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Meropenem/colistin synergy testing for multidrug-resistant *Acinetobacter baumannii* strains by a two-dimensional gradient technique applicable in routine microbiology

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Objectives: Precise assessment of potential therapeutic synergy, antagonism or indifference between antimicrobial agents currently depends on time-consuming and hard-to-standardize *in vitro* chequerboard titration methods. We here present a method based on a novel two-dimensional antibiotic gradient technique named Xact™.

Methods: We used a test comprising a combination of perpendicular gradients of meropenem and colistin in a single quadrant. We compared test outcomes with those obtained with classical chequerboard microbroth dilution testing in a study involving 27 unique strains of multidrug-resistant *Acinetobacter baumannii* from diverse origins.

Results: We were able to demonstrate 92% concordance between the new technology and classical chequerboard titration using the *A. baumannii* collection. Two strains could not be analysed by Xact™ due to their out-of-range MIC of meropenem (>128 mg/L).

Conclusions: The new test was shown to be diagnostically useful, easy to implement and less labour intensive than the classical method.

Keywords: antibiotic resistance, susceptibility testing, two-dimensional Etest

Introduction

Treatment of patients suffering from severe infections is impacted by the speed and accuracy of the assessment of the antimicrobial resistance profile of the causative agent.[1] Hence, the differentiation between susceptible and (multi)drug-resistant bacteria is of immediate clinical relevance. The current threat posed by multidrug resistance urges the development of laboratory methods that document the susceptibility of certain human bacterial pathogens to combinations of antibiotics. Combination therapy may delay the emergence of resistance, widen the spectrum of action of drug combinations and may also diminish the duration of treatment. Confronted with multidrug-resistant bacteria, physicians may ask clinical microbiologists to supply them with such synergy/antagonism testing data.

Whereas conventional, classical 'chequerboard synergy testing' does provide such information, this time-consuming and labour-intensive method cannot be easily performed on a daily routine basis.[2,3] The methods usually lack sufficient reproducibility and the results may be difficult to interpret.[4] This is especially evident when confronted with multidrug-resistant isolates of Gram-negative bacterial species such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa* or other non-fermentative organisms. In several cases, expensive clinical studies intended to show whether or not different drug combinations were synergistic failed to demonstrate such an effect.[5] Costs might have been much less if such studies could have been effectively performed in an *in vitro* setting. There is therefore a clear need for innovative technologies regarding synergism or antagonism testing for combinations of antimicrobial agents. New approaches have been proposed, e.g. Etest synergy assessment methods.[6,7] Pankey *et al.*[6] and Sopirala *et al.*[7] have shown that when different combinations and coordinations of Etests are used, some of these approaches compare favourably with classical broth dilution-based

chequerboard methods. However, the data presented cannot be considered conclusive at that stage, i.e. the stage at which people used two Etests to come to a result (since Etests were not designed to be used in combination, the 'double Etest approach' could only be considered indicative and certainly not final). A new format of the classical Etest, named Xact™, was described some years ago and here we present a validation of that technology in a routine clinical microbiology setting.[8–11]

Xact™ comprises a 50×50 mm plastic carrier immobilized with a gradient quadrant of two antibiotics of choice arranged in perpendicular alignment, thus giving a predefined concentration pattern with hundreds of unique drug ratios in 1 or 0.5 dilution increments. The carrier needs to be placed onto a bacteria-inoculated agar surface and left for 1 h to transfer the gradient imprint to the agar, after which the carrier is removed (see Figure 1). After overnight incubation, fractional inhibitory concentration indices (FICIs) are read along the inhibition isobole (see Figure 2).[12] Previously, Xact™ has been compared with chequerboard titration using bacterial isolates for several species that were successfully tested in numerous replicates.[9–11] The reproducibility of Xact™ isoboles and FICI values ($n=50$) was acceptable with a standard deviation equivalent to <25% variation from the mean FICI value. Xact™ appeared to be simple to use for the quantification of drug–drug interactions over a wide range of drug ratios and it was concluded that it deserved further investigation as a potential tool for combination testing. To date, such data are still lacking.

