ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2009, p. 4809–4815 Vol. 53, No. 11
0066-4804/09/$12.00 doi:10.1128/AAC.00269-09

Comparison of Methods To Test Antibiotic Combinations against Heterogeneous Populations of Multiresistant *Pseudomonas aeruginosa* from Patients with Acute Infective Exacerbations in Cystic Fibrosis▽

Juliet E. Foweraker,[1]* Christian R. Laughton,[1] Derek F. Brown,[2] and Diana Bilton[1]†

Department of Microbiology, Papworth Hospital, Papworth Everard, Cambridge CB3 8RE, United Kingdom,[1] *and Clinical Microbiology and Public Health Laboratory, Addenbrookes Hospital, Cambridge CB2 2QW, United Kingdom*[2]

Received 26 February 2009/Returned for modification 18 May 2009/Accepted 14 August 2009

Multiresistant *Pseudomonas aeruginosa* isolates can chronically infect patients with cystic fibrosis. Acute infective exacerbations are treated with combinations of two antipseudomonal antibiotics. Patients may respond clinically even if the bacteria are resistant, possibly due to antimicrobial synergy. The challenge for testing for synergy in vitro is that there is no standardized method, and the antibiotic susceptibility in a population of *P. aeruginosa* isolates in a single sputum sample can vary. We therefore compared (i) antibiotic combinations with different examples of resistant bacteria from the same sputum sample and (ii) the results of synergy testing by different methods. Antibiotic synergy was tested by using resistant *P. aeruginosa* isolates recovered from sputum samples taken just before the start of treatment for an acute infective exacerbation. Several examples of each morphotype of *P. aeruginosa* were tested by cidal checkerboard, time-kill curve, and multiple-combination bactericidal testing. The isolates were typed by pulsed-field gel electrophoresis (PFGE). The results were compared with the clinical and microbiological responses to 14 days of antibiotic treatment. Forty-four resistant isolates from nine patients were tested. Some *P. aeruginosa* isolates with the same morphotype and PFGE pulsotype had different results by synergy testing. There was a poor correlation between the results of the different methods of synergy testing, and no one method would have predicted the response to treatment in all patients. The in vitro effects of antibiotic combinations against different isolates from the same sputum sample can vary, and the results depend on the methodology used. The role of combination testing for the treatment of antibiotic-resistant *P. aeruginosa* in acute exacerbations of chronic infection in patients with cystic fibrosis needs to be reviewed.

Cystic fibrosis (CF) is an inherited condition that changes the function of the CF transmembrane regulator, leading to abnormal salt and water transport across the secretory epithelia. The resulting reduction in the volume of the periciliary layer of the airway epithelia leads to poor ciliary function and mucus plugging of the airways. Patients initially develop infections with *Staphylococcus aureus* and common respiratory pathogens. With time, chronic infections develop, and these are most commonly due to *Pseudomonas aeruginosa*. The patient with a chronic infection periodically suffers from acute exacerbations, marked by a sharp decline in lung function and an increase in purulent sputum.

Early infection with *P. aeruginosa* in patients with CF is treated with antibiotics to try to eradicate the bacteria. Once chronic infection with *P. aeruginosa* is established, lung function decreases with repeated acute exacerbations, and the aim of treatment is to reduce lung damage (4). The usual markers of acute respiratory infection, such as a rise in the C-reactive protein concentration, pyrexia, and an increase in the numbers of peripheral blood neutrophils, may not be seen in exacerbations of chronic infection in CF patients; and the response to treatment is often measured by the improvement in lung function or the time to the next acute exacerbation.

Intravenous antibiotics are usually given for 2 weeks to treat acute exacerbations, with the commonest combination being an antibiotic acting on the bacterial cell wall (e.g., ceftazidime or meropenem) plus an aminoglycoside, such as tobramycin. The use of two antipseudomonal antibiotics has been recommended not only to discourage the emergence of resistance (11, 17) but also with the hope that they may act synergistically, i.e., that each antibiotic would reduce the concentration of the other needed to inhibit or kill the bacteria (10). The combination of an aminoglycoside with a β-lactam in clinical practice may not, however, prevent the emergence of *P. aeruginosa* resistant through the derepression of AmpC production (18). Multiresistant strains of *P. aeruginosa* are becoming more prevalent, in particular in patients with CF (32). They limit the options for the treatment of acute exacerbations and may be associated with a faster decline in lung function (26). The use of synergy testing to identify drug combinations that may be clinically effective for multidrug-resistant organisms has been proposed, and some reference laboratories provide a synergy testing service using the checkerboard titration or the multiple-combination bactericidal test (MCBT) (35).

There are no animal studies in CF that compare the results of synergy testing by any method with the effectiveness of treatment of *P. aeruginosa* infection, and there has been only one significant human trial, and that used the MCBT (2).

We recently performed a detailed study of antibiotic treat-

* Corresponding author. Mailing address: Department of Microbiology, Papworth Hospital, Papworth Everard, Cambridge CB3 8RE, United Kingdom. Phone: 44 1480 830541. Fax: 44 1480 364780. E-mail: juliet.foweraker@papworth.nhs.uk.

† Present address: Department of Cystic Fibrosis, Royal Brompton Hospital, London SW3 6NP, United Kingdom.

▽ Published ahead of print on 24 August 2009.

ment of acute infective exacerbation in CF patients chronically infected with *P. aeruginosa*. We tested each preexacerbation sputum sample extensively and showed a wide variability in susceptibility between isolates of *P. aeruginosa* from the same sputum sample, including differences between bacteria with the same colonial appearance (morphotype) (15). We found resistant isolates that had been missed by the use of standard methods in the hospital laboratory and identified patients harboring isolates resistant to both of the antibiotics used for treatment; some of these patients had responded to 14 days of intravenous antibiotics, and some had not.

