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Routine Susceptibility Testing of Four Antibiotic Combinations for Improvement of Laboratory Guide to Therapy of Cystic Fibrosis Infections Caused by *Pseudomonas aeruginosa*

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Previous studies have demonstrated synergy between an aminoglycoside and a beta-lactam for treating *Pseudomonas aeruginosa* infections. Cystic fibrosis patients are prone to infection by this bacterium, which becomes very resistant with recurrent antibiotic treatments. The purpose of this study was to evaluate the susceptibility patterns of 122 isolates of *P. aeruginosa* isolated from cystic fibrosis patients to five individual antibiotics (tobramycin, ceftazidime, piperacillin, ticarcillin, and imipenem) and to four antibiotic combinations (tobramycin associated with one of the other antibiotics). Strains were selected because of their resistance to individual antimicrobial agents, which ranged from 21.3% for imipenem to 56.5% for tobramycin. By using an automated broth microdilution method, we were able to demonstrate synergy against 39 strains (32%) with tobramycin-ticarcillin, against 38 strains (31%) with tobramycin-piperacillin, against 47 strains (39%) with tobramycin-ceftazidime, and against 23 strains (19%) with tobramycin-imipenem. Of the 122 isolates, 77 (63%) were rendered significantly susceptible to at least one of the four antibiotic combinations by synergy. These results suggest that when appropriate technology is available, susceptibility to antibiotic combinations greatly improves the guide to antibiotic therapy for infections due to *P. aeruginosa* in cystic fibrosis patients.

Cystic fibrosis (CF) is the most common inherited genetic disease affecting people in the western world, with an incidence of 1 in 1,600 to 2,000 live births among caucasians (4). In 1989, a major breakthrough occurred when the CF gene was identified on the long arm of chromosome 7, bringing hope that a cure will be found (13). Overall, in the last 2 decades, many achievements have greatly improved patients' survival, so that more and more people with CF now reach adulthood. American Cystic Fibrosis Foundation data show that the proportion of adult patients rose from 8% in 1969 to 33% in 1990 (7). The dramatic life expectancy improvement is due to a combination of several factors, especially the availability of powerful new antimicrobial agents against *Pseudomonas aeruginosa*. This bacterium is by far the most common pathogen in CF patients, with a prevalence reaching 80% in certain studies (11, 12). However, success has been hampered by the emergence of highly resistant *P. aeruginosa* strains. As already very well described in the medical literature, *P. aeruginosa* is very commonly implicated in recurrent respiratory infections (9). It is known that continuous and recurrent damage to the respiratory tract epithelium caused by bacterial enzymes, the bacterium itself, and the inflammatory response to the aggression leads to thickening of the bronchial wall and bronchiectasis (5). After repeated episodes of mainly *P. aeruginosa*-associated respiratory tract infections, patients start to develop pulmonary insufficiency, which gradually worsens and finally leads to end stage respiratory failure and death (6).

The main treatment goal is to eradicate *P. aeruginosa*; however, this is seldom achieved in this patient population. An important decrease of the bacterial burden with subsequent lesser airway inflammation is accepted as therapeutic success (11, 14, 17, 20). As patients are often treated for infectious exacerbations and receive broad-spectrum antibiotics, *P. aeruginosa* strains tend to become rapidly resistant to antibiotics commonly used in this setting, such as tobramycin or beta-lactams. In our institution, because of the increasing rate of microorganism resistance to individual antibiotics, we had to devise a checkerboard-based susceptibility testing technique (1, 15) aimed at finding potential synergy between various antibiotic combinations, especially when resistance to individual antibiotics is present. The main objective of this study was to test an easily applicable, partially automated method for determining synergy between two antibiotics in a clinically oriented laboratory.

