DIAGNOSTIC MICROBIOLOGY

Rapid antimicrobial susceptibility testing of urinary tract isolates and samples by flow cytometry

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A multiparametric flow cytometry antimicrobial susceptibility test was developed and its performance was evaluated on clinical urine isolates and samples in comparison with standard methods. Alterations in cytoplasmic membrane integrity were monitored by propidium iodide, and the anionic probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) was used to measure changes in membrane potential. Microbial size and cellular content were analysed by light scattering. Twelve antibiotics were tested on 6 ATCC control strains, 22 urine isolates and 19 clinical urine samples, variously containing *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, *Staphylococcus aureus*, *S. saprophyticus* and *S. epidermidis*. Agreement between the flow cytometry results, broth microdilution and disk diffusion tests was 93.9% (n = 328 tests). Of the 20 discrepancies observed, 18 were for species other than *E. coli*. Perfect correlation was obtained with five antibiotics, whereas norfloxacin, nitrofurantoin and tetracycline were responsible for 13(65%) of the 20 discrepancies.

Introduction

Antimicrobial susceptibility testing (AST) of bacteria isolated from urinary tract infection (UTI) samples is usually done by standardised disk diffusion or broth dilution. An alternative strategy is to detect directly the effects of an antibiotic on bacterial cells by using specific fluorescent molecules [1]. Propidium iodide (PI) can enter microbial cells that have lost their membrane integrity following antimicrobial exposure, and can bind to nucleic acids [2, 3]. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) is a negatively charged hydrophobic molecule that binds to membranes and the intracellular proteins of depolarised cells, resulting in increased fluorescence emission [4, 5]. The fluorescent cells can then be detected by epifluorescence microscopy or laser flow cytometry. The advantages of flow cytometry (FC) are that it can detect and enumerate thousands of cells in a few seconds, and can monitor up to four fluorescent probes at the same time. Light scattering and fluorescence

measurements are indicative both of the morphological and physiological states of the individual cells, and are acquired and analysed in real time by a computer system [6, 7].

This study aimed to use FC to reduce the analysis time of ASTs. A flow cytometric susceptibility testing (FC-AST) method was established by monitoring the antimicrobial effects on bacterial cells with two fluorescent dyes, PI and DiBAC₄(3). This method was applied to urine isolates and urine samples, and the results were compared with those obtained by two conventional AST methods.

Materials and methods

Bacterial strains and media

Control strains were from the American Type Culture Collection (ATCC). Bacterial isolates from urine, and infected urine samples (Table 2) were collected from UTIs and were provided by the Department of Infectious Diseases and Microbiology at the Hôpital Maisonneuve-Rosemont (University of Montreal, Montreal, QC, Canada). Infected urine samples contained >10⁵ cfu/ml and were kept at 4°C in sterile tubes for no longer than 24 h before assays. Bacteria were cultured in Mueller-Hinton Broth (BBL, Franklin Lakes, NJ, USA) supplemented with cations (MHSC:

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CaCl₂ 50 mg/L, MgCl₂ 20 mg/L). Bacterial viability was assessed by performing spread-plate counts on Trypticase Soy Agar (TSA); Difco Laboratories, Detroit, MI, USA.

Antibiotics and fluorescent dyes

Ampicillin, gentamicin and tetracycline were from ICN (Mississauga, ON, Canada), carbenicillin from Pfizer (Ste-Anne-de-Bellevue, QC, Canada), ceftazidime from Lilly (Scarborough, ON, Canada), ceftriaxone from Hoffman-La Roche (Mississauga, ON, Canada), nitrofurantoin, norfloxacin, oxacillin, penicillin G, trimethoprim, sulphamethoxazole and vancomycin from Sigma. Antibiotic stock solutions (5 mg/ml) were made with the appropriate diluents, as listed by the NCCLS [8] and kept at -20°C. DiBAC₄(3) (Molecular Probes, Eugene, OR, USA) was dissolved initially in dimethyl sulphoxide and diluted in ethanol to 1 mg/ml. The PI (Sigma) stock solution (1 mg/ml) was made with Milli-Q water. Solutions of fluorescent probes were kept at 4°C and were stable for up to 6 months when protected from light.

Reference susceptibility tests

Disk susceptibility tests were performed at the Department of Infectious Diseases and Microbiology (Hôpital Maisonneuve-Rosemont) by the NCCLS protocol [8]. Broth microdilution susceptibility tests were done to determine the minimum inhibitory concentration (MIC), again according to the NCCLS guidelines [8].