In this study, we used the *P. aeruginosa* isolates from patients with carefully characterized exacerbations and retrospectively tested for synergy, comparing different methods (the time-kill [TK] curve, cidal checkerboard, and MCBT methods). As we had shown a wide variability in susceptibility to single antibiotics between isolates of *P. aeruginosa* from the same sputum sample, we investigated whether different bacteria from the same sputum sample responded differently to antibiotic combinations in vitro.

(Preliminary research findings were presented at the 2006 North American Cystic Fibrosis Conference, Denver, CO.)

MATERIALS AND METHODS

Patients. This study had ethics approval. The *P. aeruginosa* isolates were cultured from adult patients at Papworth Hospital, Cambridge, United Kingdom, who had CF and chronic infection with *P. aeruginosa* (defined as greater than 10^5 *P. aeruginosa* cells per ml sputum, cultured on at least three occasions in the previous year). Strict criteria were used to define an acute exacerbation of infection (16), and sputum was obtained for culture just before the start of intravenous antibiotic treatment. Two antipseudomonal antibiotics were chosen according to the local protocol, which took into account standard recommended combinations, the patient's allergy history, and what had successfully treated a previous exacerbation. The patients were regularly reviewed, and as is common in CF practice, if the clinical laboratory identified resistance in vitro, the antibiotics were changed only if the patient was not responding to treatment. Antibiotic combinations were tested retrospectively and therefore had no impact on the treatment decisions made during the clinical study.

The primary clinical response to 14 days of antibiotic treatment was measured by the change in lung function by using the following criteria for the forced expiratory volume in 1 s (FEV_1). The response was considered to be good when FEV_1 was restored to greater than 95% of the stable baseline level (measured at the annual review). The response was considered to be poor when FEV_1 was less than 95% of the baseline level. A bacteriological response was measured by comparing the number of bacteria in a sputum sample taken just before treatment with the number at day 14 (see "Microbiological materials and methods"). A change in the numbers of CFU equal to or more than 10-fold was considered significant.

Microbiological materials and methods. (i) Materials. All liquid and solid bacteriology media for single and combination antibiotic testing were obtained from Oxoid, Basingstoke, United Kingdom. Standard 96-well round-bottom microtiter trays were supplied by Triple Red Laboratory Technology, United Kingdom.

The following antibiotics were provided by the indicated suppliers: aztreonam, Bristol-Myers Squibb, Princeton, NJ; ceftazidime, GlaxoSmithKline, Barnard Castle, United Kingdom; meropenem, Astra Zeneca, Wilmington, DE; piperacillin-tazobactam, Wyeth Manufacturing, Havant, United Kingdom; and tobramycin, Sigma Aldrich, Poole, United Kingdom.

(ii) Sputum culture. Sputum was homogenized and cultured by standard methods, as described previously (15). The numbers of CFU were measured with a spiral plater (Don Whitley Scientific, Shipley, United Kingdom).

The total viable counts (in CFU) of *P. aeruginosa* were calculated from the spiral plates by using a Sorcerer automated colony counter (Perceptive Instruments, Haverhill, United Kingdom). Sputum was also cultured on Iso-Sensitest agar containing each of the two antibiotics used for treatment. The antibiotics were used at the British Society for Antimicrobial Chemotherapy (BSAC) breakpoint concentrations in order to detect resistant subpopulations. *P. aeruginosa* was identified by phenotypic and genotypic methods, and different morphotypes were distinguished by colonial size, texture (including mucoidity), and pigmentation (15).

(iii) Disk diffusion susceptibility testing. The bacteria were subcultured from nonselective agar and antibiotic-incorporated agar. Four colonies of each morphotype of *P. aeruginosa* were individually tested for their susceptibilities to 12 antipseudomonal antibiotics by the BSAC standardized disk diffusion method (www.bsac.org.uk). Zone diameters were measured with electronic calipers (Bowers Metrology, West Drayton, United Kingdom). Isolates resistant to both antibiotics used for treatment were stored at −80°C in a cryopreservative (15% glycerol broth). They were later revived and tested for their susceptibilities to each antibiotic alone (by determination of the MIC and minimum bactericidal concentration [MBC] by the broth microdilution method) and to the antibiotic combinations.

(iv) Broth microdilution MIC and MBC determination. MICs and MBCs were measured by the broth microdilution method (3) with Iso-Sensitest broth (ISB) in microtiter wells and a final inoculum of 5×10^5 CFU/ml. The bacterial count was verified by culturing serial dilutions of the final suspension onto blood agar (BA) in triplicate. Wells with no visible growth at 24 h were subcultured onto BA, incubated at 37°C for up to 40 h, and examined for 99.9% killing of the original inoculum. The BSAC MIC breakpoints for resistance were as follows: ceftazidime, >8 mg/liter; aztreonam, >8 mg/liter; piperacillin-tazobactam, >16/2 mg/liter; meropenem, >8 mg/liter (intermediate, 4 to 8 mg/liter); and tobramycin, >4 mg/liter.

(v) Broth microdilution bactericidal checkerboard method. The broth microdilution bactericidal checkerboard method was used as described previously (31). In brief, all combinations of eight twofold dilutions of the two antibiotics were dispensed into a microtiter tray in a checkerboard fashion. Growth and sterility controls were included in all plates. A bacterial inoculum of 5×10^5 CFU/ml was used, and the count was verified. Microtiter trays were incubated at 37°C, and wells with no visible growth at 24 h were subcultured onto BA at 37°C for up to 40 h and examined for 99.9% killing of the original inoculum. The fractional bactericidal concentration index (ΣFBCI) of each antibiotic was calculated. Synergy was defined as an ΣFBCI of ≤0.5, antagonism was defined as an ΣFBCI of >4, and indifference was defined as an ΣFBCI of >0.5 but ≤4.

