

## Application of a spectrophotometric method for the determination of post-antibiotic effect and comparison with viable counts in agar

M. Carmen Domínguez<sup>a\*</sup>, Manuel de la Rosa<sup>a</sup> and M. Victoria Borobio<sup>b</sup>

<sup>a</sup>Microbiology Service, General Specialty Hospital 'Virgen de las Nieves', Granada;

<sup>b</sup>Department of Microbiology, University Hospital 'Virgen Macarena', Seville, Spain

**The post-antibiotic effects of gentamicin and ciprofloxacin at 1×, 2× and 4× MIC on *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 were studied using a spectrophotometric method and the classic method of viable counts on agar as a reference. Monitoring of the growth kinetics was carried out by viability counting on the plate every hour and by means of the optical density of the cultures measured by spectrophotometry at a wavelength of 450 nm. No statistically significant differences were found between the results obtained with the spectrophotometric method and the reference method. The former method was much quicker, much easier to use and to replicate.**

### Introduction

Post-antibiotic effect (PAE) is the suppression or slowing down of bacterial growth after a short exposure to an antimicrobial agent, which is then eliminated from the culture.<sup>1</sup> Traditionally, viability counting on agar has been used to monitor the kinetics of growth of a culture treated with antibiotic and that of a control.<sup>1–5</sup> Using this methodology, the PAE is the time required for the number of colony-forming units (cfu) in the test culture to increase by 1 log<sub>10</sub> (from the count observed immediately after drug removal), minus the time required for the number of cfu in an untreated control culture to increase by 1 log<sub>10</sub> (from the count observed immediately after completion of the same procedure used on the test culture for drug removal). The reason for arbitrarily choosing a 10-fold growth rate is that, beyond that level, the rate of growth of the organisms exposed to the antibiotic is the same as that of the untreated controls.<sup>4</sup>

The laborious nature of this technique is its biggest drawback. For this reason, other techniques have been developed and applied to the study of PAE, such as bioluminescence,<sup>6–10</sup> electrical impedance,<sup>11,12</sup> bacterial morphology,<sup>13</sup> infrared spectroscopy,<sup>14</sup> radiometry,<sup>15</sup> electrical counting,<sup>16,17</sup> fluorometry<sup>18</sup> and spectrophotometry. The spectrophotometric method involves either determining the time difference for a 5% decrease in transmittance, between the antibiotic-exposed and unexposed bacterial

cultures,<sup>19–21</sup> or measuring the difference in time to reach a chosen point on the absorbance curve between the treated and control cultures.<sup>22,23</sup>

The objectives of this study were to develop a spectrophotometric method for determining PAE, and to validate it by comparison with the traditional method of viable counts on agar. With this spectrophotometric method, the PAE was calculated by measuring the separation of the spectrophotometric logarithmic growth curves of the control and treated cultures; the time of exposure of the cultures to the antimicrobial agent and the regrowth time, or the theoretical time that it takes for the exposed culture to recover from the bactericidal effect of the antibiotic and reach the initial inoculum, was subtracted from the separation time.

### Materials and methods

#### *Bacterial strains and media*

The strains used in this study were *Escherichia coli* ATCC 25922; *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213. The strains were cultured in Mueller–Hinton broth (Difco Laboratories, Detroit, MI, USA) to monitor the growth kinetics by spectrophotometry. Mueller–Hinton agar (Oxoid, Basingstoke, UK) was used for determination of viable counts.

\*Correspondence address. Lopez Tarruellas St. 6 A, 41940 Tomares (Sevilla), Spain.

Tel: +34-954152607; Fax: +34-953024315; E-mail: cdiagnostico@hsa.sas.cica.es

### Antimicrobial agents

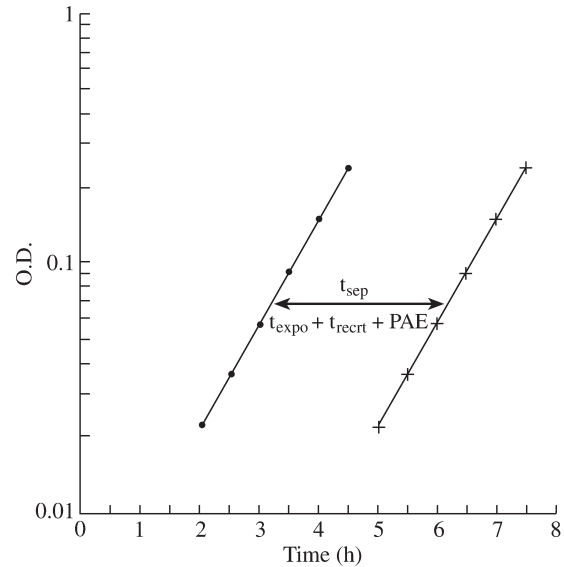
The antimicrobial agents used were gentamicin (Instituto Llorente, Madrid, Spain) and ciprofloxacin (Bayer AG, Leverkusen, Germany) at  $1 \times$ ,  $2 \times$  and  $4 \times$  MIC. The MIC was determined for each isolate using the macrodilution method.<sup>24</sup>

