

Breakpoints for Predicting *Pseudomonas aeruginosa* Susceptibility to Inhaled Tobramycin in Cystic Fibrosis Patients: Use of High-Range Etest Strips

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Inhaled administration of tobramycin assures high concentrations in cystic fibrotic lungs, improving the therapeutic ratio over that of parenteral tobramycin levels, particularly against *Pseudomonas aeruginosa*. Conventional Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) breakpoints only consider parenteral levels and do not take into account these high antimicrobial concentrations. The Spanish Antibiogram Committee (The MENSURA Group) has tentatively defined specific breakpoint values for inhaled tobramycin when testing *P. aeruginosa* isolates from cystic fibrosis (CF) patients (susceptible, ≤ 64 $\mu\text{g/ml}$; resistant, ≥ 128 $\mu\text{g/ml}$). The antimicrobial susceptibilities of 206 prospectively collected CF *P. aeruginosa* isolates were determined by the reference agar dilution method. For tobramycin, the performance of high range tobramycin Etest strips (AB Biodisk, Solna, Sweden) and conventional tobramycin disks were assessed with the same collection. Applying MENSURA proposed breakpoints, 95.1% of the strains were categorized as susceptible to tobramycin, either using agar dilution or Etest high-range strips (99% categorical agreement between both methods). With CLSI breakpoints, susceptibility rates decreased to 79.1 and 81.1% for agar dilution and Etest strips, respectively (83.5% categorical agreement). Minor, major, and very major errors for Etest strips (CLSI criteria) were 13.6, 1.2, and 14.8%, respectively. Upon applying the new proposed criteria for inhaled tobramycin, only one major and one very major error were observed with Etest strips. Whenever inhaled tobramycin is considered for therapy, we suggest that *P. aeruginosa* strains from CF patients categorized as intermediate or resistant to tobramycin according to the CLSI criteria should be retested with high-range Etest strips and recategorized using MENSURA interpretive criteria. CLSI breakpoints should still be followed when intravenous tobramycin is used in CF patients, particularly during the course of exacerbations.

Pseudomonas aeruginosa has been recognized as a major pathogen in cystic fibrosis (CF) (12, 17). Isolation of this organism from sputum samples in CF patients during follow-up is regarded as a risk factor for lung function deterioration (15, 31). *P. aeruginosa* in CF airways mainly grows in a biofilm disposition with an inherent resistance that may be, at least in part, overcome with high antimicrobial concentrations similar to those reached with the inhaled form of administration (19, 34).

The most recent treatment guidelines for primarily chronically colonized or *P. aeruginosa*-infected CF patients recommend the use of aerosolized antimicrobials, including tobramycin or colistin, while intravenous treatment is reserved for acute exacerbations (6, 8, 9, 27, 35). As stated, the main reason for inhaled treatments is to achieve high antimicrobial concentrations in the lungs. Nevertheless, a decrease in the potential side effects of the long-term treatment schedules frequently used in these patients and a lower risk of organism resistance development has also been advocated (27, 35). Inhaled tobramycin has been optimized for use in CF patients (11). As all

aminoglycosides, tobramycin is a concentration-dependent antimicrobial agent with a long postantimicrobial effect. Its safety profile with inhaled administration allows the delivery of high doses, which in turn improve the efficiency of local concentrations of bioactive antimicrobial (14, 29). The multiple antimicrobial resistances and the frequent development of hypermutation as a result of prolonged treatments (24) are a matter of major concern in CF patients. The intermittent schedule of administration of nebulized antimicrobial solution is a possible strategy that may contribute to preserve susceptibility among *P. aeruginosa* isolates (14, 28, 29).