We here focused on a panel of multidrug-resistant *A. baumannii* clinical isolates and, using an Xact™ assay, tested them for potentially synergistic susceptibility to meropenem and colistin. We selected the combination between meropenem and colistin since this is one of the preferred therapeutic combinations against such strains. In addition, colistin resistance fortunately is still rare and in combination with a carbapenem such as meropenem therapeutic success is frequent.

Materials and methods

Strains

Escherichia coli ATCC 25922, *E. coli* Biodisk Culture Collection (BCC) 2, *Staphylococcus aureus* ATCC 29213, *Enterobacter cloacae* BCC 44/75 and

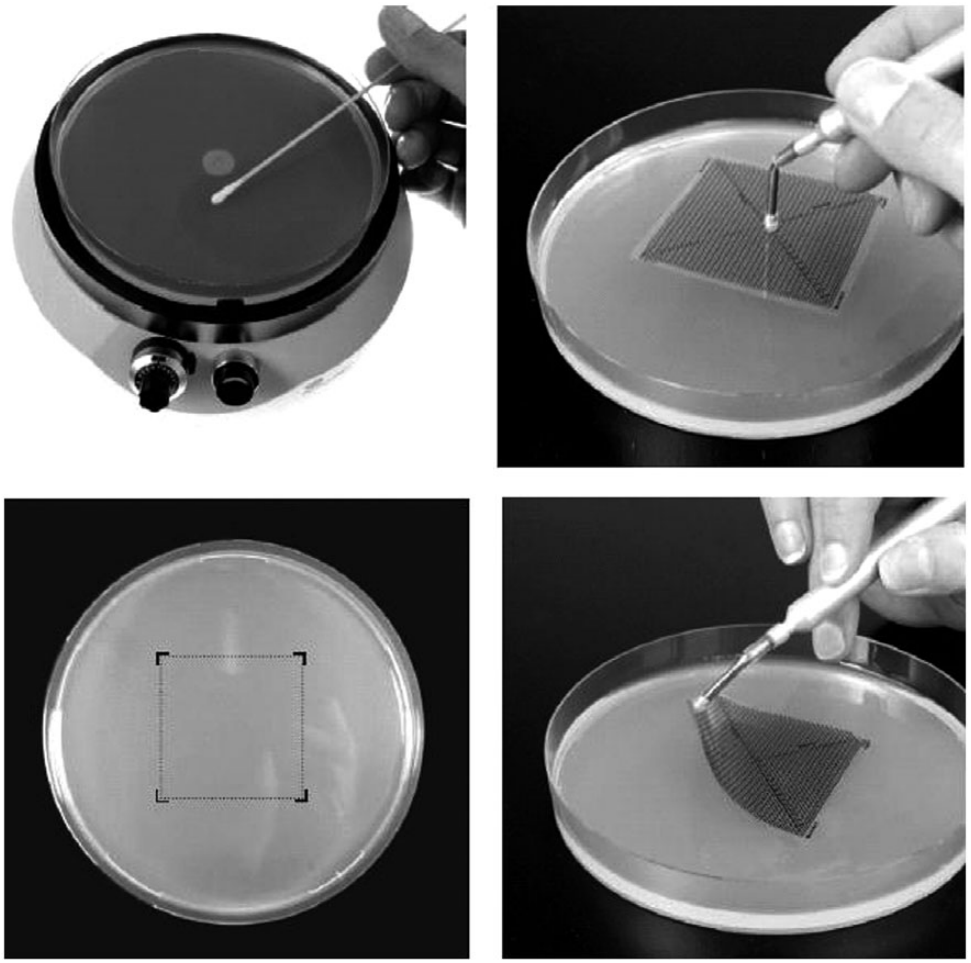

Figure 1. Xact™ procedure. (Top left) Inoculate: the agar surface is streaked with Retro C80™ and then dried completely. The same result can be achieved by flooding of the plate and drying afterwards. (Top right) Apply Xact™: the Xact™ quadrant is applied to the agar surface using the vacuum pen Nema C88™. (Bottom left) Mark Xact™ position: the position is marked on the back of the plate and left in place for 1 h. (Bottom right) Remove Xact™ and incubate: the quadrant is removed after 1 h and the plate incubated, usually at 37°C.