### PAE determination

Two to three colonies of a 20 h growth on Mueller–Hinton agar of the organism to be studied were suspended in 50 mL prewarmed ( $37^\circ\text{C}$ ) Mueller–Hinton broth. The suspension was incubated overnight at  $37^\circ\text{C}$ , diluted  $1/2500$  in the same prewarmed medium and incubated in a waterbath at  $37^\circ\text{C}$  with agitation (50 rpm). The absorbance of the culture was monitored with a spectrophotometer (Spectronic 20, Milton Roy Company, PACISA, Madrid, Spain) using a wavelength of 450 nm and 19 mm diameter spectrophotometer tubes (Perkin-Elmer Hispania S.A., Granada, Spain), until an absorbance of 0.1 was reached (equivalent to  $2.5\text{--}3.0 \times 10^7$  cfu/mL for *E. coli* and *P. aeruginosa* and to  $1.8\text{--}2.0 \times 10^7$  cfu/mL for *S. aureus*). Equal volumes of the control culture and the cultures to be treated with the antibiotic were separated and a viability count was taken to determine the exposed bacterial inoculum ( $N_{\text{inic}}$ ). The bacteria–antibiotic contact lasted 1 h ( $t_{\text{expo}}$ ), at the end of which drug activity was stopped by placing a  $10^{-3}$  dilution of the bacterial suspension in drug-free prewarmed Mueller–Hinton broth. In preliminary experiments this dilution was shown to be sufficient as the residual drug activity resulted in no significant deviation of the growth curve. Colony counts were taken at time zero ( $N_{\text{inic}}$ ), after removal of the antibiotic ( $N_{\text{anti}}$ ) and then at hourly intervals for 6 h for PAE determination by viable count. The control culture was also subjected to the  $1/1000$  dilution and growth rate was determined under identical conditions without antibiotic exposure. All the cultures were incubated at  $37^\circ\text{C}$  with agitation and the absorbance was measured every 30 min.

### Calculation of the PAE

Once  $N_{\text{inic}}$  and  $N_{\text{anti}}$  were determined and the growth of the control and exposed cultures were spectrophotometrically monitored, we proceeded with the following steps. (i) Plotting, on semi-logarithmic paper, of the spectrophotometric growth curves of the control and post-exposure cultures, representing optical density (OD) along the y-axis and time along the x-axis (Figure 1). Customarily the first meaningful reading of OD can be taken for the control culture at 120 or 150 min after the initial time ( $t_{\text{inic}} = 0$ ). (ii) Determination of the generation time ( $t_g$ ) or duplication time. The  $t_g$  is calculated by the spectrophotometric monitoring of the control culture and of a  $1/8$  dilution of the same culture. This dilution is separated in time by three generations



**Figure 1.** Theoretical graph of PAE determination. Symbols: v, control; +, exposed.

( $2^1$ ,  $2^2$ ,  $2^3$ ); therefore, by dividing the separation time between both growth curves by 3, the generation time is obtained. (iii) The calculation of bactericidal effect ( $r$ ):

$$r = N_{\text{inic}}/N_{\text{anti}} \quad (1)$$

(iv) Graphical determination of the time separation of the spectrophotometric growth curves of the control culture and the post-exposure culture ( $t_{\text{sep}}$ ). (v) Calculation of the PAE according to the general formula:

$$\text{PAE} = t_{\text{sep}} - t_{\text{expo}} - t_{\text{recre}} \quad (2)$$

where  $t_{\text{sep}}$  is the separation time of the spectrophotometric growth curves of the control culture and the post-exposure culture;  $t_{\text{expo}}$  is the exposure time equivalent to 1 h duration and  $t_{\text{recre}}$  is the theoretical time that the treated culture takes for its viability count ( $N_{\text{anti}}$ ) to match the initial count ( $N_{\text{inic}}$ ); this depends on the succeeding generations ( $n$ ) and the generation time ( $t_g$ ) of the bacteria.

Under constant conditions, after a long enough time, when cell–cell interaction is small, growth measured by any method is expected to proceed according to:

$$N_t = N_0 e^{\lambda t} \quad (3)$$

Where  $N_0$  and  $N_t$  are the counts at times zero and  $t$ , respectively.  $\lambda$  has been used for the specific growth rate.

$$\ln N_t = \ln N_0 + \lambda t \quad (4)$$

$$\log N_t = \log N_0 + \lambda t/2.303 \quad (5)$$

or

$$N_t = N_0 2^{t/t_g} \quad (6)$$

$$n = t/t_g \quad (7)$$

## Spectrophotometric determination of PAE

thus

$$N_{\text{inic}} = N_{\text{anti}} 2^n \quad (8)$$

$$N_{\text{inic}}/N_{\text{anti}} = 2^n \quad (9)$$

$$r = 2^n \quad (10)$$

$$\log r = n \log 2 \quad (11)$$

$$n = \log r / \log 2 \quad (12)$$

$$t_{\text{reert}} = n t_g = \log r / \log 2 t_g \quad (13)$$

$$t_{\text{sep}} = t_{\text{expo}} + t_{\text{reert}} + \text{PAE} \quad (14)$$

$$\text{PAE} = t_{\text{sep}} - t_{\text{expo}} - t_{\text{reert}} = t_{\text{sep}} - 1 \text{ h} - t_g \log r / \log 2 \quad (15)$$

At the same time, the growth kinetics were monitored by viable counts in agar, determining PAE with the standard formula of Craig & Gudmundsson,<sup>1</sup> as the difference in time, between test (T) and control (C) cultures, for organisms to increase in number by a factor of 10:

$$\text{PAE} = T - C \quad (16)$$

Colony counts were determined with Mueller–Hinton agar pour plates in the initial inoculum, after dilution to remove the antibiotic, and every hour for 6 h.

### Statistical analysis

To compare both methods, Student's *t*-test at the 0.05 level of significance was used for matching data.