(vi) MCBT. MCBT was performed as described previously (25). In brief, each antibiotic combination was tested (by using a single concentration of each antibiotic) and compared with the activity of each antibiotic alone. All testing was done in a microtiter tray. A final inoculum of 5×10^5 CFU/ml was used, and the count was verified in triplicate. Growth and sterility control wells were included in all plates. The microtiter trays were incubated at 37°C for 48 h, and each well with no visible growth was subcultured to establish whether 99.9% killing was achieved. An MCBT was scored as positive if the bacteria were killed by the combination or by a single antibiotic (as in the published method). The antibiotic concentration used in the published MCBT method was the peak level measured in blood after a single intravenous dose. As this is higher than would be expected in the lung, we also tested concentrations of antibiotics at the BSAC breakpoint. The original method is referred to as the "high-concentration MCBT," and the modification is referred to as "the low-concentration MCBT." The antibiotic concentrations were as follows for the high- and low-concentration MCBTs: for ceftazidime, 32 and 8 mg/liter, respectively; for aztreonam, 32 and 8 mg/liter, respectively; for piperacillin-tazobactam, 32/4 and 16/2 mg/liter, respectively; for meropenem, 32 and 4 mg/liter, respectively; and for tobramycin, 4 mg/liter for both the high- and the low-concentration MCBTs.

(vii) TK curves. TK studies were performed as described previously (31) by testing each antibiotic alone and the two antibiotics in combination with each antibiotic at 0.25× MIC. Tubes containing each antibiotic at twice the MIC and broth without antibiotic were included as controls. The bacteria were grown overnight in ISB and diluted in 10 ml ISB to achieve a final inoculum of 1×10^6/ml. Broths were cultured at 37°C in a shaking incubator at 140 rpm. Aliquots of 100 μl were taken after 8, 12, and 24 h of culture and serially diluted in sterile distilled water; and three 20-μl volumes from each dilution were immediately inoculated onto BA plates. These were incubated at 37°C for up to 40 h, and the colonies were counted. Killing curves were drawn by plotting the $\log_{10}$ CFU/ml against time. Synergy was defined as a $\geq 10^2$-CFU/ml reduction in the bacterial numbers with the antibiotic combination compared with the numbers achieved with the most active single antibiotic plus a reduction of the original inoculum of $\geq 10^2$ CFU/ml.

(viii) Typing by PFGE. Isolates of *P. aeruginosa* from the same sputum sample with different synergy results were typed by pulsed-field gel electrophoresis (PFGE) (24). The restriction fragment patterns were used to calculate the Dice coefficient of similarity for each isolate. Isolates with similarity coefficients greater than 85% were considered indistinguishable.

TABLE 1. Activities of single antibiotics (MICs and MBCs) and antibiotic combinations (MCBT, checkerboard, or TK curve methodology) against isolates of *P. aeruginosa*

Patient no.	*P. aeruginosa* isolate[a]	Activity of treatment antibiotics[b]						Combination bactericidal activity (MCBT)[c]		Result of combination synergy testing	
		Antibiotic 1 in the combination			Antibiotic 2 in the combination						
		Agent	MIC (mg/liter)	MBC (mg/liter)	Agent	MIC (mg/liter)	MBC (mg/liter)	High	Low	Checkerboard result	TK curve analysis result at 24 h
1	1	ATM	128	>256	TOB	8	16	Yes	No	Synergy	Indifference
2	1a	ATM	32	64	TOB	8	16	Yes	Yes	Indifference	Indifference
	1b	ATM	64	128	TOB	16	16	No	No	Synergy	Synergy
3	1	CAZ	16	64	TOB	8	32	No	No	Synergy	Indifference
	2	CAZ	16	64	TOB	8	32	Yes	No	Synergy	Indifference
	3	CAZ	16	64	TOB	8	16	No	No	Synergy	Indifference
4	1a	CAZ	32	32	TOB	8	8	Yes	Yes	Indifference	Indifference
	1b	CAZ	8	32	TOB	4	4	Yes	Yes	Synergy	Indifference
	1c	CAZ	8	8	TOB	4	8	Yes	Yes	Synergy	Indifference
5	1	MEM	8	16	TOB	4	4	Yes	No	Indifference	Indifference
6	1a	MEM	8	16	TOB	16	16	Yes	No	Synergy	Indifference
	1b	MEM	8	16	TOB	32	32	Yes	No	Indifference	Indifference
	2	MEM	8	16	TOB	8	16	Yes	No	Indifference	Indifference
	3a	MEM	32	32	TOB	32	32	Yes	No	Indifference	Synergy
	3b	MEM	16	32	TOB	32	32	Yes	No	Synergy	Synergy
7	1	TZP	256	128	TOB	32	32	No	No	Indifference	Indifference
	2	TZP	>256	64	TOB	8	16	No	No	Indifference	Indifference
8	1	CAZ	128	>256	TZP	>256	>256	No	No	Indifference	Not tested
9	1	CAZ	32	>128	TOB	4	8	Yes	No	Indifference	Indifference
	2a	CAZ	32	>128	TOB	4	8	Yes	Yes	Indifference	Indifference
	2b	CAZ	32	>128	TOB	8	16	No	No	Indifference	Indifference
	3	CAZ	32	>128	TOB	8	16	No	No	Synergy	Indifference

[a] The number refers to the morphotype, and the letter (a, b, or c) refers to the different isolates of that morphotype.
[b] ATM, aztreonam; CAZ, ceftazidime; MEM, meropenem; TOB, tobramycin; TZP, piperacillin-tazobactam.
[c] The result of combination bactericidal activity are yes, killed by the combination or a high concentration of a single antibiotic, and no, not killed. The concentrations used for the high-concentration MCBT were 32 mg/liter for aztreonam, 32 mg/liter for ceftazidime, 32 mg/liter for meropenem, 4 mg/liter for tobramycin, and 32/4 mg/liter for piperacillin-tazobactam; and those used for the low-concentration MCBT were 8 mg/liter for aztreonam, 8 mg/liter for ceftazidime, 4 mg/liter for meropenem, 4 mg/liter for tobramycin, and 16/2 mg/liter for piperacillin-tazobactam.