MATERIALS AND METHODS

Bacterial strains. We prospectively collected 122 *P. aeruginosa* strains isolated from the respiratory tracts of different CF patients during a 2-year period (1991 to 1993) for susceptibility testing against five individual antibiotics and four antibiotic combinations. These strains were chosen with regard to their susceptibility patterns, which demonstrated resistance to either tobramycin or individual beta-lactams used to treat CF exacerbations and sometimes to all antibiotics. Once isolated, strains were quickly submitted to the checkerboard-based susceptibility testing technique.

Measurement of MICs. MICs were determined by a partially automated, broth-based microdilution technique. The MIC-2000 Plus Dispensor (Dynatech Laboratories, Chantilly, Va.) is a semiautomated system which has the ability to prepare a 96-U-well tray in accordance with our needs. Each well can be automatically filled with a desired antibiotic or antibiotic combination at a given concentration prepared on the same day in 200-ml bottles connected to volume-reducing heads. In accordance with the manufacturer's instructions, each well was inoculated with the MIC-2000 Plus Dispensor by adding 100 μl of an antimicrobial preparation containing either one of five antibiotics or one of the three ratios of four antibiotic combinations. Mueller-Hinton broth (Difco, Detroit, Mich.) supplemented with adequate amounts of Ca^{2+} and Mg^{2+} was used as the support medium throughout the experiment. Once the broth was prepared, sterility was controlled for each batch by incubating two trays at 35°C for 24 h and then replicating the 96 U wells onto Mueller-Hinton agar plates. All antibiotic-containing microdilution trays were plastic wrapped and frozen on the same day at −85°C until used. For quality control, *P. aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 were used. A quality control test was per-

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TABLE 1. Concentrations of antibiotic preparations[a] on the checkerboard microdilution tray

Concn(s) (μg/ml) on plate 1									Concn(s) (μg/ml) of plate 2								
Tic	Tic-Tob	Tic-Tob	Tic-Tob	Tob	Cef	Cef-Tob	Cef-Tob	Cef-Tob	Imi	Imi-Tob	Imi-Tob	Imi-Tob	Tob	Pip	Pip-Tob	Pip-Tob	Pip-Tob
256	32, 16	128, 16	512, 16	32	64	8, 16	32, 16	128, 16	32	4, 16	16, 16	64, 16	32	256	32, 16	512, 16	128, 16
128	16, 8	64, 8	256, 8	16	32	4, 8	16, 8	64, 8	16	2, 8	8, 8	32, 8	16	128	16, 8	256, 8	64, 8
64	8, 4	32, 4	128, 4	8	16	2, 4	8, 4	32, 4	8	1, 4	4, 4	16, 4	8	64	8, 4	128, 4	32, 4
32	4, 2	16, 2	64, 2	4	8	1, 2	4, 2	16, 2	4	0.5, 2	2, 2	8, 2	4	32	4, 2	64, 2	16, 2
16	2, 1	8, 1	32, 1	2	4	0.5, 1	2, 1	8, 1	2	0.25, 1	1, 1	4, 1	2	16	2, 1	32, 1	8, 1
8	1, 0.5	4, 0.5	16, 0.5	1	2	0.25, 0.5	1, 0.5	4, 0.5	1	0.12, 0.5	0.5, 0.5	2, 0.5	1	8	1, 0.5	16, 0.5	4, 0.5
4	0.5, 0.25	2, 0.25	8, 0.25	0.5	1	0.12, 0.25	0.5, 0.25	2, 0.25	0.5	0.06, 0.25	0.25, 0.25	1, 0.25	0.5	4	0.5, 0.25	8, 0.25	2, 0.25
2	0.25, 0.12	1, 0.12	4, 0.12	0.25	0.5	0.06, 0.12	0.25, 0.12	1, 0.12	0.25	0.03, 0.12	0.12, 0.12	0.5, 0.12	0.25	2	0.25, 0.12	4, 0.12	1, 0.12

[a] Tic, ticarcillin; Tob, tobramycin; Cef, ceftazidime; Imi, imipenem; Pip, piperacillin.