## Results

### MIC determination

The MICs of gentamicin for *E. coli* ATCC 25922; *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213 were 0.5, 0.5 and 0.25 mg/L, respectively. The MIC of ciprofloxacin was 0.007 mg/L for *E. coli* ATCC 25922 and 0.25 mg/L for *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213.

### Time–kill and PAE experiments

Gentamicin demonstrated a marked bactericidal activity against the three standard strains when cultures in the logarithmic phase of growth with an inoculum of  $3\text{--}5 \times 10^7$  cfu/mL were exposed to  $4 \times \text{MIC}$ . In the case of *S. aureus* a fall in the initial inoculum of  $2.91 \pm 0.12 \log_{10}$  cfu/mL was detected. The PAE was not calculated in cases where a large decrease in viability was observed. No reductions in the inoculum exposed to  $1 \times$  and  $2 \times \text{MIC}$  were observed when this antibiotic was used against *E. coli*, evidence of bacteriostatic activity being observed at both concentrations.

The PAE induced by gentamicin on the three standard strains was concentration dependent, with a duration of between  $0.52 \pm 0.12$  h against *E. coli* and  $1 \times \text{MIC}$  of

gentamicin and  $1.77 \pm 0.14$  h with *S. aureus* and  $2 \times \text{MIC}$  of gentamicin (Table).

The bactericidal activity of ciprofloxacin was very marked and concentration dependent when it was in contact with the bacterial cultures, with quite noticeable reductions in the initial inoculum when assayed at  $4 \times$  and  $8 \times \text{MIC}$ ; this was the reason for opting to carry out the test only at low concentrations ( $1 \times$  and  $2 \times \text{MIC}$ ).

The duration of the PAE of ciprofloxacin was also dose dependent, being  $0.14 \pm 0.09$  h and  $0.41 \pm 0.10$  h against *E. coli*;  $0.88 \pm 0.08$  h and  $1.27 \pm 0.15$  h against *P. aeruginosa*; and  $0.55 \pm 0.10$  h and  $0.65 \pm 0.05$  h against *S. aureus*, at  $1 \times$  and  $2 \times \text{MIC}$ , respectively (Table).

No statistically significant differences ( $P > 0.05$ ) were detected in the duration of PAE between the spectrophotometric method developed by us and the traditional viability count in any of the combinations assayed. The results obtained with the latter method appear in the Table.

Figures 2, 3 and 4 show the growth curves monitored by spectrophotometry and by viability counting, for the control cultures and for those exposed to the antibiotic, once they had all been subjected to the same procedure of elimination of the antibiotic. As can be seen, the exponential growth phases of each culture run in parallel and the greater the concentration of the antimicrobial agent to which the bacterial culture has been exposed, the longer the time separation from the growth curve of the control culture.

## Discussion

The importance of the study of PAE lies in the fact that intermittent dosing regimes may be as effective as continuous dosing, with a concomitant reduction in costs and toxicity, for those antimicrobial agent–microorganism combinations with which a prolonged PAE is obtained.<sup>1,25</sup>

A variety of techniques have been applied to the study of PAE *in vitro*, as well as models for its determination *in vivo*.

The spectrophotometric technique that we have developed enables results to be available in 24 h, for various antimicrobial–microorganism combinations, easily and conveniently and with results that can be replicated. For every antimicrobial agent–microorganism association and for every antimicrobial concentration tested, the results obtained are not significantly different from those obtained with the viable count technique.

Other authors have measured PAE using spectrophotometry and have compared it with the standard method. Bergan *et al.*<sup>26</sup> observed discrepancies between turbidity and colony count with *E. coli*–gentamicin and *E. coli*– $\beta$ -lactam antibiotics in contrast to the chloramphenicol and oxytetracycline combinations. However, with flucloxacillin and *S. aureus*, the cfu/mL and OD curves are essentially parallel.<sup>26</sup> Rescott *et al.*<sup>21</sup> demonstrated a considerable

**Table.** Relationship of drug concentration to bactericidal activity and PAE, determined photometrically and by viable count, after 1 h exposure to the antimicrobial agent

Drug concentration mg/L ( $\times$ MIC)	Decrease in the inoculum ( $\log_{10} r$ ) <sup>a</sup>	PAE (h) <sup>a</sup>	
		photometry	viable counts
<i>E. coli</i> ATCC 25922			
gentamicin			
0.5 (1)	$-0.58 \pm 0.12$	$0.52 \pm 0.12$	$0.52 \pm 0.14$
1.0 (2)	$-0.38 \pm 0.03$	$0.62 \pm 0.14$	$0.65 \pm 0.12$
2.0 (4)	$0.88 \pm 0.92$	$1.13 \pm 0.07$	$1.05 \pm 0.04$
ciprofloxacin			
0.007 (1)	$0.04 \pm 0.39$	$0.14 \pm 0.09$	$0.18 \pm 0.12$
0.014 (2)	$0.77 \pm 0.24$	$0.41 \pm 0.10$	$0.42 \pm 0.12$
<i>P. aeruginosa</i> ATCC 27853			
gentamicin			
0.5 (1)	$-0.03 \pm 0.08$	$0.31 \pm 0.05$	$0.32 \pm 0.05$
1.0 (2)	$-0.03 \pm 0.04$	$0.80 \pm 0.08$	$0.81 \pm 0.09$
2.0 (4)	$0.89 \pm 0.24$	$1.57 \pm 0.16$	$1.57 \pm 0.16$
ciprofloxacin			
0.25 (1)	$1.03 \pm 0.18$	$0.88 \pm 0.08$	$0.83 \pm 0.03$
0.50 (2)	$1.87 \pm 0.15$	$1.27 \pm 0.15$	$1.22 \pm 0.04$
<i>S. aureus</i> ATCC 29213			
gentamicin			
0.25 (1)	$-0.21 \pm 0.13$	$0.82 \pm 0.14$	$0.71 \pm 0.22$
0.50 (2)	$0.82 \pm 0.65$	$1.77 \pm 0.14$	$1.80 \pm 0.20$
1.00 (4)	$2.91 \pm 0.12$	ND	ND
ciprofloxacin			
0.25 (1)	$0.49 \pm 0.35$	$0.55 \pm 0.10$	$0.54 \pm 0.11$
0.50 (2)	$1.68 \pm 0.24$	$0.65 \pm 0.05$	$0.70 \pm 0.02$