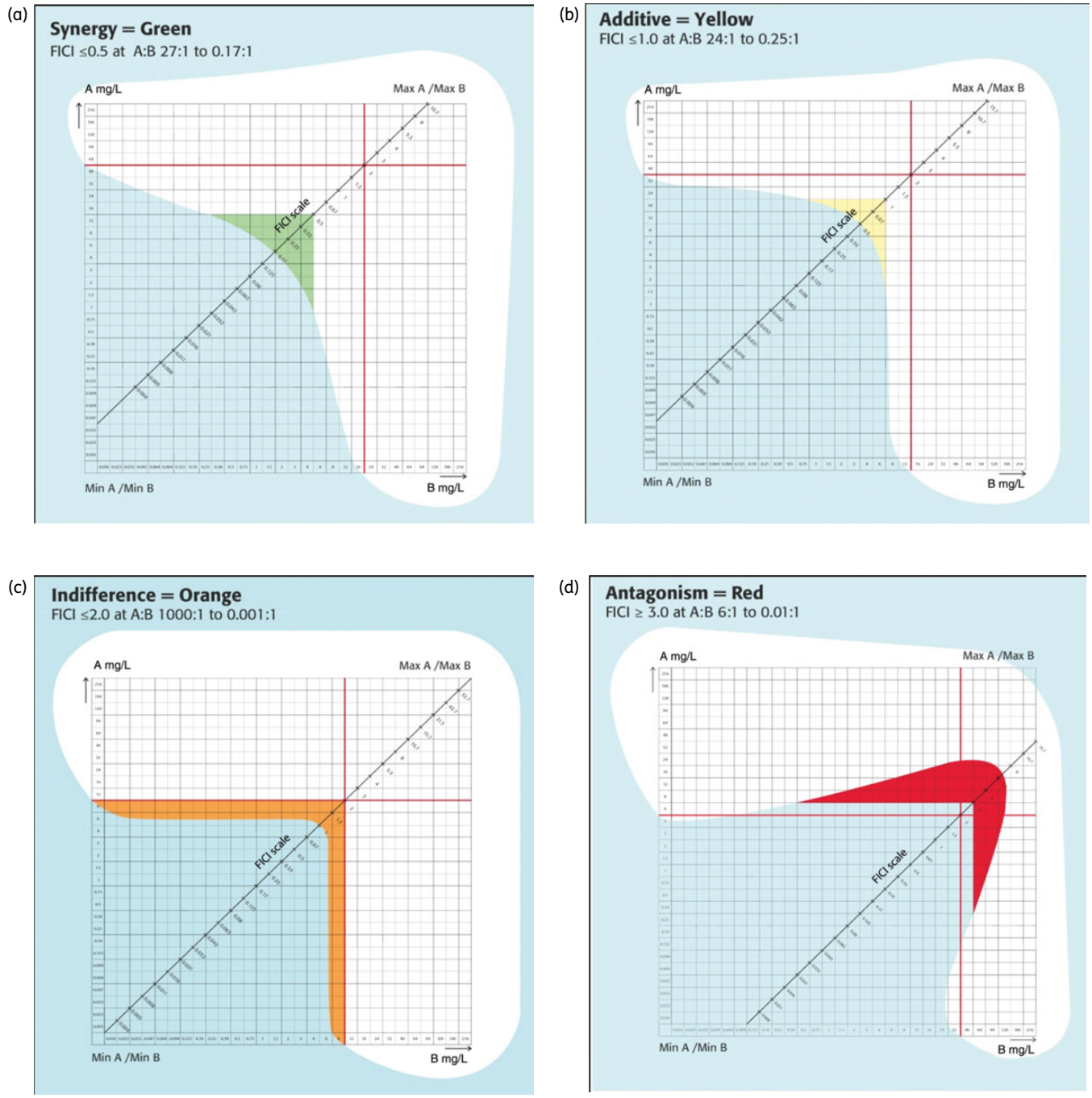

Figure 2. Design of the Xact™ combination gradient quadrant. The antibiotic gradients A and B are perpendicularly aligned. Each square equals 0.5 dilution, essentially leading to a total of 900 unique ratios of A+B. The diagonal 'Max A to Max B' gives a gradient ratio of A:B from 16000 (256/0.016) to 0.00006 (0.016/256). The diagonal 'Max A/Max B to Min A/Min B' equals a concentration gradient of A+B from 512 to 0.024 mg/L. The FICI scale is based on calculations of FICs according to the formula: FICI=FICA+FICB=A(B)/ICA+B(A)/ICB, where A(B) is the lowest concentration of A in the presence of B, and vice versa for B(A), and ICA is the concentration of A at ≤Min B, and vice versa for ICB. The scale can be transposed in a parallel direction to read the FICI of various inhibition isoboles at different interaction points. For examples of the isoboles see (a) to (d). (a) Xact™ synergy isobole (FICI ≤0.5). (b) Xact™ additive isobole (FICI >0.5 and ≤1.0). (c) Xact™ indifference isobole (FICI >1.0 and ≤2.0). (d) Xact™ antagonism isobole (FICI ≥3.0).

Staphylococcus epidermidis 1-1478 were used as control strains during the preliminary validation experiments.[9–11] The *A. baumannii* strains used in the present validation study (n=27) are described in Table 1. This table shows the results of the synergy testing by chequerboard titration and Xact™ testing. Single-plex PCR assays were used to detect the following β-lactamase genes: four carbapenem-hydrolysing oxacillinases (bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24} and bla_{OXA-58}), four metallo-β-lactamase genes (bla_{IMP}, bla_{VIM}, bla_{SPM} and bla_{NDM}), bla_{KPC} and bla_{GES}.

Table 1. Overview of the results obtained for chequerboard titrations and Xact™ analysis for 27 strains of *A. baumannii*

	Chequerboard			
No.	MIC CST (mg/L)	MIC MEM (mg/L)	synergy/additivity	Xact™
1	4	2	synergy	synergy
2	4	32	additivity	additivity
3	4	16	additivity	synergy
4	4	32	additivity	additivity