RESULTS

We identified isolates of *P. aeruginosa* resistant to both antibiotics used for treatment in nine patients. A mean of three to four morphotypes of *P. aeruginosa* were found in the sputum sample from each of these patients, and on average 14 isolates were picked from each sample for testing for their antimicrobial susceptibilities by the disk diffusion method. Although isolates with different morphotypes often had the same antibiotic susceptibility pattern, bacteria with the same morphotype often had different antibiograms, as already described (15).

A total of 128 susceptible and resistant isolates from these patients were then stored at −80°C for later analysis. For one patient (patient 9), only susceptible bacteria were picked from the standard agar plates, but *P. aeruginosa* grew from sputum plated directly onto antibiotic-incorporated agar; up to four isolates of each morphotype (a total of 21) were also stored at −80°C for further work.

Thirty-five of the 128 stored isolates from the standard plates were resistant by disk diffusion to both antibiotics used for treatment. The MICs and MBCs for these isolates were then measured by broth microdilution. The 21 isolates cultured from antibiotic-containing agar grew too slowly for disk diffusion testing, and therefore, disk diffusion could not be used as a screening test. Susceptibility was tested just by measuring the MICs and the MBCs.

P. aeruginosa isolates resistant to the two treatment antibiotics were mixed with susceptible *P. aeruginosa* isolates in eight patients and with an antibiotic-susceptible strain of *Achromobacter xylosoxidans* in one patient (patient 8).

Four of the 35 *P. aeruginosa* isolates resistant by disk diffusion and 8 of the 21 isolates recovered from antibiotic-containing agar had a broth microdilution MIC more than 1 $\log_2$ below the breakpoint and were excluded from further analysis. The remaining 44 resistant and borderline isolates were then tested for their susceptibilities to antibiotic combinations.

Isolates of *P. aeruginosa* from an individual patient were differentiated by consideration of all the susceptibility results, irrespective of the colonial morphotype, as bacteria with the same morphotype may have different antibiotic susceptibilities. One isolate was considered to be distinct from another isolate from the same patient if the MIC or the MBC differed by ≥2 $\log_2$ and/or if any of the synergy test results differed. By these criteria there were 1 to 4 isolates from each patient with distinct patterns of susceptibility, giving a total of 22 unique isolates. The results for these isolates are shown in Table 1.

Synergy was not tested by TK curve analysis with the *P. aeruginosa* isolates from patient 8. The MICs of both antibiotics were so high that even if we had showed synergy at 0.25× MIC, those antibiotic levels would not have been achievable in the patient.

The high-concentration MCBT included single antibiotics at concentrations that in some cases were higher than the MBC

TABLE 2. Summary of results for each patient, giving the size of the resistant subpopulation and a comparison of results of antibiotic combination testing with the clinical and bacterial response to antibiotics[a]

Patient no.	Antibiotics used for treatment[b]	*P. aeruginosa* count (CFU/g) at day 0		MCBT[c]		Checkerboard result	TK curve analysis result	Response to treatment[d]	
		Total	Resistant	High	Low			FEV_1	CFU/g sputum
1	TOB + ATM	6.3×10^7	1.1×10^7	Yes	No	Synergy	Indifference	Not valid[e]	Not valid
2	TOB + ATM	2.2×10^8	4.4×10^7	No	No	Indifference	Indifference	Yes	No change
3	TOB + CAZ	1.0×10^8	2.3×10^7	No	No	Synergy	Indifference	No	No change
4	TOB + CAZ	7.1×10^8	1.9×10^8	Yes	Yes	Indifference	Indifference	Yes	No change
5	TOB + MEM	4.0×10^8	1.0×10^8	Yes	No	Indifference	Indifference	Yes	No change
6	TOB + MEM	4.2×10^7	4.1×10^7	Yes	No	Indifference	Indifference	Not done[f]	No change
7	TOB + TZP	1.8×10^8	7.0×10^7	No	No	Indifference	Indifference	Not valid	Not valid
8[g]	CAZ + TZP	2.0×10^8	1.7×10^7	No	No	Indifference	Not tested	No	No change
9	TOB + CAZ	3.0×10^8	6.0×10^6	No	No	Indifference	Indifference	Yes	No change

[a] For the results the most resistant isolate (i.e., the organism least affected by the antibiotic combinations) is quoted.
[b] TOB, tobramycin; ATM, aztreonam; CAZ, ceftazidime; MEM, meropenem; TZP, piperacillin-tazobactam.
[c] The results of combination bactericidal activity are yes, killed by the combination or a high concentration of a single antibiotic, and no, not killed. The concentrations used for the high-concentration MCBT were 32 mg/liter for aztreonam, 32 mg/liter for ceftazidime, 32 mg/liter for meropenem, 4 mg/liter for tobramycin, and 32/4 mg/liter for piperacillin-tazobactam; and those used for the low-concentration MCBT were 8 mg/liter for aztreonam, 8 mg/liter for ceftazidime, 4 mg/liter for meropenem, 4 mg/liter for tobramycin, and 16/2 mg/liter for piperacillin-tazobactam.
[d] The response is that after 14 days of antibiotic treatment. See Materials and Methods for the full definition of the clinical and microbiological responses to treatment after 14 days of antibiotic treatment.
[e] The antibiotic treatment was changed at day 7 because of a poor response to the initial treatment regimen.
[f] This patient developed a pneumothorax, and lung function could not be measured at day 14.
[g] The total CFU for patient 8 was a combination of *P. aeruginosa* and an antibiotic-susceptible strain of *Achromobacter xylosoxidans.* The resistant subpopulation was just *P. aeruginosa*.

of the bacteria tested (as the criterion used to select bacteria for study was the breakpoint MIC). In these cases, the bacterial killing was due to the effect of the single antibiotic and not the antibiotic combination. The antibiotic concentrations used for the low-concentration MCBT were always less than the MBC of the bacteria tested.