TABLE 2. Antibiotic susceptibilities[a] of 122 selected *P. aeruginosa* strains isolated from CF patients

Antibiotic	No. (%) of isolates				*P* value[c]
	Susceptible	Intermediate	Resistant	Synergy[b]	
Ticarcillin	53 (43.4)		69 (56.5)	69 (56.5)	0.02
Piperacillin	55 (45.0)		67 (54.9)	70 (57.3)	0.02
Ceftazidime	54 (44.2)	16 (13.0)	52 (42.6)	76 (62.2)	0.0002
Imipenem	66 (54.0)	30 (24.5)	26 (21.3)	75 (61.4)	NS
Tobramycin	47 (38.5)	21 (17.2)	54 (44.2)	85 (69.6)	<0.0001

[a] MIC interpretive standards (19): piperacillin and ticarcillin susceptibility and resistance, ≤64 and ≥128 μg/ml, respectively; tobramycin susceptibility, intermediacy, and resistance, ≤4, 8, and ≥16 μg/ml; ceftazidime, ≤8, 16, and ≥32 μg/ml; imipenem, ≤4, 8, and ≥16 μg/ml.

[b] Total number of susceptible strains for each individual antibiotic, obtained by adding those categorized as susceptible to those rendered susceptible when another antibiotic was added.

[c] Fisher's exact test. NS, not significant.

formed every month with these strains on randomly selected plates of residual batches.

For this study, two types of trays were specifically designed. The first tray contained eight twofold dilutions of ticarcillin, tobramycin, and ceftazidime. Antibiotic combinations were devised in the following way: three ratios of eight twofold dilutions of ticarcillin-tobramycin and three ratios of ceftazidime-tobramycin. The second tray contained eight twofold dilutions of imipenem, tobramycin, and piperacillin and three ratios of eight twofold dilutions of imipenem-tobramycin and piperacillin-tobramycin. The exact configurations of the two types of trays are described in Table 1. Generic antibiotic combinations were designed for all strains without knowledge of the MICs of the individual antibiotics.

A MacFarland no. 1 bacterial suspension of a freshly isolated *P. aeruginosa* strain, checked for the actual number of CFU per milliliter, was simultaneously inoculated by a fully automated system (MIC-2000 Plus Inoculator; Dynatech Laboratories) onto the previously described two tailored sets. They were then incubated at 35°C for 24 h in an aerobic environment before MIC determination.

The susceptibilities of the 122 strains were determined in accordance with National Committee for Clinical Laboratory Standards guidelines (19); synergy between two agents was defined as a fourfold decrease in the MIC of each antibiotic in the combination, which corresponds to a fractional inhibitory concentration index (Σ) of less than or equal to 0.5. Moreover, the MIC of each antibiotic included in the combination had to be in the range considered susceptible by National Committee for Clinical Laboratory Standards criteria for that particular antimicrobial agent.

RESULTS AND DISCUSSION

As shown in Table 2, the individual rates of resistance of these 122 strains to various antibiotics were quite high, ranging from 21.3 to 56.5%, and these rates were still higher when intermediate strains were added. The rates of resistance to the individual antibiotics are in relation to their chronological use in the hospital, and a high frequency of cross-resistance between ticarcillin, piperacillin, and ceftazidime was observed. Imipenem, which was the last drug to be introduced, had the lowest resistance rate among these strains. The high rate of antibiotic resistance was nonetheless foreseeable, because these strains were chosen because of their particular resistance

TABLE 3. Numbers of strains with which antibiotic combinations showed relevant synergy

No. of strains/total (%)	No. of combinations showing synergy
45/122 (37.0)	0
34/122 (28.0)	1
20/122 (16.5)	2
19/122 (15.5)	3
4/122 (3.0)	4
77/122 (63.0)	At least 1

TABLE 4. Original patterns of susceptibility to individual antibiotics shown by strains against which various antibiotic combinations acted synergistically