5	4	16	synergy	synergy
6	8	>128	MIC MEM >128 mg/L	synergy
7	4	64	synergy	synergy
8	1	16	additivity	additivity
9	4	32	synergy	additivity
10	2	32	synergy	synergy
11	4	32	synergy	synergy
12	8	32	synergy	synergy
13	4	32	additivity	additivity
14	4	16	synergy	synergy
15	2	8	synergy	synergy
16	8	32	synergy	synergy
17	4	32	synergy	synergy
18	2	4	additivity	additivity
19	2	8	synergy	synergy
20	2	32	synergy	synergy
21	2	8	synergy	synergy
22	4	16	synergy	synergy
23	4	16	additivity	additivity
24	4	64	synergy	synergy
25	8	16	additivity	additivity
26	2	16	synergy	synergy
27	4	>128	MIC MEM >128 mg/L	synergy

CST, colistin; MEM, meropenem.
Strains are all epidemiologically independent. Columns indicate strain number, MICs obtained by chequerboard titration and the results of the synergy assays. Note that in two cases, results were non-interpretable due to the elevated MIC of meropenem (>128 mg/L).

Antibiotics and configuration

For susceptibility testing of the *A. baumannii* strains, concentrations of colistin and meropenem were applied in the Xact™ format that equalled the dilutions used in the chequerboard titrations in 2-fold dilutions in Mueller–Hinton broth in 96-well microtitre trays. Different lots of Xact™ quadrants were used. The quadrants were stored at −20°C in the presence of desiccant.

Procedure for the chequerboard titration

Chequerboard was performed *in duplo* using 10^6 cfu/mL in Mueller–Hinton broth (BBL). The MICs of meropenem, colistin and meropenem plus colistin at various ratios were read and the FICI calculated. Briefly, per round-bottomed 96-well ELISA plate, a single strain was tested and the inoculum was set at 10^6 cfu/mL in Mueller–Hinton broth. The core antibiotic stock solutions were set at 11 mg/mL for colistin and at 50 mg/mL for meropenem. Per well, 50 μL of the two antibiotic solutions each was mixed with 100 μL of the strain suspension. After 24 h at 37°C, plates were read and the last wells without growth were sampled to define the true negative cultures.

