Given the slow growth and relatively easy imaging of many fungi by microscopy, these may also present attractive subjects for this method.

In terms of clinical applications, only a few mm^2 of Anopore (costing a few Euro cents) is required to test an antibiotic. Fluorescent stains can be expensive, but are used in small quantities. Well-characterized and relatively low-cost stains, such as acridine orange, are also usable. Taken together, these considerations suggest that the method can be economic, particularly if a large number of tests are combined on a single strip of Anopore. The ability to distribute controlled and variable amounts of compound into very many defined areas of the substrate may also allow break-point testing, and synergistic or antagonistic interactions by means of 'micro' chessboard titrations. In terms of workflow, streamlining the staining method by incorporating low-toxicity fluorescent stains into the growth medium will be advantageous in reducing hands-on time. Non-fluorescent stains or contrast-enhancing techniques for conventional light microscopy could be used to adapt the method to the developing world, where conventional light microscopes are more common than those capable of fluorescence (Olympus Europe, personal communication). For analysis, publicly available image-processing software (Rasband, 2004) is relatively easily tailored to microcolony analysis, with only minor scripting or macro development. Finally, we suggest that there is greater potential for the automation of culture with Anopore than with conventional microbial growth methods such as agar-based culture.

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