FC-AST

Three-to-five colonies from TSA, or 100 µl of infected urine, were added to 5 ml of MHSC broth and incubated for *c.* 2 h with shaking at 37°C and 250 rpm. These cultures were then adjusted to the turbidity of a 0.5 McFarland standard and diluted five-fold in MHSC broth; 500 µl of this dilution was then mixed with 500 µl of MHSC containing the appropriate concentration of antibiotic. The inoculum size was *c.* 5 × 10⁶ cfu/ml. The final antibiotic concentrations in the samples were equivalent to the NCCLS MIC breakpoints for susceptible interpretation (Table 2). The cultures were incubated for 2 h at 37°C and 250 rpm, centrifuged at 10 000 *g* for 2 min, then resuspended in 1 ml of phosphate-buffered saline (PBS, 130 mM sodium chloride, 10 mM sodium phosphate buffer) pH 7.2 containing both DiBAC₄(3) (10 µg/ml) and PI (10 µg/ml). Before use this diluent was filter-sterilised through a 0.22-µm pore membrane. After incubation for 10 min in the dark at room temperature, the cultures were diluted in PBS to yield *c.* 10⁶ cells/ml. The controls were: (i) untreated and unstained bacterial cells for autofluorescence, (ii) untreated and stained bacterial cells (live control) and (iii) bacterial cells treated for 5 min with ethanol 70% (dead control) and stained with both dyes.

Flow cytometry

The Coulter Epics XL-MCL (Beckman Coulter, Fullerton, CA, USA) was used. The DiBAC₄(3) fluorescent signal was collected in the FL1 photomultiplier with a 525-nm bandpass filter (adjusted at 720 V) and the PI fluorescent signal was collected in the FL3 photomultiplier with a 620-nm bandpass filter (adjusted at 850 V). Signal compensation that eliminates optical overlap between both dyes was <10%. The samples were run at a low pressure setting, corresponding to a flow rate of 100–200 events/s during the analysis; 10 000 events were acquired for each analysis.

Interpretative criteria for FC-AST

Two acquisition regions were defined. The first was applied to the light-scattering profiles (region I; Fig. 1a, c, e, g), and was used to exclude events related to electronic noise and cell debris. The mean fluorescence intensity (MFI) of the PI and DiBAC₄(3) signals was measured only from events recorded within this region. The second acquisition region was defined on the bi-parametric fluorescence histograms (region II in Fig. 1b, d, f, h). Events recorded in this region were fluorescent with both dyes and allowed the exclusion of non-fluorescent events (live cells) and events labelled with only one dye. Fluorescent events in acquisition region II represented cells that have lost both membrane integrity and polarisation.

The numbers of fluorescent events in region II and the MFI for the PI and DiBAC₄(3) signals were compared between the live controls and the antibiotic-treated samples. For each sample, a value *V_i* was derived, defined by equation 1:

$$V_i = N_i \times \text{MFI}_{\text{PI}} \times \text{MFI}_{\text{DiBAC4(3)}} \quad (1)$$

where *N_i* was the number of fluorescent events recorded in acquisition region II, and *MFI_{PI}* and *MFI_{DiBAC4(3)}* were the MFI values in acquisition region I for each dye. The comparative factor, *C_i* was determined by equation 2:

$$C_i = V_i/V_L \quad (2)$$

where *V_L* was the *V* value determined by equation 1 for the live control sample.

A discrepancy was considered minor when: (1) intermediate resistance was indicated by the FC-AST and one of the standard antimicrobial tests (disk diffusion or microdilution) indicated resistance or susceptibility; (2) the FC-AST results indicated resistance and one of the standard tests indicated resistance and the other susceptibility; or (3) the FC-AST results indicated resistance or susceptibility and the standard tests indicated intermediate resistance. A discrepancy was considered major when: (1) susceptibility was indicated by FC-AST whereas one of the standard tests indicated susceptibility and the other resistance; or (2) FC-AST results indicated

Fig. 1. Light-scattering profiles (forward scatter, FS, versus side scatter, SS) (a, c, e, g) and bi-parametric histograms (b, d, f, h) (DiBAC₄(3) versus PI) for *E. coli* 25922 untreated (a, b) or treated for 2 h with ampicillin (c, d), gentamicin (e, f) or cotrimoxazole (g, h), and analysed by FC. Events recorded in the acquisition region I in the light scattering-profiles were measured for fluorescence. Fluorescent events recorded in acquisition region II were taken as dead cells. The proportions of dead cells recorded in panels b, d, f and h were 0.5%, 89%, 79% and 9.6%, respectively.

resistance whereas both standard tests indicated susceptibility. Finally, a discrepancy was considered very major when susceptibility was indicated by FC-AST and resistance by both the standard tests. The susceptibility interpretations for the two reference tests were based on the NCCLS performance standards (M100-S9) [8] and were controlled with the appropriate ATCC strains.