Synergistic effects were seen with some antibiotic combinations by some methods. There were no examples of antagonism. In many instances there were differences between the results of the four methods for antibiotic combination testing for a single isolate of *P. aeruginosa*.

In some cases, isolates of *P. aeruginosa* with the same morphotype cultured from a single sputum sample had different synergy results. Examples are isolates 1a and 1b for patient 4 and isolates 3a and 3b for patient 6 (Table 1). These pairs were indistinguishable by PFGE.

As more than one isolate of *P. aeruginosa* from each sputum sample was tested, we compared the isolate with the least favorable result with the antibiotic combination with the clinical and microbiological outcome after treatment. For example, if the antibiotics acted synergistically for one isolate of *P. aeruginosa* but were indifferent for another, the overall result for that patient was classified as indifference. Even in this small group of patients, none of the synergy testing methods predicted the clinical outcome in terms of FEV_1 for all of the patients, nor did any synergy testing method predict the bacteriological outcome in terms of reduction in sputum bacterial numbers in CFU after treatment (Table 2).

DISCUSSION

While this was a small clinical study, it is the first to evaluate synergy testing by different methods and by using multiple isolates of resistant *P. aeruginosa* from sputum samples cultured at the time of exacerbation in CF patients. We have shown both that dissimilar results can be obtained by different methods and that the results can vary depending on the bacteria selected for testing. Bacteria of the same morphotype in the same sputum sample can have different susceptibilities to antibiotics used in combination. While the conclusions drawn are limited by the small numbers of patients studied, the synergy testing methods appeared to be poor at predicting the clinical or microbiological response in these nine patients.

As this study was specifically designed to look at the practice of testing the activities of combinations of antibiotics against resistant isolates, we did not look at whether combinations could be antagonistic to susceptible bacteria. In addition, although we tested far more isolates from each patient than have been tested in other studies of synergy, we may still have missed isolates that responded differently to antibiotic combinations in vitro. Another criticism of the study could be that the outcome measure of improvement in FEV_1 (to at least 95% of the preexacerbation baseline FEV_1) was too stringent. There is no consensus on the clinical end points to be used for studies of exacerbations of chronic infection, but with the improvements in treatment, many CF clinicians aim to restore lung function to preexacerbation levels. If a 90% level had been chosen, it would have altered the outcome for only one patient (patient 8), whose FEV_1 was restored to 93% of the preexacerbation level, and this result does not affect the general conclusions drawn from this study.

Studies of antibiotic combinations and strains *of P. aeruginosa* from patients with CF and other clinical conditions by the use of single methods (checkerboard, TK curve analysis, MCBT, or Etest) have shown in vitro synergy with a ß-lactam plus an aminoglycoside, a ß-lactam plus a quinolone, and even some dual ß-lactam combinations (6, 40). Antagonism is rare and may be a result of the use of a different definition of antagonism (ΣFICI, less than 4). There is, however, no refer-

ence or standard technique; and the various methods differ in the antibiotic concentrations and time points used and the criteria used to determine synergy.

The microdilution plate MIC checkerboard method has the advantages that it can be automated, it is suitable for use for the testing of multiple isolates, and multiple concentrations of each antibiotic combination can be tested; but the result is read at one time point (usually 24 h). Some isolates of *P. aeruginosa* from patients with CF grow slowly, and the result of the checkerboard method cannot be read after 24 h of incubation. We therefore used a bactericidal checkerboard method rather than prolong the incubation and risk the degradation of antibiotics and the consequent false susceptibility. This is more labor-intensive but had the added advantage that it enabled us to compare four methods of determination of the bactericidal activities of combinations of antibiotics.

TK curve analysis is far more labor-intensive and uses each antibiotic at a single concentration, but it follows bacterial killing over 24 h. The effect of combinations at times relating to dosing intervals can be seen, e.g., after 6, 8, or 12 h. In our study, however, many isolates had not sufficiently grown in the control tube before 24 h to be able to assess synergy at the earlier time points. The TK method poorly reflects what happens in vivo, as antibiotic concentrations do not decrease with time. There are pharmacodynamic models; but the use of those models is even more labor-intensive (20) and they may be of limited significance, as pharmacokinetics vary between different patients, especially those with CF (4).

The MCBT does not measure true synergy, as a ΣFBCI cannot be calculated. It was published as a simpler alternative to the TK method and examines the effects of a single concentration at a fixed time point of 48 h (25). It has the advantage that it uses only one tube per test, allowing many double and even triple combinations to be assessed. While a TK curve analysis uses the antibiotic at a concentration that is a fraction of the MIC, the original MCBT method uses the average peak serum antibiotic concentration. This leads to the anomaly that an isolate resistant by the conventional methods of testing single antibiotics may be susceptible to the antibiotic at the concentration used in the MCBT. The results of this test are therefore a mixture of the impacts of combinations and the results of testing higher concentrations of single antibiotics. The low-concentration, modified MCBT has the advantage that antibiotics are tested at levels at or near the susceptibility breakpoint and closer to the concentrations found in the lungs of CF patients.

A poor concordance between TK analysis and the checkerboard method has been shown in several studies. However, the numbers of bacteria tested in those studies were small, and only one isolate of *P. aeruginosa* from a patient with CF was described (7, 9, 39). However, a large study with CF patients that compared an Etest method with the checkerboard method showed a good correlation (>90%) (5).