Qualitative interpretative categories (susceptible, intermediate, and resistant) used in conventional susceptibility testing procedures are generally adapted for parenteral and/or oral treatments and do not consider the high local antimicrobial concentrations obtained when inhaled therapy is used (7). Considering both sputum and epithelial lining fluid concentrations (11, 30) and cumulative clinical experience (11, 14, 28, 29), the Spanish Antibiogram Committee (The MENSURA Group) has tentatively defined specific breakpoints for inhaled tobramycin (susceptible, ≤ 64 $\mu\text{g/ml}$, and resistant, ≥ 128 $\mu\text{g/ml}$) when *P. aeruginosa* isolates from CF patients are tested (20). These breakpoints are significantly higher than those currently recommended by the Clinical and Laboratory Standards Institute (CLSI [formerly National Committee for Clin-

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ical Laboratory Standards]; susceptible, ≤ 4 $\mu\text{g/ml}$; intermediate, 8 $\mu\text{g/ml}$; and resistant, ≥ 16 $\mu\text{g/ml}$) (7), which do not take into account the specific pharmacology of inhaled antimicrobials (10). The MENSURA Group proposal is in agreement with other authors (4, 16, 32).

We determined here the in vitro antimicrobial susceptibilities of a large collection of *P. aeruginosa* isolates prospectively collected from CF patients attending our CF unit. With this aim and according to CLSI recommendations (7), the agar dilution method with commonly used antipseudomonal antimicrobials, including colistin, was performed. Simultaneously and for comparative purposes, the performance of high-range tobramycin Etest strips, specifically designed for susceptibility testing of *P. aeruginosa* from CF patients, and the standard diffusion test with tobramycin disks were assessed with the same collection. Tobramycin CLSI susceptibility criteria for *P. aeruginosa* (7) and the breakpoints proposed for inhaled tobramycin by The MENSURA Group were used for this aim (20).

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MATERIALS AND METHODS

Patients and bacterial strains. We studied a total of 206 *P. aeruginosa* isolates comprising different morphotypes and prospectively collected in 2003 and 2004 from sputum samples of 56 patients attending our CF Unit (Hospital Universitario Ramón y Cajal, Madrid, Spain). *P. aeruginosa* ATCC 27853 was used as a quality control strain.

Antimicrobial agents. Tested antimicrobials, tobramycin, gentamicin, amikacin, and piperacillin alone or in combination with a fixed amount of tazobactam, ceftazidime, imipenem, meropenem, ciprofloxacin, and colistin (4 $\mu\text{g/ml}$) were purchased from Sigma Chemical Co. (St. Louis, Mo.) or were provided by their respective manufacturers.

Antimicrobial susceptibility testing. MICs were determined by the agar dilution procedure with Mueller-Hinton agar (Oxoid, Ltd., Basingstoke, Hampshire, United Kingdom) according to the NCCLS M7-A6 standard (22). *P. aeruginosa* ATCC 27853 was included as control strain in each run. MIC breakpoints recommended by the CLSI M100-S15 informational supplement (7) were used for all antimicrobials, including colistin, whose breakpoint values were extrapolated from those of polymyxin B (7, 10). In the case of tobramycin, MICs were categorized considering the breakpoints proposed by The MENSURA Group for inhaled tobramycin.

Susceptibility testing with the high range tobramycin Etest strips (0.064 to 1,024 $\mu\text{g/ml}$; AB Biodisk, Solna, Sweden) was performed according to the NCCLS M7-A6 standard (22) and technical guides provided by the manufacturer. Evaluation of results was also performed considering both the MIC interpretive criteria of the CLSI (M100-S15) (7) and those proposed by The MENSURA Group (20). The disk diffusion method with 10- μg tobramycin-containing disks (Oxoid) was carried out according to the NCCLS M2-A6 standard (23). The indication to extend incubation up to 24 h for susceptibility tests of *P. aeruginosa* isolates from CF patients before they were reported as susceptible (CLSI M100-S15) was followed in all determinations.

Analysis of the results. Tobramycin MIC results obtained with the standard agar dilution method and Etest were compared by regression analysis. Pearson correlation coefficients (r values) and regression slope equations were obtained by using the Whonet computer program (WHO/CSR/DRS/99.1 [World Health Organization]). Etest MICs were converted to the currently double-dilution scale used in conventional methods (rounded up to the next highest dilution when necessary).