Results

Interpretative criteria

For each antibiotic, a specific range of the C_i values (Table 1) was determined to define the interpretative

criteria for resistance, susceptibility and intermediate resistance and to compare with standard tests. Bacteria with resistance to ampicillin and carbenicillin as determined by standard tests gave C_i values ranging from 0 to 10, whereas susceptible isolates usually gave very high C_i values ($>10^3$), as dead cells were highly fluorescent with both dyes. The bottom limit of the C_i value for susceptibility was arbitrarily established at 500, as some susceptible isolates or samples had C_i values just below 1000. Unfortunately, no bacteria were resistant to ceftriaxone, ceftazidime, gentamicin, or vancomycin; instead the interpretative criteria for ampicillin and carbenicillin were applied also to these four antibiotics. The ranges of C_i values associated

Table 1. Ranges of C_i values determining the interpretative categories for susceptibility, intermediate resistance and resistance

Antibiotics	C_i indicating		
	Susceptibility	Intermediate resistance	Resistance
Ampicillin	> 500	10–500	0–10
Carbenicillin	> 500	10–500	0–10
Ceftriaxone	> 500	10–500	0–10
Ceftazidime	> 500	10–500	0–10
Gentamicin	> 500	10–500	0–10
Nitrofurantoin	> 10	—	0–10
Norfloxacin	> 20	10–20	0–10
Oxacillin	> 500	50–500	0–50
Penicillin G	> 100	50–100	0–50
Tetracycline	> 50	10–50	0–10
Co-trimoxazole	> 50	20–50	0–20
Vancomycin	> 500	10–500	0–10

with resistance and susceptibility to nitrofurantoin, norfloxacin, oxacillin, penicillin G, tetracycline and co-trimoxazole were different from those for ampicillin and carbenicillin. Resistance to nitrofurantoin, as determined by standard tests, was associated with C_i values ranging from 0 to 10, and susceptibility with values >10; resistance to tetracycline was associated with C_i values of 0–10 and susceptibility with values >50. The C_i values for intermediate resistance were defined as being those associated with resistance but below those associated with susceptibility.

FC-AST on control strains

E. coli ATCC 25922. The light-scattering profile of untreated *E. coli* cultures (Fig. 1a) showed a homogeneous population of cells with intact membranes, with >99% of these organisms impermeable to fluorescent dyes (Fig. 1b). After incubation for 2 h with ampicillin, the light-scattering profile became more dispersed (Fig. 1c), indicating important morphological changes; >90% of the cells detected became permeable to the fluorescent dyes. Examination by epifluorescent microscopy revealed a high proportion of elongated and clustered cells (not shown). Bacteria

treated with ceftazidime and ceftriaxone generated similar results (data not shown). These changes were easily detected and quantified by FC, confirming several other reports [2, 5, 9, 10]. Exposure to gentamicin did not significantly affect the light-scattering profile (Fig. 1e); nevertheless, susceptibility to this antibiotic could be shown, as >80% of the cells detected by FC were fluorescent (Fig. 1f). Interestingly, two fluorescent populations appeared on the bi-parametric histogram, one was brighter with PI, whereas the other was brighter with DiBAC₄(3). With co-trimoxazole (trimethoprim:sulphamethoxazole at a 1:19 ratio), the light-scattering profile changed to generate a comet-tale pattern, reflecting the presence of enlarged cells (Fig. 1g). Although a significant number of fluorescent cells were detected in acquisition region II (9.6%), a large percentage remained unstained after the 2 h of treatment (Fig. 1h).

Pseudomonas aeruginosa ATCC 27853. In contrast to *E. coli*, most untreated *P. aeruginosa* ATCC 27853 cells had an intrinsic permeability to the fluorescent dyes, with 43% of cells being detected in the acquisition region II designated for the dead cells (Fig. 2b). Similar results were observed with the two clinical

Fig. 2. Light-scattering profile (a) and bi-parametric histogram (b) (DiBAC₄(3) versus PI) for untreated *P. aeruginosa* ATCC 27853. No significant basal fluorescence was recorded (untreated and unstained control; data not shown). The proportion of dead cells (region II), recorded in panel b, was 43%.