P. aeruginosa has a wide range of antimicrobial resistance mechanisms, including the production of antibiotic-modifying enzymes, target modification, and altered permeability or efflux (27). The mechanisms of synergy proposed for *P. aeruginosa* include the effects of β-lactams, which increase the uptake of tobramycin, and aminoglycosides, which increase β-lactam uptake by displacing Mg^{2+} (21, 29); but more work is needed to understand how antibiotic combinations act and to determine the best way to test interactions in vitro.

Our previous work showed a wide variation in the susceptibilities of individual colonies of *P. aeruginosa* in the sputum from patients with an acute exacerbation of CF to single antibiotics (15). Phenotypic variation in *P. aeruginosa* isolates of the same genotype from patients with chronic infections is not restricted to antibiotic susceptibility but is also seen in type III secretion and pyocyanin production (14, 23). As we have now shown that bacteria from the same sputum sample differ in their susceptibilities to combinations of antimicrobial agents as well as to single antibiotics, we recommend that laboratories offering synergy testing examine multiple examples of resistant bacteria from an individual patient. The use of a single resistant bacterium or even an example of each resistant morphotype for testing may not be adequate as a means of sampling this population.

It has been shown that patients may respond to antibiotic treatment for acute exacerbations of infection even when their isolates of *P. aeruginosa* are resistant (37), and this may be because the antibiotic combination used is synergistic. However, there is very little published evidence of the utility of current synergy testing methods for determination of the choice of antibiotics. One study of serious infection with *P. aeruginosa* in patients without CF reported that TK curve analysis was more predictive of the clinical response than the checkerboard method. In three of four patients for whom there was antagonism by TK analysis, combination treatment with a β-lactam plus an aminoglycoside failed. That small study, however, included both susceptible and resistant bacteria; and the patients had infections at different body sites, so it is difficult to draw firm conclusions (9). A survey from a CF referral center that tests multiresistant *P. aeruginosa* by the checkerboard method found that the majority of physicians believed that the results were helpful in determining the appropriate antibiotic treatment (34). The only prospective study evaluating the use of synergy testing with isolates from patients with CF used the MCBT (2). That was a randomized double-blind controlled study conducted over 5 years and compared antibiotic choice for acute exacerbation by using the local hospital conventional susceptibility testing results or the MCBT. A total of 132 patients had chronic infection with multiresistant gram-negative bacteria (*P. aeruginosa*, *Stenotrophomonas maltophilia*, *Burkholderia cepacia* complex, *Achromobacter xylosoxidans*). No difference in treatment success was found (by clinical measures or a change in the sputum bacterial density after treatment). The limitations of the study were that the susceptibility results were for isolates from sputum samples taken within 3 months of the exacerbation, and each center used its local method to culture the sputum samples and measure susceptibility. Although MCBT was done centrally, the investigators relied on the local laboratory to select the bacteria to be used for testing. As discussed above, the use of peak serum antibiotic concentrations does question the choice of MCBT as the best method for choosing antibiotics that will act in the lung.

There are many reasons other than synergy that may explain why patients harboring resistant bacteria improve when they receive antibiotics that are ineffective in vitro. Resistant *P. aeruginosa* isolates may be less fit and less pathogenic than the susceptible organisms in the same sputum sample (36). *P.*

aeruginosa isolates are thought to grow as biofilms in the airways of patients with CF, whereas we measured susceptibility in the planktonic phase. There are in vitro methods that may be used to test the antibiotic susceptibilities of biofilms (1, 8, 30), and the clinical relevance of those tests needs investigation.

A clinical response to antibiotics may not be accompanied by a significant decrease in bacterial numbers in patients with chronic infections (28, 41). In the MCBT study, there was, on average, less than a 1-$\log_{10}$ reduction in the bacterial density (CFU/ml sputum) with treatment on the basis of the MCBT results, even though the test measured bacterial killing. The use of antibiotics at concentrations below the MIC can affect pathogenicity factors (e.g., adherence, alginate synthesis, quorum sensing, and biofilm formation) without killing or inhibiting the bacterial growth (13, 19, 22). This could explain the in vivo effects of antibiotics against bacteria that are resistant by conventional test methods. Alternatively, a range of species, many of which are considered part of the normal flora of the upper respiratory tract, can be found in sputum in significant numbers and at numbers greater than could be explained by contamination with saliva (33, 38). Some of these have been shown to increase the pathogenicity of *P. aeruginosa* by modulating gene expression through quorum sensing (12). Antibiotics could therefore have an indirect effect on the pathogenicity of antibiotic-resistant *P. aeruginosa* isolates by their action on these cofactor species.

Successful treatment for exacerbation in patients with CF may depend on more than the choice of antibiotic. Host factors such as the specific immune response, nutritional state, drug handling (pharmacokinetics), and comorbidities (such as diabetes or renal failure) are all thought to affect the outcome. In addition, pharmacodynamic factors are more complex for patients with CF than for patients with some other infections, given the altered behavior of the bacteria in chronic infection and the specific conditions in the sputum. Current in vitro synergy methods do not model this.

In conclusion, because of the phenotypic variability of *P. aeruginosa* in the sputum of patients with CF, synergy testing needs to be done with multiple examples of resistant bacteria from each patient to get a more complete picture of the activity of antibiotic combinations. Future studies are needed to determine the most clinically relevant method for prediction of the best antibiotic combinations for use in patients with CF. Such a method should be designed to take into account the variability of bacteria in individual sputum samples.

ACKNOWLEDGMENTS

We thank C. Docherty, University of Edinburgh, for typing strains by PFGE (reference service funded by the United Kingdom Cystic Fibrosis Trust) and John Perry, Microbiology Department, Freeman Hospital, Newcastle, United Kingdom, for his description of the low-concentration MCBT.

This study was supported in part by grants from the United Kingdom Cystic Fibrosis Trust and from Chiron Corporation (now Novartis).

None of us declares any financial interests that relate to this study.