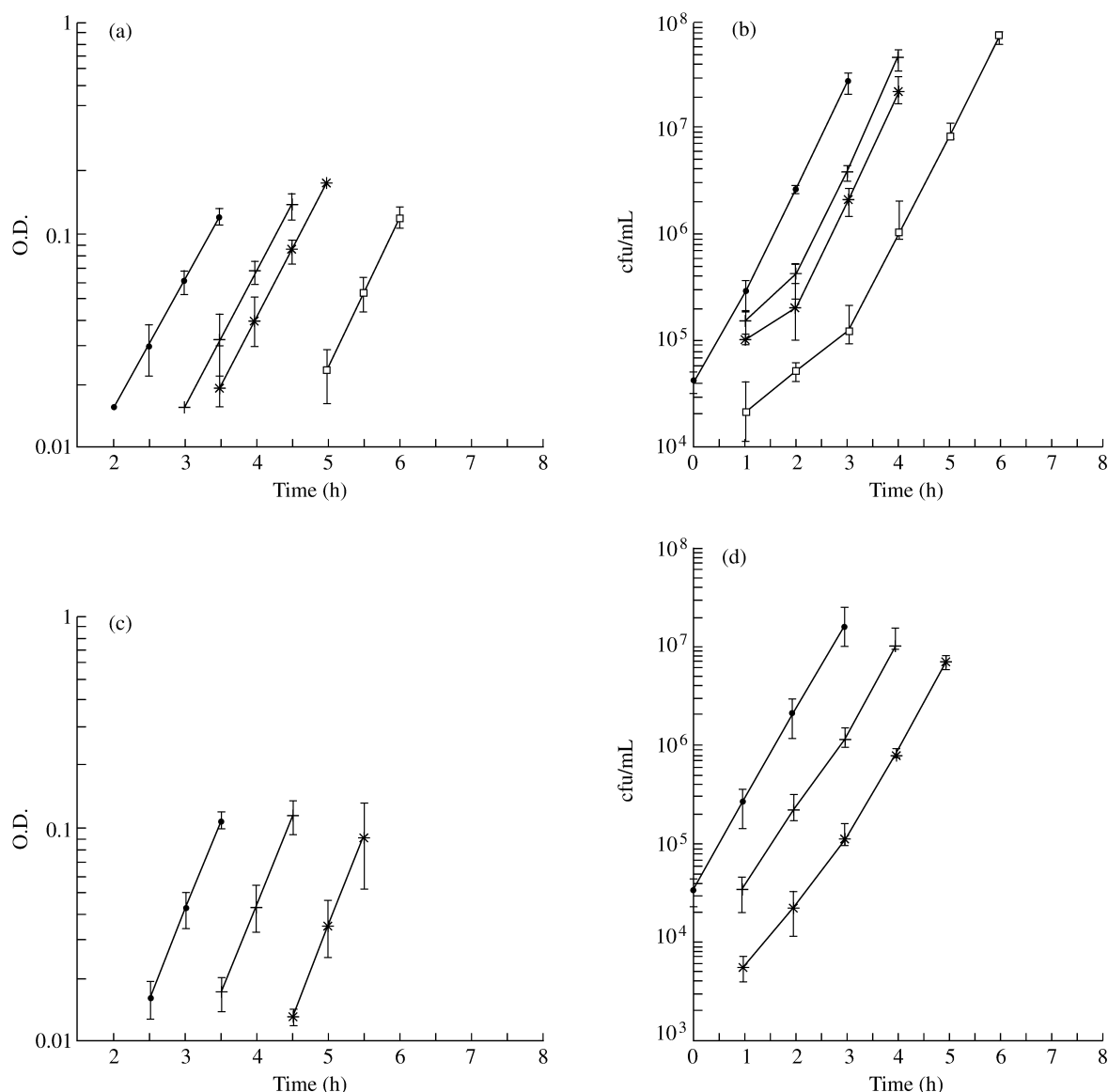
<sup>a</sup>Expressed as the mean  $\pm$  S.D. of four determinations. $r = N_{\text{init}}/N_{\text{anti}}$ ; ND, not determined.