P. aeruginosa strains with 15 and 70% of the cells in the untreated stained samples becoming fluorescent. No significant fluorescence was detected in the absence of fluorescent dyes, indicating that this behaviour could not be attributed to endogenous fluorescent molecules or organelles. Because of the high proportion of 'dead' cells in untreated samples, results with the treated samples were inconsistent and were excluded from analysis.

Gram-positive control bacteria. A high proportion (>50%) of live log-phase gram-positive bacterial cells, including two *S. aureus* strains and two *Ent. faecalis* strains, were slightly permeable to PI (Fig. 3). However, these fluorescent events were not included in the acquisition region II. *S. aureus* ATCC 29213 is resistant to penicillin G and susceptible to the other five antibiotics

Fig. 3. Bi-parametric histograms (DiBAC₄(3) versus PI) for *S. aureus* ATCC 29213 untreated (a), permeabilised for 5 min with ethanol 70% (b), or treated for 2 h with vancomycin (c), oxacillin (d), penicillin G (e), nitrofurantoin (f), norfloxacin (g) or co-trimoxazole (h), and analysed by FC. No significant basal fluorescence was recorded (data not shown). The proportions of dead cells recorded (region II) in panels a–h were 3.1%, 99%, 97%, 84%, 14%, 56%, 61% and 43%, respectively.

tested as determined by standard ASTs. As illustrated in Fig. 3, the FC-AST correctly identified this pattern.

FC-AST on UTI specimens

Several clinical bacterial specimens were collected from UTIs. Although *E. coli* accounts for >80% of UTIs, this study aimed to gather representatives of many different types of bacterial UTIs. Fig. 4 shows the susceptibility of *E. coli* 15462, a urine isolate, to ceftriaxone, gentamicin, norfloxacin and nitrofurantoin, and its resistance to ampicillin and co-trimoxazole, as determined by FC-AST. Both conventional susceptibility test methods supported these results.

To bypass the overnight incubation period to obtain single colonies, FC-AST was applied directly to 19 urine samples infected with >10⁵ cfu/ml, and representing four different species. With urine samples infected with *E. coli*, the FC-AST correlated at 98% (13 specimens, 114 tests) and gave only two minor errors. With urine samples infected with organisms other than *E. coli* (six specimens, 48 tests), five

Fig. 4. Bi-parametric histograms (DiBAC₄(3) versus PI) for the urine isolate *E. coli* 15462 treated for 2 h with ampicillin (a), ceftriaxone (b), gentamicin (c), norfloxacin (d), nitrofurantoin (e) or co-trimoxazole (f), and analysed by FC. The proportion of non-fluorescent cells in the untreated sample was 99.1%, whereas 99.8% of the cells in the ethanol-treated sample were fluorescent with both dyes (data not shown). The proportions of dead cells recorded (region II) in panels a–f were 0.8%, 94%, 94%, 88%, 70% and 0.7%, respectively.