REFERENCES

1. **Aaron, S. D., D. Kottachchi, W. J. Ferris, K. L. Vandemheen, M. L. St. Denis, A. Plouffe, S. P. Doucette, R. Saginur, F. T. Chan, and K. Ramotar.** 2004. Sputum versus bronchoscopy for diagnosis of Pseudomonas aeruginosa biofilms in cystic fibrosis. Eur. Respir. J. **24:**631–637.
2. **Aaron, S. D., K. L. Vandemheen, W. Ferris, D. Fergusson, E. Tullis, D. Haase, Y. Berthiaume, N. Brown, P. Wilcox, V. Yozghatlian, P. Bye, S. Bell, F. Chan, B. Rose, A. Jeanneret, A. Stephenson, M. Noseworthy, A. Freitag, N. Paterson, S. Doucette, C. Harbour, M. Ruel, and N. MacDonald.** 2005. Combination antibiotic susceptibility testing to treat exacerbations of cystic fibrosis associated with multiresistant bacteria: a randomised, double-blind, controlled clinical trial. Lancet **366:**463–471.
3. **Andrews, J. M.** 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. **48**(Suppl. 1)**:**5–16.
4. **Anonymous.** 2002. Antibiotic treatment for cystic fibrosis. Report of the United Kingdom Cystic Fibrosis Trust's Antibiotic Group, 2nd ed. Cystic Fibrosis Trust, Bromley, United Kingdom.
5. **Balke, B., M. Hogardt, S. Schmoldt, L. Hoy, H. Weissbrodt, and S. Haussler.** 2006. Evaluation of the E test for the assessment of synergy of antibiotic combinations against multiresistant Pseudomonas aeruginosa isolates from cystic fibrosis patients. Eur. J. Clin. Microbiol. Infect. Dis. **25:**25–30.
6. **Bosso, J. A., B. A. Saxon, and J. M. Matsen.** 1990. In vitro activities of combinations of aztreonam, ciprofloxacin, and ceftazidime against clinical isolates of *Pseudomonas aeruginosa* and *Pseudomonas cepacia* from patients with cystic fibrosis. Antimicrob. Agents Chemother. **34:**487–488.
7. **Cappelletty, D. M., and M. J. Rybak.** 1996. Comparison of methodologies for synergism testing of drug combinations against resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **40:**677–683.
8. **Ceri, H., M. E. Olson, C. Stremick, R. R. Read, D. Morck, and A. Buret.** 1999. The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J. Clin. Microbiol. **37:**1771–1776.
9. **Chandrasekar, P. H., L. R. Crane, and E. J. Bailey.** 1987. Comparison of the activity of antibiotic combinations in vitro with clinical outcome and resistance emergence in serious infection by Pseudomonas aeruginosa in non-neutropenic patients. J. Antimicrob. Chemother. **19:**321–329.
10. **Doring, G., S. P. Conway, H. G. Heijerman, M. E. Hodson, N. Hoiby, A. Smyth, and D. J. Touw.** 2000. Antibiotic therapy against Pseudomonas aeruginosa in cystic fibrosis: a European consensus. Eur. Respir. J. **16:**749–767.
11. **Drago, L., E. De Vecchi, L. Nicola, L. Tocalli, and M. R. Gismondo.** 2005. In vitro selection of resistance in Pseudomonas aeruginosa and Acinetobacter spp. by levofloxacin and ciprofloxacin alone and in combination with beta-lactams and amikacin. J. Antimicrob. Chemother. **56:**353–359.
12. **Duan, K., C. Dammel, J. Stein, H. Rabin, and M. G. Surette.** 2003. Modulation of Pseudomonas aeruginosa gene expression by host microflora through interspecies communication. Mol. Microbiol. **50:**1477–1491.
13. **Fonseca, A. P., C. Extremina, A. F. Fonseca, and J. C. Sousa.** 2004. Effect of subinhibitory concentration of piperacillin/tazobactam on Pseudomonas aeruginosa. J. Med. Microbiol. **53:**903–910.
14. **Fothergill, J. L., S. Panagea, C. A. Hart, M. J. Walshaw, T. L. Pitt, and C. Winstanley.** 2007. Widespread pyocyanin over-production among isolates of a cystic fibrosis epidemic strain. BMC Microbiol. **7:**45.
15. **Foweraker, J. E., C. R. Laughton, D. F. Brown, and D. Bilton.** 2005. Phenotypic variability of Pseudomonas aeruginosa in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. J. Antimicrob. Chemother. **55:**921–927.
16. **Fuchs, H. J., D. S. Borowitz, D. H. Christiansen, E. M. Morris, M. L. Nash, B. W. Ramsey, B. J. Rosenstein, A. L. Smith, M. E. Wohl, et al.** 1994. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. N. Engl. J. Med. **331:**637–642.
17. **Giwercman, B., P. A. Lambert, V. T. Rosdahl, G. H. Shand, and N. Hoiby.** 1990. Rapid emergence of resistance in Pseudomonas aeruginosa in cystic fibrosis patients due to in-vivo selection of stable partially derepressed beta-lactamase producing strains. J. Antimicrob. Chemother. **26:**247–259.
18. **Giwercman, B., C. Meyer, P. A. Lambert, C. Reinert, and N. Hoiby.** 1992. High-level beta-lactamase activity in sputum samples from cystic fibrosis patients during antipseudomonal treatment. Antimicrob. Agents Chemother. **36:**71–76.
19. **Govan, J. R., J. A. Fyfe, and N. R. Baker.** 1983. Heterogeneity and reduction in pulmonary clearance of mucoid Pseudomonas aeruginosa. Rev. Infect. Dis. **5**(Suppl. 5)**:**S874–S849.
20. **Gunderson, B. W., K. H. Ibrahim, L. B. Hovde, T. L. Fromm, M. D. Reed, and J. C. Rotschafer.** 2003. Synergistic activity of colistin and ceftazidime against multiantibiotic-resistant *Pseudomonas aeruginosa* in an in vitro pharmacodynamic model. Antimicrob. Agents Chemother. **47:**905–909.
21. **Hancock, R. E., V. J. Raffle, and T. I. Nicas.** 1981. Involvement of the outer membrane in gentamicin and streptomycin uptake and killing in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **19:**777–785.
22. **Ichimiya, T., T. Yamasaki, and M. Nasu.** 1994. In-vitro effects of antimicrobial agents on Pseudomonas aeruginosa biofilm formation. J. Antimicrob. Chemother. **34:**331–341.
23. **Jain, M., D. Ramirez, R. Seshadri, J. F. Cullina, C. A. Powers, G. S. Schulert, M. Bar-Meir, C. L. Sullivan, S. A. McColley, and A. R. Hauser.** 2004. Type III secretion phenotypes of *Pseudomonas aeruginosa* strains change during infection of individuals with cystic fibrosis. J. Clin. Microbiol. **42:**5229–5237.