variation between the reference method and spectrophotometry using the Abbott MS-2 system to quantify the increase in OD of the cultures with tobramycin-*E. coli*. With the MS-2 method, there is a threshold for growth detection at approximately  $10^6$ – $10^7$  cfu/mL. When the initial colony count was significantly below this threshold concentration, there was an apparent growth lag measured by MS-2. During this apparent growth lag, log-phase growth may be present. It is particularly important to stress that with the utilization of this spectrophotometric method, the observed lag in growth is dependent on the initial number of cfu/mL as well as on the growth rate of the organism during the monitoring period. With potent bactericidal drugs such as tobramycin, there was considerable variability in both the reference and MS-2 methods.<sup>21</sup> These same authors found no differences between either method in the combinations of *E. coli* and ampicillin or ciprofloxacin. PAE durations for both the reference and MS-2

methods for *E. coli* ATCC 25922 exposed at  $2 \times$  MIC for 1 h were 0.0 and 26.0 min for ampicillin, 56.0 and 70.3 min for ciprofloxacin, 98.0 and 111.4 min for tobramycin.<sup>21</sup> Li *et al.*<sup>19</sup> established an excellent concordance between the reference method and the turbidimetric assay only with antibiotics that have a minimal or no PAE on the bacteria assayed. This assertion contrasts with our results and those indicated by Meng *et al.*<sup>27</sup> and Rescott *et al.*,<sup>21</sup> using the spectrophotometric technique.

Meng *et al.*<sup>27</sup> developed a spectrophotometric method for determining PAE that was unaffected by the detection threshold of the OD of the culture. The PAE was quantified on the basis of the mean recovery time of the bacterial population exposed to the antimicrobial agent. To validate this technique, they studied the PAE of ciprofloxacin on *E. coli* ATCC 25922, comparing their method with the traditional one of viable count. They found no statistically significant difference between them. PAEs

## Spectrophotometric determination of PAE



**Figure 2.** Growth curves monitored by spectrophotometry and by viable count of *E. coli* ATCC 25922 culture after 1 h exposure to concentrations equal to and higher than the MIC of gentamicin [(a) and (b); —●—, control; —+—, 1 × MIC (0.5 mg/L); —\*—, 2 × MIC (1 mg/L); —□—, 4 × MIC (2 mg/L)] and ciprofloxacin [(c) and (d); —●—, control; —+—, 1 × MIC (0.007 mg/L); —\*—, 2 × MIC (0.014 mg/L)]. Data points are expressed as the mean ± S.D. of four determinations.

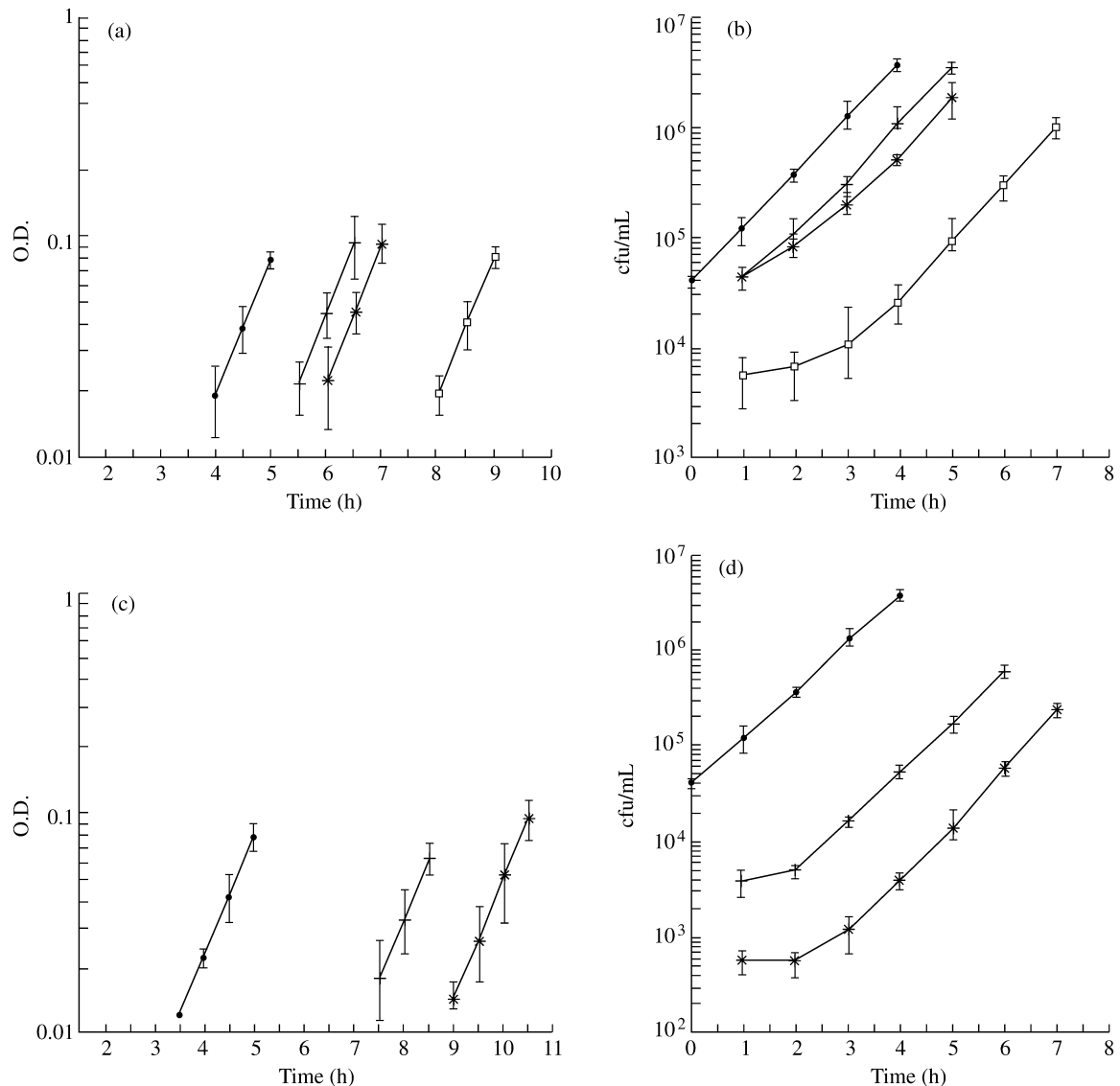
induced by a 1.5 h exposure of *E. coli* ATCC 25922 to ciprofloxacin at 2 × and 3 × MIC were 1.6 and 2.1 h, respectively.<sup>28</sup>