Table 2. FC-AST on control strains, urine isolates and urine samples

Organism	Ampicillin 8*			Carbenicillin 16			Ceftriaxone 8			Ceftazidime 8			Gentamicin 4			Nitrofurantoin 32			Norfloxacin 4			Oxacillin 2			Penicillin G 0.125			Tetracycline 4			Co-trimoxazole 2/38			Vancomycin 4		
	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E	n	R	E
Control																																				
<i>E. coli</i>	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	0	–	–	0	–	–	1	0	0	1	0	0	0	–	–
<i>Ent. faecalis</i>	2	0	0	0	–	–	0	–	–	0	–	–	0	–	–	2	0	0	2	0	0	0	–	–	2	0	0	1	1	1 [§]	0	–	–	2	1	0
<i>S. aureus</i>	0	–	–	0	–	–	0	–	–	0	–	–	0	–	–	2	0	0	2	2	1 [‡]	2	1	1 [†]	2	2	0	0	–	–	2	0	0	2	0	0
Urine isolates																																				
<i>E. coli</i>	11	10	0	1	0	0	11	0	0	1	0	0	11	0	0	11	0	0	0	–	–	0	–	–	1	0	0	11	1	0	0	–	–			
<i>K. pneumoniae</i>	1	1	0	0	–	–	1	0	0	0	–	–	1	0	0	1	1	0	1	1	1 [†]	0	–	–	0	–	–	0	–	–	1	1	0	0	–	–
<i>Proteus mirabilis</i>	1	0	0	0	–	–	1	0	0	0	–	–	1	0	1 [†]	1	1	1	0	1	0	0	0	–	–	0	–	–	1	0	0	0	–	–		
<i>Ent. faecalis</i>	2	0	0	0	–	–	0	–	–	0	–	–	0	–	–	2	0	0	2	2	1 [†]	0	–	–	2	1	1 [‡]	1	0	0	0	–	–	2	1	0
<i>Staphylococcus</i>	0	–	–	0	–	–	0	–	–	0	–	–	0	–	–	7	0	2 [‡]	7	5	1 [§]	7	5	1 [‡]	7	6	0	0	–	–	7	1	2 [‡]	7	0	0
Urine samples																																				
<i>E. coli</i>	13	4	0	12	3	0	13	0	0	13	0	0	12	0	0	13	1	1	13			0	–	–	0	–	–	12	1	1 [†]	13	2	0	0	–	–
<i>K. pneumoniae</i>	4	4	1 [†]	3	3	0	4	0	0	4	0	0	3	0	0	4	3	3 [§]	4	0	0	0	–	–	0	–	–	3	0	0	4	0	0	0	–	–
<i>Pr. mirabilis</i>	1	0	0	1	0	0	1	0	0	1	0	0	1	0	0	1	1	0	1	0	0	0	–	–	0	–	–	1	0	1 [‡]	1	0	0	0	–	–
<i>Enterococcus</i>	0	–	–	0	–	–	0	–	–	0	–	–	0	–	–	1	0	0	1	0	0	1	1	0	1	1	0	0	–	–	1	0	0	1	0	0
Control	Assays 32			Minor error 1			Major error 1			Very major error 1			90.6%																							
Urine isolates	134			3			6			1			92.5%																							
Urine samples	162			3			1			3			95.7%																							
Total	328			7			8			5			93.9%																							

n, number of samples tested; R, number of samples tested resistant or intermediate by the disk AST or the broth microdilution AST, or both; E, number of errors (indicated by superscripts): [†]minor error, [‡]major error, [§]very major error.

Of the four *K. pneumoniae* urine samples, three were very major errors and one minor (not mentioned in table).

Penicillin G was used at 8 mg/L for enterococci.

*Concentration (mg/L) of antibiotic used.

Fig. 5. Bi-parametric histograms (DiBAC₄(3) versus PI) for a urine sample, identified later as containing *K. pneumoniae* 43450, treated for 2 h with ampicillin (a), carbenicillin (b), ceftriaxone (c), gentamicin (d), norfloxacin (e), nitrofurantoin (f), tetracycline (g) or co-trimoxazole (h), and analysed by FC. The proportion of non-fluorescent cells in the untreated sample was 94.4%, whereas 99.6% of the cells in the ethanol-treated sample were fluorescent with both dyes (data not shown). The proportions of dead cells recorded (region II) in panels a–h were 2.2%, 2.8%, 85%, 54%, 30%, 22%, 28% and 43%, respectively.

discrepancies were observed with the conventional assays (90% agreement) (Table 2). Three of these were very major errors and involved nitrofurantoin and *Klebsiella pneumoniae*. Fig. 5 shows FC-AST results on one of these samples. Susceptibility was observed for this strain to ceftriaxone, ceftazidime (not shown), gentamicin, norfloxacin, nitrofurantoin, tetracycline and co-trimoxazole, and resistance to ampicillin and carbenicillin with both the FC-AST and standard tests, whilst disagreement was seen only for nitrofurantoin (Fig. 5f).

Incubation of 100 μ l of urine (containing $>10^5$ cfu/ml from UTI patients) in 5 ml of MHSC consistently

yielded visible growth after 90–120 min at 37°C, whereas no visible growth was observed with urine from a healthy individual. The number of events recorded by FC of these urine samples was equivalent to that of filtered PBS (data not shown).