Categorical error rates were generated for tobramycin after Etest results were compared with those obtained with the reference agar dilution method. Essential agreement was defined to be when MIC results obtained with both methods were identical or agreed within ± 1 -log₂ dilution. In addition, acceptable levels of error were determined according to the NCCLS criteria (21). When the MIC was

TABLE 1. Comparative in vitro activity (agar dilution technique) of antimicrobials tested against the 206 CF *P. aeruginosa* isolates

Antimicrobial agent	MIC ($\mu\text{g/ml}$)			% Of fully ^a :	
	Range	50%	90%	Susceptible isolates	Resistant isolates
Tobramycin	0.25->1,024	2	16	79.1	13.1
Gentamicin	0.5-512	8	64	41.7	33.0
Amikacin	0.5-512	8	64	68.4	13.1
Piperacillin	0.5-1,024	8	256	79.1	20.9
Piperacillin-tazobactam	0.5-1,024	4	256	83.0	17.0
Ceftazidime	0.25-1,024	4	64	78.6	14.6
Imipenem	0.12-128	4	64	58.3	35.0
Meropenem	0.03-64	0.5	16	75.7	13.6
Ciprofloxacin	0.25-256	1	8	51.0	28.6
Colistin	≤ 0.06 -512	0.5	0.5	97.1	2.9

^a CLSI breakpoints (document M100-S15) were used.

categorized as susceptible with the Etest and as resistant by the reference method, the result was classified as a very major error. Conversely, a major error was considered when the Etest result corresponded to the resistant category but the result with the reference agar dilution method was susceptible. Minor errors were defined as an intermediate result for either the Etest or the reference method. All of these considerations were also followed when disk diffusion was evaluated versus reference agar dilution results.

RESULTS

Susceptibility profiles. Comparative antimicrobial susceptibilities of the 206 *P. aeruginosa* isolates obtained using the reference agar dilution technique are presented in Table 1. According to CLSI interpretive categories (7), colistin, tobramycin, meropenem, and amikacin were the most active agents tested with percentages of susceptible isolates of 97.1, 79.1, 75.7, and 68.4%, respectively. The corresponding values for ceftazidime, piperacillin and piperacillin-tazobactam were 78.6, 79.1, and 83.0%, respectively. Imipenem was the least active antimicrobial, with 35.0% of resistant isolates followed by gentamicin (33.0%) and ciprofloxacin (28.6%) (Table 1)

The overall distribution of different morphotypes was as follows: mucoid, 40.7%; metallic, 18.5%; rough, 16.5%; enterobacterial, 12.6%; and dwarf, 11.7%. As in previous reports, mucoid strains were slightly more susceptible than the other morphotypes. By the agar dilution method, 77.0% of nonmucoid isolates were susceptible to tobramycin; this value reached 82.1% for mucoid isolates.

With the high-range tobramycin Etest strips, the percentage of tobramycin susceptible isolates of the entire collection was 81.1% (77.0% for nonmucoid and 86.9% for mucoid strains) when CLSI criteria were adopted. This background varies considerably if the new tentative breakpoints (susceptible, ≤ 64 $\mu\text{g/ml}$, and resistant, ≥ 128 $\mu\text{g/ml}$) for inhaled tobramycin, proposed by The MENSURA Group (20) and also suggested by Schülin (32), are applied. According to these values and considering the whole studied population, susceptibility to tobramycin comprised 95.1% of total isolates with both the agar dilution and the Etest methods (Table 2). Tobramycin MIC distributions obtained when reference agar dilution and high-range Etest techniques were used for the 206 *P. aeruginosa* isolates are shown in Fig. 1. At least one bimodal distribution was observed with both methods. The first peak of this distri-