Odenholt-Tornqvist<sup>23</sup> determined the PAE of meropenem for different strains of *E. coli* and *P. aeruginosa* with viable counts and then measured by continuous monitoring of OD in a BioScreen C apparatus. Again, no major difference was seen between the two methods. However, BioScreen C was found to present certain problems with the setting-up temperature and OD stability, and needs at least 1 h to reach the set-point temperature: the time lag is affected, but not the growth rate. Also there were perturbations of the measurements recorded at the beginning of the experiment.<sup>29</sup>

Consequently, there has been no basis for a uniform comparison of PAEs obtained from growth curves measured by colony counting and by OD. The limitation of sensitivity and changes in bacterial morphology are two major problems involved in the quantification of PAE using OD measurements for determination of bacteria growth.

The Spectronic 20 has an effective OD scale much too short for high precision assays. It follows that if the shorter wavelength (450 nm) is used, scattering will be greater and the absorbance measured more accurate.

We have established that the OD of a culture is directly proportional to the viable counts in agar at an interval that varies, depending on the organism being studied, between



**Figure 3.** Growth curves monitored by spectrophotometry and by viable count of *P. aeruginosa* ATCC 27853 culture after 1 h exposure to concentrations equal to and higher than the MIC of gentamicin [(a) and (b); ●—, control; +—, 1 × MIC (0.5 mg/L); \*—, 2 × MIC (1 mg/L); □—, 4 × MIC (2 mg/L) and ciprofloxacin [(c) and (d); ●—, control; +—, 1 × MIC (0.25 mg/L); \*—, 2 × MIC (0.5 mg/L)]. Data points are expressed as the mean ± s.d. of four determinations..

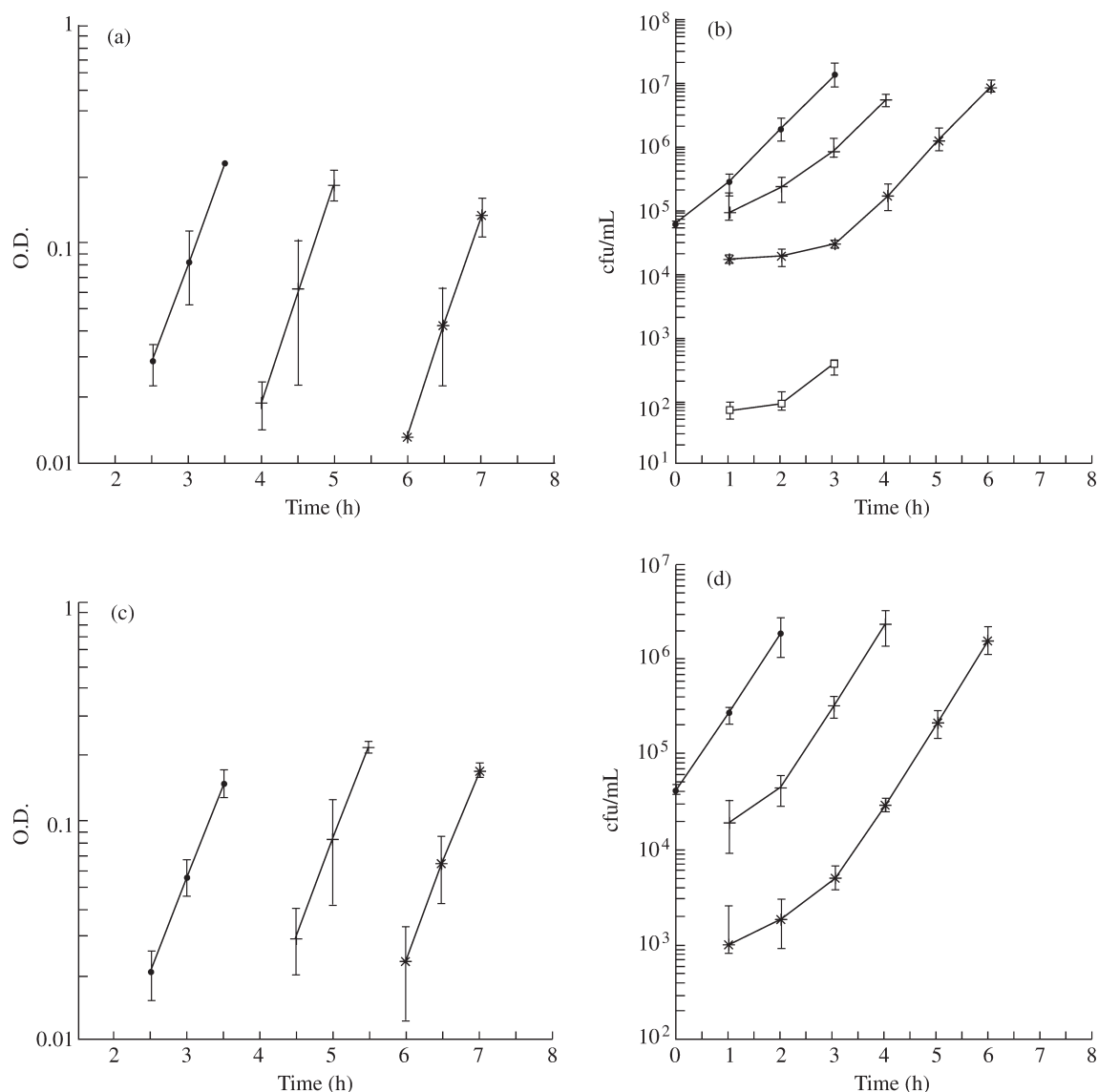
$4 \times 10^6$  and  $3 \times 10^8$  cfu/mL with *E. coli* ATCC 25922; between  $3 \times 10^6$  and  $10^8$  cfu/mL with *P. aeruginosa* ATCC 27853; and between  $4 \times 10^6$  and  $7 \times 10^7$  cfu/mL with *S. aureus* ATCC 29213. In our case, the sensitivity threshold of the spectrophotometer did not interfere with the calculation of PAE. This is because our method is based first on the linearity of the spectrophotometry ratio and viable counts in the range mentioned earlier, and secondly on the parallelism of the phases of logarithmic growth, measured by spectrophotometry, of the control cultures and of those exposed to the antibiotic. The change in size of the bacteria (e.g. with ciprofloxacin–*E. coli*) is too small to influence the spectrophotometric growth curves. The photometric method measures cell mass, not cell number. Neither was the bactericidal activity affected by filamentation gener-