Discussion

The capacity of the fluorescent probes to detect antibiotic effects on individual cells, and the use of flow cytometry to measure and enumerate these effects rapidly was a major factor in establishing the FC-AST protocol. Other reports also indicated the superiority of PI and DiBAC₄(3) over other probes to determine bacterial mortality [11, 12]. Although not all antibiotics target the membrane, their effects – such as the inhibition of protein synthesis – can have rapid but indirect effects on this structure [2]. The use of two mortality dyes increases the confidence that cells harbouring double fluorescence are non-viable. Thus, Nebe-von Caron *et al.* [13] showed that some bacteria stained only by DiBAC₄(3) may have lost membrane potential but not membrane integrity, and that a significant proportion of these could be recovered on solid media. However, cells stained with both PI and DiBAC₄(3) showed no recovery. The very short incubation time (5–10 min) required for staining, and the fact that no washing, permeabilising or fixation steps are necessary before FC analysis are also strong advantages for the use of these two fluorochromes.

The incubation time with antibiotics is an important factor to consider in the development of a rapid FC-AST. Although morphological changes can be observed by FC within 30 min with β -lactam agents, the present study found that 2 h at 37°C was the minimum period required to allow reliable results with all the bacteria–antibiotic combinations tested. This short exposure was sufficient to produce a significant decrease of bacterial concentrations as determined by plate counting, from 10^8 cfu/ml to 10^3 – 10^6 cfu/ml, depending on the type of antibiotic (10^3 cfu/ml was the detection limit) (not shown).

The interpretation criteria for susceptibility and resistance are well defined with the two conventional methods, but interpretative criteria for the FC-AST had to be established. While susceptibility could be observed qualitatively with some antibiotics (e.g., β -lactams) in the form of significant changes in the light-scattering profiles, a quantitative measurement was sought to give a more objective determination. This measure was based on the number of cells fluorescent for both dyes and the mean fluorescence intensity (MFI) of events measured in acquisition region I, and was compared between antibiotic-treated and -non-treated samples giving the ratio C_i . The ranges of the C_i values that defined resistance, susceptibility and

intermediate resistance for FC-AST were established by comparison with standard tests. However, some of the C_i range were not defined, because resistant isolates were not available (e.g., with gentamicin, vancomycin, ceftriaxone, ceftazidime). C_i values between 0 and 10 correlated well with resistance and C_i values >1000 correlated with susceptibility. Nine (45%) of the 20 discrepancies were caused by C_i values that were between 10 and 1000.

FC-AST was first evaluated with six ATCC control strains representing four bacterial species frequently encountered in UTIs: *E. coli*, *P. aeruginosa*, *Ent. faecalis* and *S. aureus*. The method was not suitable for *P. aeruginosa*, where live cells stained with both dyes, especially PI. *P. aeruginosa* is a strict aerobe and it rapidly de-energises when deprived of an energy source, resulting in enhanced permeability of its outer membrane (R. E. W. Hancock, Department of Microbiology and Immunology, University of British Columbia, Vancouver, Canada, personal communication). This bacterium is also sensitive to temperature changes such as arise when standing without agitation at room temperature or when incubated on ice [14]. Such conditions occurred during the FC-AST. Therefore, modifications of the protocol might be tested, such as bypassing the PBS washing step, by adding the fluorescent probes directly in the cultures at the end of the antibiotic treatment, and by staining for ≤ 5 min at 37°C.

The changes in light-scattering and fluorescence profiles were specific for each antibiotic or class. The four β -lactam antibiotics – ampicillin, carbenicillin, ceftriaxone and ceftazidime that were used mainly in this study against gram-negative bacteria – produced similar changes to one another in the light-scattering profile, usually with $>80\%$ of the cells becoming fluorescent with one or both dyes. Two other β -lactam antibiotics, penicillin G and oxacillin, are used against gram-positive bacteria, and showed a different pattern: untreated logarithmic phase cells displayed a significant (but weak) PI fluorescence, whereas treated, susceptible cells showed a bright staining with DiBAC₄(3), thus underscoring the advantage of using a combination of mortality dyes. Although the light-scattering profile was not affected by gentamicin, a large percentage of cells became fluorescent with both dyes. In contrast to the behaviour with β -lactam agents, gentamicin generated two populations as resolved by fluorescence profiles. This probably reflects the mechanism of action of gentamicin, which first causes the loss of membrane potential, then affects membrane integrity.

Taking the FC-AST results for the five control strains together with those for urine samples and isolates, there were 20 discrepancies among 328 individual comparisons (93.9% agreement). Non-*E. coli* strains accounted for 18 of those discrepancies, and 13 (65%) of these 20 occurred when norfloxacin, nitrofurantoin and tetracy-

cline were tested. Low percentages of fluorescent cells (5–50%) were generally obtained from susceptible strains incubated with nitrofurantoin and tetracycline, which are bacteriostatic, and this could have resulted in a misinterpretation of susceptibility.