Xact™ procedure

Mueller–Hinton (BBL) agar plates (150 mm) were flooded or streaked with a bacterial inoculum (a 10× diluted 0.5 McFarland suspension) and dried completely at 35°C for 15 min. The Xact™ quadrant was applied to the agar surface while its exact position was marked on the back of the plate (Figure 1). The quadrant was removed after 1 h. The plate was incubated at 35°C for 16–20 h. The effect of inoculum variation (~10^5–10^9 cfu/mL) was studied with *E. coli* ATCC 25922. Digital pictures of the results were transferred to a computer and interpreted using a reading grid. Then, the demarcation zone of the isobole could be defined by clicking on the screen. The software then automatically calculates the various FICIs for the respective drug ratios. The program also provides a table surveying all results as well as a graph showing FICI versus the ratios of the two drugs used. Plates were also read manually and independently by two technicians with a grid placed on the back of the plate. Inhibition zones provide information on synergistic, additive or antagonistic effects along various concentration ratios of antimicrobials A and B, respectively. The inhibition curves for meropenem and colistin were read where the inhibition curvature intersected the outer edge of the grid (Figure 2). The FICI was read where the inhibition isobologram intersected the diagonal FICI scale with 2.0 on the scale positioned on the inhibition curve/inhibition

curve intersection point (Figure 2). Sometimes, manual reading using a ruler was preferred for the sake of convenience. The FICI at different antibiotic ratios was read by transposing the FICI scale in a parallel direction. The area of the inhibition isobole that fell in various FICI interpretive sections showed the different types of interactions that occurred at different meropenem/colistin ratios.

Interpretative definitions

Arbitrary definitions were defined—before experiments—as follows: when the FICI was $\leq$0.5 the antibiotic combination was considered to be synergistic; when the FICI was >0.5 and $\leq$1.0 the effect of the two antibiotics was considered to be additive; when the FICI was >1.0 and $\leq$2.0 the antibiotic interaction was indifferent; and, finally, when the FICI was $\geq$3.0 the antibiotic combination was defined as antagonistic.[13] When groups of strains were compared, the two-sided Fisher exact test was used and $P<0.05$ was considered to be significant.

Results

Control assays with the dedicated strains indicated that both antibiotics were present in an active form. Previous analyses of inoculum effects on the Xact™ FICI as measured for *E. coli* ATCC 25922 revealed that within a range between a 1:100 dilution of a 0.5 McFarland and a 5 McFarland suspension there was consistent measurement of both the inhibition curve and the FICI. It was concluded that at the level of Xact™, the inoculum effect was negligible. Similar results were obtained previously with different combinations of antibiotics.[9–11]

This work examined 27 multidrug-resistant *A. baumannii* strains, most of them isolated from rectal swabs and all collected between 2006 and 2013 in the Hôpital Universitaire de Geneve. All study isolates were resistant to amikacin, ciprofloxacin, ceftazidime and piperacillin/tazobactam. All strains were positive for $bla_{\text{OXA-51}}$, 13/27 strains carried $bla_{\text{OXA-23}}$, 2/27 strains carried a $bla_{\text{OXA-24-like}}$ gene and 4/27 strains had a $bla_{\text{OXA-58}}$ gene. GES-type carbapenemases were found in three strains. A strain harbouring NDM-1 was identified from two patients. KPC, VIM, IMP and SPM assays were uniformly negative. The MIC of imipenem ranged from 0.38 to 256 mg/L. Of note, the presence of any of these carbapenemase-encoding genes did not correlate with whether a strain's phenotype would show synergism or not. The MIC of meropenem ranged from 0.5 to 256 mg/L. Twenty-six out of 27 isolates were resistant according to EUCAST clinical breakpoints as published for 2014. All study isolates were susceptible to colistin. The MIC of colistin ranged from 0.064 to 0.5 mg/L.