ated in *E. coli* after ciprofloxacin exposure because, in contrast to results with the  $\beta$ -lactams, this morphological alteration did not appear until after drug removal.<sup>30</sup>

The duration of the PAE was concentration dependent for all the combinations studied. This would be related to the degree of bactericidal activity at the different concentrations. Therefore, increasing concentrations were associated with a larger  $t_{\text{sep}}$  between the spectrophotometric growth curves of the control and the post-exposure cultures, due to a longer  $t_{\text{recl}}$  time being required for cellular recovery from drug-induced damage.

In conclusion, the spectrophotometric method that we have developed for measuring PAE is a less laborious and speedier alternative than the traditional one of viable counts. Only two counts are required, one initially and a

## Spectrophotometric determination of PAE



**Figure 4.** Growth curves monitored by spectrophotometry and by viable count of *S. aureus* ATCC 27853 culture after 1 h exposure to concentrations equal to and higher than the MIC of gentamicin [(a) and (b); ●—, control; +, 1 × MIC (0.25 mg/L); \*—, 2 × MIC (0.5 mg/L); □—, 4 × MIC (1 mg/L)] and ciprofloxacin [(c) and (d); ●—, control; +, 1 × MIC (0.25 mg/L); \*—, 2 × MIC (0.5 mg/L)]. Data points are expressed as the mean ± s.d. of four determinations.

second after the elimination of the antibiotic. An excellent correlation was demonstrated between the PAEs quantified by this system and by viable counts for gentamicin and ciprofloxacin with standard strains of *E. coli*, *P. aeruginosa* and *S. aureus*. The equipment needed is a standard item in most clinical microbiology laboratories. In addition, this method overcomes the lack of sensitivity of other photometric methods.

## Acknowledgements

The authors wish to thank Dr Luis Martínez for his review of the manuscript.

## References

1. Craig, W. A. & Gudmundsson, S. (1996). Postantibiotic effect. In *Antibiotics in Laboratory Medicine*, 4th edn, (Lorian, V., Ed.), pp. 296–329. Williams & Wilkins, Baltimore, MD.
2. Bundtzen, R. W., Gerber, A. U., Cohn, D. L. & Craig, W. A. (1981). Postantibiotic suppression of bacterial growth. *Reviews of Infectious Diseases* **3**, 28–37.
3. Howard, B. M., Pinney, R. J. & Smith, J. T. (1993). Contributions of post-antibiotic lag and repair-recovery to the post-antibiotic effects of ciprofloxacin on *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Streptococcus pyogenes*. *Chemotherapy* **39**, 22–31.
4. McDonald, P. J., Craig, W. A. & Kunin, C. M. (1977). Persistent effect of antibiotics on *Staphylococcus aureus* after exposure for limited periods of time. *Journal of Infectious Diseases* **135**, 217–23.