24. **Kaufman, M. E.** 1998. Pulsed field gel electrophoresis. Methods Mol. Med. **15:**33–50.
25. **Lang, B. J., S. D. Aaron, W. Ferris, P. C. Hebert, and N. E. MacDonald.** 2000. Multiple combination bactericidal antibiotic testing for patients with cystic fibrosis infected with multiresistant strains of Pseudomonas aeruginosa. Am. J. Respir. Crit. Care Med. **162:**2241–2245.
26. **Lechtzin, N., M. John, R. Irizarry, C. Merlo, G. B. Diette, and M. P. Boyle.** 2006. Outcomes of adults with cystic fibrosis infected with antibiotic-resistant Pseudomonas aeruginosa. Respiration **73:**27–33.
27. **Livermore, D. M.** 2002. Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: our worst nightmare? Clin. Infect. Dis. **34:**634–640.
28. **McLaughlin, F. J., W. J. Matthews, Jr., D. J. Strieder, B. Sullivan, A. Taneja, P. Murphy, and D. A. Goldmann.** 1983. Clinical and bacteriological responses to three antibiotic regimens for acute exacerbations of cystic fibrosis: ticarcillin-tobramycin, azlocillin-tobramycin, and azlocillin-placebo. J. Infect. Dis. **147:**559–567.
29. **Miller, M. H., S. A. Feinstein, and R. T. Chow.** 1987. Early effects of beta-lactams on aminoglycoside uptake, bactericidal rates, and turbidimetrically measured growth inhibition in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **31:**108–110.
30. **Moskowitz, S. M., J. M. Foster, J. Emerson, and J. L. Burns.** 2004. Clinically feasible biofilm susceptibility assay for isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis. J. Clin. Microbiol. **42:**1915–1922.
31. **Pillai, S., R. Moellering, and G. Eliopoulos.** 2005. Antimicrobial combinations, p. 365–440. *In* V. Lorian (ed.), Antibiotics in laboratory medicine, 5th ed. Lippincott Williams & Wilkins, Baltimore, MD.
32. **Pitt, T. L., M. Sparrow, M. Warner, and M. Stefanidou.** 2003. Survey of resistance of Pseudomonas aeruginosa from UK patients with cystic fibrosis to six commonly prescribed antimicrobial agents. Thorax **58:**794–796.
33. **Rogers, G. B., M. P. Carroll, D. J. Serisier, P. M. Hockey, G. Jones, V. Kehagia, G. J. Connett, and K. D. Bruce.** 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. J. Clin. Microbiol. **44:**2601–2604.
34. **Saiman, L.** 2007. Clinical utility of synergy testing for multidrug-resistant Pseudomonas aeruginosa isolated from patients with cystic fibrosis: 'the motion for'. Paediatr. Respir. Rev. **8:**249–255.
35. **Saiman, L., F. Mehar, W. W. Niu, H. C. Neu, K. J. Shaw, G. Miller, and A. Prince.** 1996. Antibiotic susceptibility of multiply resistant Pseudomonas aeruginosa isolated from patients with cystic fibrosis, including candidates for transplantation. Clin. Infect. Dis. **23:**532–537.
36. **Sanchez, P., J. F. Linares, B. Ruiz-Diez, E. Campanario, A. Navas, F. Baquero, and J. L. Martinez.** 2002. Fitness of in vitro selected Pseudomonas aeruginosa *nalB* and *nfxB* multidrug resistant mutants. J. Antimicrob. Chemother. **50:**657–664.
37. **Smith, A. L., S. B. Fiel, N. Mayer-Hamblett, B. Ramsey, and J. L. Burns.** 2003. Susceptibility testing of Pseudomonas aeruginosa isolates and clinical response to parenteral antibiotic administration: lack of association in cystic fibrosis. Chest **123:**1495–1502.
38. **Tunney, M. M., T. R. Field, T. F. Moriarty, S. Patrick, G. Doering, M. S. Muhlebach, M. C. Wolfgang, R. Boucher, D. F. Gilpin, A. McDowell, and J. S. Elborn.** 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. Am. J. Respir. Crit. Care Med. **177:**995–1001.
39. **Visalli, M. A., M. R. Jacobs, and P. C. Appelbaum.** 1998. Determination of activities of levofloxacin, alone and combined with gentamicin, ceftazidime, cefpirome, and meropenem, against 124 strains of *Pseudomonas aeruginosa* by checkerboard and time-kill methodology. Antimicrob. Agents Chemother. **42:**953–955.
40. **Weiss, K., and J. R. Lapointe.** 1995. Routine susceptibility testing of four antibiotic combinations for improvement of laboratory guide to therapy of cystic fibrosis infections caused by *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **39:**2411–2414.
41. **Wolter, J. M., S. D. Bowler, and J. G. McCormack.** 1999. Are antipseudomonal antibiotics really beneficial in acute respiratory exacerbations of cystic fibrosis? Aust. N. Z. J. Med. **29:**15–21.