Chequerboard titration and Xact™ testing revealed concordant synergism in 16/25 strains (64%), with two strains being non-testable due to an elevated meropenem MIC. In two strains, discordant results were obtained. Finally, 7/25 (28%) strains were reported as additive in perfect concordance by the two methods. Overall, 23/25 isolates (92%) for which both synergy tests were performed successfully showed full concordance in the comparison between the gold standard method and Xact™.

Discussion

It has been shown by comparative *in vitro* testing for carbapenem-resistant *A. baumannii* isolates with multiple resistance traits that synergy between imipenem, amikacin, tobramycin and colistin could be demonstrated for significant percentages of isolates but not for all.[14–16] The analyses were performed by chequerboard titration and time–kill assays and it was concluded that synergy testing, though technically challenging, might lead to better selection of more adequate therapy. Fortunately, besides the chequerboard titration methods for assessing synergistic activities for antibiotic combinations, simpler alternatives have been proposed over the past years. Still, these methods all share two big shortcomings: first, they only provide information for a fixed antibiotic mixture; and, second, they usually require the very precise positioning of discs or strips that contain fixed amounts of gradients of antibiotics. This is usually cumbersome and non-precise and alternatives are still being sought. We here show that according to chequerboard reference data, the newly developed Xact™ test showed 92% identity with the gold standard assay, a very good level of concordance for testing of *Acinetobacter* strains. In addition, underscoring the relevance of the Xact™ format, not all strains belonged to a single category of isolates, showing the relevant specificity of the method. However, there was not a single example of antagonism and if this can be verified in more extensive studies, then this would suggest that a combination of colistin and meropenem would be a good antibiotic therapy to start with for treatment of infections caused by multidrug-resistant *Acinetobacter*. In support of this suggestion, it has been demonstrated that combination therapy with colistin and carbapenems is among the most successful known to date.[17] Finally, the concentrations where synergy was observed were deemed quite compatible with the concentration range across which these antibiotics can be used in patients. However, in the case of the combination of colistin and meropenem the Xact™ format does not allow for the precise assessment of MICs of the individual antibiotics due to interference between the compounds. We have preliminary evidence that the method also works for *Burkholderia* spp., *P. aeruginosa*, MSSA, MRSA and a variety of other bacterial and even yeast species (A. van Belkum, D. Halimi, E.-J. Bonetti, G. Renzi, A. Cherkaoui, V. Sauvonnet, R. Martelin, G. Durand, S. Chatellier, G. Zombardi, A. Engelhardt, Å. Karlsson and J. Schrenzel, unpublished results).[18] The Xact™ method is easy to set up and the data generated were convenient to analyse. The continuous and stable gradient generated high precision levels and adequate reproducibility. The test is inherently high throughput since drug–drug interactions could be studied across 225 or 900 drug ratios in 1 or 0.5 dilution increments. One disadvantage is that it will remain unclear whether the effect seen in the growth curves is due to bacteriostatic or bactericidal activity, a feature important in the therapy of multidrug-resistant organisms as a whole.

In comparison with chequerboard titration, Xact™ allowed us to perform three times more analyses during a similar amount of working time, not even including the preparation time for the plates. The assay offered clear potential and added value for routine antibiotic synergy testing and deserves further investigation for combination testing of various drugs and organisms in different configurations. Also, the interaction between antibiotics and enzyme inhibitors (e.g. β-lactams and β-lactamase inhibitors) or antibiotics and antifungals can be subjected to Xact™-mediated analysis, although this will still require a detailed analysis of the shelf-life of the products. Optimization of the assays can be pursued via intelligent imaging and automation. Finally, the current format offers clear opportunities for the assessment of

interactions between antibiotics and other medications including anticancer drugs or cardiac medication.

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Transparency declarations

Xact™ is a trademark; both the product and the technology have been patented by AB BIODISK, Sweden. AB BIODISK was acquired by bioMérieux SA in June 2008.

A. v. B., D. H., V. S., R. M., G. D., S. C., G. Z., A. E. and A. K. are employees of bioMérieux, the company currently holding the rights to the development of Xact™ into a product. All other authors: none to declare.

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