5. Parker, R. F. & Luse, S. (1948). The action of penicillin on *Staphylococcus*: further observations on the effect of a short exposure. *Journal of Bacteriology* **56**, 75–84.
6. Hanberger, H., Svensson, E., Nilsson, M., Nilsson, L. E., Hörnsten, E. G. & Maller, R. (1993). Effects of imipenem on *Escherichia coli* studied using bioluminescence, viable counting and microscopy. *Journal of Antimicrobial Chemotherapy* **31**, 245–60.
7. Isaksson, B., Maller, R., Nilsson, L. E. & Nilsson, M. (1993). Postantibiotic effect of aminoglycosides on staphylococci. *Journal of Antimicrobial Chemotherapy* **32**, 215–22.
8. MacKenzie, F. M., Gould, I. M., Chapman, D. G. & Jason, D. (1994). Comparison of methodologies used in assessing the post-antibiotic effect. *Journal of Antimicrobial Chemotherapy* **34**, 223–30.
9. MacKenzie, F. M., Gould, I. M., Chapman, D. G. & Jason, D. (1994). Postantibiotic effect of meropenem on members of the family *Enterobacteriaceae* determined by five methods. *Antimicrobial Agents and Chemotherapy* **38**, 2583–9.
10. Winstanley, T. G. & Hastings, J. G. (1990). Synergy between penicillin and gentamicin against enterococci. *Journal of Antimicrobial Chemotherapy* **25**, 551–60.
11. Baquero, F., Culebras, E., Patrón, C., Pérez Díaz, J. C., Medrano, J. C. & Vicente, M. F. (1986). Postantibiotic effect of imipenem on gram-positive and gram-negative micro-organisms. *Journal of Antimicrobial Chemotherapy* **18**, Suppl. E, 47–59.
12. Gould, I. M., Milne, K., Harvey, G. & Jason, C. (1991). Ionic binding, adaptive resistance and post-antibiotic effect of netilmicin and ciprofloxacin. *Journal of Antimicrobial Chemotherapy* **27**, 741–8.
13. Lorian, V., Ernst, J. & Amaral, L. (1989). The post-antibiotic effect defined by bacterial morphology. *Journal of Antimicrobial Chemotherapy* **23**, 485–91.
14. Gottfredsson, M., Erlendsdottir, H. & Gudmundsson, S. (1991). Quantitation of postantibiotic effect by measuring CO<sub>2</sub> generation of bacteria with the BACTEC blood culture system. *Antimicrobial Agents and Chemotherapy* **35**, 2658–61.
15. Gottfredsson, M., Erlendsdottir, H., Gudmundsson, A. & Gudmundsson, S. (1989). DNA synthesis in *S. aureus* and *E. coli* during the postantibiotic effect (PAE) phase. In *Programs and Abstracts of the Twenty-Ninth Interscience Conference on Antimicrobial Agents and Chemotherapy, 1989*. Abstract 935, p. 258. American Society for Microbiology, Washington, DC.
16. Nadler, H. L., Curby, W. A., Forgacs, P. & Rosenberg, F. (1989). Comparison of electronic and viability counting methods for determination of postantibiotic effect of oxacillin on *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy* **33**, 2155–6.
17. Li, R. C., Lee, S. W. & Lam, J. S. (1996). Novel method for assessing postantibiotic effect by using the Coulter counter. *Antimicrobial Agents and Chemotherapy* **40**, 1751–3.
18. Weihaun, F. (1996). A novel fluorometric method for evaluation of the postantibiotic effect of antibacterial drugs on mastitis-causing *Staphylococcus aureus* and *Escherichia coli*. *Journal of Microbiological Methods* **26**, 151–9.
19. Li, R. C., Nix, D. E. & Schentag, J. J. (1993). New turbidimetric assay for quantitation of viable bacterial densities. *Antimicrobial Agents and Chemotherapy* **37**, 371–4.
20. Ravizzola, G., Caruso, A., Manca, N., Savoldi, E. & Turano, A. (1983). In-vitro activity of cefotetan and other cephalosporins on *Klebsiella* and resistance to inactivating bacterial enzymes. *Journal of Antimicrobial Chemotherapy* **11**, Suppl. A, 133–8.
21. Rescott, D. L., Nix, D. E., Holden, P. & Schentag, J. J. (1988). Comparison of two methods for determining in vitro postantibiotic effects of three antibiotics on *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **32**, 450–3.
22. Hošťacká, A. & Karellová, E. (1997). Outer membrane proteins and elastase of *Pseudomonas aeruginosa* after the postantibiotic effect induced by amikacin. *Chemotherapy* **43**, 118–22.
23. Odenholt-Törnqvist, I. (1993). Studies on the postantibiotic effect and the postantibiotic sub-MIC effect of meropenem. *Journal of Antimicrobial Chemotherapy* **31**, 881–92.
24. National Committee for Clinical Laboratory Standards. (1993). *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically—Third Edition: Approved Standard M7-A3*. NCCLS, Villanova, PA.
25. Gilbert, D. N. (1997). Meta-analyses are no longer required for determining the efficacy of single daily dosing of aminoglycosides. *Clinical Infectious Diseases* **24**, 816–9.
26. Bergan, T., Carlsen, I. B. & Fuglesang, J. E. (1980). An in vitro model for monitoring bacterial responses to antibiotic agents under simulated in vivo conditions. *Infection* **8**, Suppl. 1, S96–S102.
27. Meng, X., Nightingale, C. H. & Sweeney, K. R. (1991). Quantification of in-vitro post-antibiotic effect based on the mean recovery-time. I: Theoretical perspectives and a practical procedure. *Journal of Antimicrobial Chemotherapy* **28**, 505–14.
28. Meng, X., Nightingale, C. H. & Sweeney, K. R. (1991). Quantification of in-vitro post-antibiotic effect based on the mean recovery-time. II: A comparison of colony counting versus photometric methods for determination of bacterial growth. *Journal of Antimicrobial Chemotherapy* **28**, 515–21.
29. Begot, C., Desnier, I., Daudin, J. D., Labadie, J. C. & Lebert, A. (1996). Recommendations for calculating growth parameters by optical density measurements. *Journal of Microbiological Methods* **25**, 225–32.
30. Gottfredsson, M., Erlendsdottir, H., Sigfússon, A. & Gudmundsson, S. (1998). Characteristics and dynamics of bacterial populations during postantibiotic effect determined by flow cytometry. *Antimicrobial Agents and Chemotherapy* **42**, 1005–11.

Received 5 January 2000; returned 2 May 2000; revised 3 August 2000; accepted 3 November 2000