Antibiotic combination	No. of strains synergistically inhibited/total (%)	Original pattern of susceptibility to β-lactam antibiotic alone	Original pattern of susceptibility to tobramycin[a]		
			S	I	R
Ticarcillin-tobramycin	39/122 (32)	Ticarcillin susceptible	12	7	4
		Ticarcillin resistant	12	2	2
Piperacillin-tobramycin	38/122 (31)	Piperacillin susceptible	15	6	2
		Piperacillin resistant	3	8	4
Ceftazidime-tobramycin	47/122 (39)	Ceftazidime susceptible	20	4	1
		Ceftazidime intermediate	5	5	2
		Ceftazidime resistant	4	4	2
Imipenem-tobramycin	23/122 (19)	Imipenem susceptible	9	3	2
		Imipenem intermediate	2	2	2
		Imipenem resistant	0	2	1

[a] S, susceptible; I, intermediate; R, resistant.

to individual antibiotics and the challenge that this situation implies for patients' treatment. Furthermore, except for imipenem, there was a statistically significant increase in the total number of susceptible strains for all of the antibiotics when tobramycin was added to individual beta-lactams (Table 2; see Table 4). For tobramycin in particular, the rise of the susceptibility rate was dramatic, and this occurred mostly because intermediate strains became susceptible when another antibiotic was added (Table 2). In 77 (63%) of 122 strains, we also found synergy with at least one of the four combinations (Table 3). Multiple synergy ([12]i.e., synergy with at least two combinations) was present in 35% of the cases. The combination of ceftazidime and tobramycin showed the highest percentage of synergy (47 [39%] of 122), in contrast to imipenem, for which synergy was significantly less frequent (Table 4), although imipenem resistance was a less frequent event (21.3%) in the 122 isolates studied.

As previously mentioned, resistance of *P. aeruginosa* in CF patients is continuously increasing worldwide (8, 18), so we had to design a method which enabled us to improve our available antimicrobial agents. The purpose of this study was to create an easily applicable susceptibility testing technique to help us in our choice of therapy. The fact that our hospital is a referral center for CF patients in the Province of Quebec, with many older patients already challenged with many antibiotics, can explain the high incidence of multiresistant strains. A checkerboard-based technique was chosen because it could be easily implemented in a diagnostically oriented laboratory.

Nevertheless, several drawbacks have to be taken into account when dealing with such a method. The first problem encountered is that in contrast to a time-kill curve method, our method measures inhibitory synergy at one point, namely, 24 h, thus losing the dynamic and bactericidal aspects of the phenomenon (3). Furthermore, the correlation between the two methods has not always been perfect; one explanation for this is that the definition of synergy is method dependent (21). Besides, no study in the field of CF pseudomonas infection has ever made a clear in vitro-in vivo correlation (2, 8, 10, 14, 16), but physicians still strongly rely on susceptibility testing for their therapeutic choices.

Despite of all these inconveniences, the technique used in our study has many evident advantages. First, this setting can give us considerable information on a strain's susceptibility to five individual antibiotics and four combinations in a 24-h period. It is mostly a semiautomated method in the sense that checkerboards are prepared in quantity with the help of a machine and later automatically inoculated. As we are using an open system, trays can be tailored to our needs, with the possibility of changing individual antibiotics or combinations; for example, ciprofloxacin, which is often used to treat CF patients, can be easily added. New antimicrobial agents can also be tested alone or in combinations when available, while older antibiotics that have very high resistance rates and are no longer efficient can be deleted.

Physicians in our institution rely on this method to treat CF patients infected with resistant *P. aeruginosa*. The life expectancy of CF patients in our institution is very similar to that usually reported in other centers. Besides, when no synergy between any pair of drugs exists for a particular strain, we can avoid using tobramycin with ceftazidime and imipenem, thus diminishing the risk of aminoglycoside-related side effects and resistance emergence.

As CF patients live longer now, antibiotic resistance is becoming a serious challenge and this checkerboard-based technique is a first step in trying to improve the available treatment options. However, animal or human models would be desirable to corroborate these data with a measurable clinical outcome.

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