The goal of this study was to evaluate the feasibility of a FC-AST in a clinical setting, not an exhaustive statistical comparison with standard antimicrobial tests. Therefore the aim was to gather many different bacterial species involved in clinical urine infections, especially those with wide resistance. The results showed that FC-AST has similar reliability when testing either urine isolates (10 discrepancies in 134 tests; 93% or urine samples (7 discrepancies in 162 tests; 96%). FC-AST applied directly to urine samples instead of isolates would accelerate antimicrobial susceptibility testing, as it does not require the overnight incubation for isolating colonies on solid media [15–17].

In our hands, the protocol required 5 h for the analysis of up to 20 samples (as cytometers in current use acquire data in a serial fashion), instead of the 24–48 h for standard tests. Therefore, FC-AST could be a rapid and accurate alternative to conventional methods, and could benefit health-care systems by accelerating urine analysis.

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References

- McFeters GA, Yu FP, Pyle BH, Stewart PS. Physiological assessment of bacteria using fluorochromes. *J Microbiol Methods* 1995; **21**: 1–13.
- Gant VA, Warnes G, Phillips I, Savidge GF. The application of flow cytometry to the study of bacterial responses to antibiotics. *J Med Microbiol* 1993; **39**: 147–154.
- Langsrud S, Sundheim G. Flow cytometry for rapid assessment of viability after exposure to a quaternary ammonium compound. *J Appl Bacteriol* 1996; **81**: 411–418.
- Deere D, Porter J, Edwards C, Pickup R. Evaluation of the suitability of bis-(1,3-dibutylbarbituric acid) trimethine oxonol, (DiBAC₄(3)-), for the flow cytometric assessment of bacterial viability. *FEMS Microbiol Lett* 1995; **130**: 165–170.
- Jepras RI, Paul FE, Pearson SC, Wilkinson MJ. Rapid assessment of antibiotic effects on *Escherichia coli* by bis-(1,3-dibutylbarbituric acid) trimethine oxonol and flow cytometry. *Antimicrob Agents Chemother* 1997; **41**: 2001–2005.
- Davey HM, Kell DB. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single cell analyses. *Microbiol Rev* 1996; **60**: 641–696.
- Shapiro HM. Practical flow cytometry, 2nd edn. New York, Alan R. Liss. 1988.
- National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk susceptibility tests M2-A6. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically M7-A4. Ninth informational supplement M100-S9. Villanova, PA, NCCLS. 1999.
- Walberg M, Gaustad P, Steen HB. Rapid flow cytometric assessment of mecillinam and ampicillin bacterial susceptibility. *J Antimicrob Chemother* 1996; **37**: 1063–1075.

10. Walberg M, Gaustad P, Steen HB. Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin susceptibility in exponentially-growing *E. coli* cells by means of flow cytometry. *Cytometry* 1997; **27**: 169–178.
11. Comas J, Vives-Rego J. Enumeration, viability and heterogeneity in *Staphylococcus aureus* cultures by flow cytometry. *J Microbiol Methods* 1998; **32**: 45–53.
12. Mason DJ, Power EGM, Talsania H, Phillips I, Gant VA. Antibacterial action of ciprofloxacin. *Antimicrob Agents Chemother* 1995; **39**: 2752–2758.
13. Nebe-Von Caron G, Stephens P, Badley RA. Assessment of bacterial viability status by flow cytometry and single cell sorting. *J Appl Microbiol* 1998; **84**: 988–998.
14. Lei Y, Satake S, Ishii J, Nakae T. Factors that influence the permeability assay of the outer membrane of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett* 1991; **80**: 337–340.
15. Gillenwater JY, Clark MM. Tentative direct antimicrobial susceptibility testing in urine. *J Urology* 1996; **156**: 149–153.
16. Johnson JR, Tiu FS, Stamm WE. Direct antimicrobial susceptibility testing for acute urinary tract infections in women. *J Clin Microbiol* 1995; **33**: 2316–2323.
17. Oakes AR, Badger R, Grove DI. Comparison of direct and standardised testing of infected urine for antimicrobial susceptibilities by disk diffusion. *J Clin Microbiol* 1994; **32**: 40–45.