TABLE 2. Comparison of tobramycin MIC susceptibility values for the 206 *P. aeruginosa* isolates as determined by the reference agar dilution method and the use of high-range tobramycin Etest strips using CLSI conventional breakpoints and MENSURA-proposed criteria for inhaled tobramycin

Method	Interpretive criteria (no. of isolates [%]) ^a					
	CLSI			MENSURA		
	S (≤ 4 $\mu\text{g/ml}$)	I (8 $\mu\text{g/ml}$)	R (≥ 16 $\mu\text{g/ml}$)	S (≤ 64 $\mu\text{g/ml}$)	I	R (≥ 128 $\mu\text{g/ml}$)
Agar dilution	163 (79.1)	16 (7.8)	27 (13.1)	196 (95.1)	NA	10 (4.9)
Etest	167 (81.1)	20 (9.7)	19 (9.2)	196 (95.1)	NA	10 (4.9)

^a S, susceptible; I, intermediate; R, resistant. NA, not applicable.

bution was slightly displaced to the left with the agar dilution method; the modal MIC for the first peak with the agar dilution method was 1 $\mu\text{g/ml}$, but for the Etest it was 2 $\mu\text{g/ml}$. Of note is the width of the first peak, a typical feature of CF *P. aeruginosa* isolates, suggesting the phenotypic heterogeneity of this population. A second peak could be observed with a modal MIC of 64 $\mu\text{g/ml}$ with the agar dilution method and 128 $\mu\text{g/ml}$ with Etest. We therefore consider that this type of population requires concentrations of tobramycin exceeding 128 $\mu\text{g/ml}$ to be inhibited. A trail of very highly resistant strains presented MICs over 512 $\mu\text{g/ml}$, even reaching MICs of $>1,024$ $\mu\text{g/ml}$.

When intermethod analysis was performed, the MIC agreement within ± 2 -log₂ dilutions between Etest technique with high-range tobramycin and agar dilution technique was 95.6% (Fig. 2). This figure decreased to 83.5% when evaluated with a ± 1 -log₂ dilution. The correlation coefficient between both methods was $r = 0.91$. Moreover, the reference agar dilution and Etest technique with high-range tobramycin displayed 83.5% of categorical agreement when CLSI interpretive criteria were applied. Agreement between categories obtained by both techniques rose up to 99% when the breakpoint

proposed by The MENSURA Group for the inhaled tobramycin formulation was considered (Table 3). Discrepancies ($n = 34$ isolates) were mainly observed among intermediate (12 of 34 [35.3%]) and resistant (11 of 34 [32.3%]) isolates when CLSI criteria were used. Minor, major, and very major errors for Etest strips using the CLSI criteria were 13.6, 1.2, and 14.8%, respectively. When the new proposed criteria for inhaled tobramycin were applied, only one major and one very major error were observed (Table 3). No minor errors were possible with the new proposed criterion because of its lack of an intermediate category (Table 3).

Categorical agreement of tobramycin disk diffusion results with agar dilution results was 84% in our CF *P. aeruginosa* collection as 16 of 27 tobramycin-resistant isolates were categorized as susceptible with the disk diffusion technique (data not shown in tables). This evaluation was performed using CLSI interpretive categories since no criteria for the disk diffusion method have been proposed by The MENSURA Group. Moreover, the correlation of disk diffusion with the reference agar dilution method was within an unacceptable range (correlation coefficient [r] = 0.68).

DISCUSSION

It is well known that the Etest, a stable antimicrobial gradient method for MIC determination, ensures accurate results and is easy to perform (3). Etest and disk diffusion methods have proven to be useful not only for susceptibility purposes but also for the detection of hypermutable *P. aeruginosa* strains and the frequently associated resistant mutant subpopulations whose presence is a common occurrence in chronically colonized CF patients (18). Significantly, these strains are prone to develop antimicrobial resistance, and their detection by microbiology laboratories may be quite helpful for the early establishment or even the redesign of adequate therapeutic regimens (25). Moreover, susceptibility testing of direct clinical

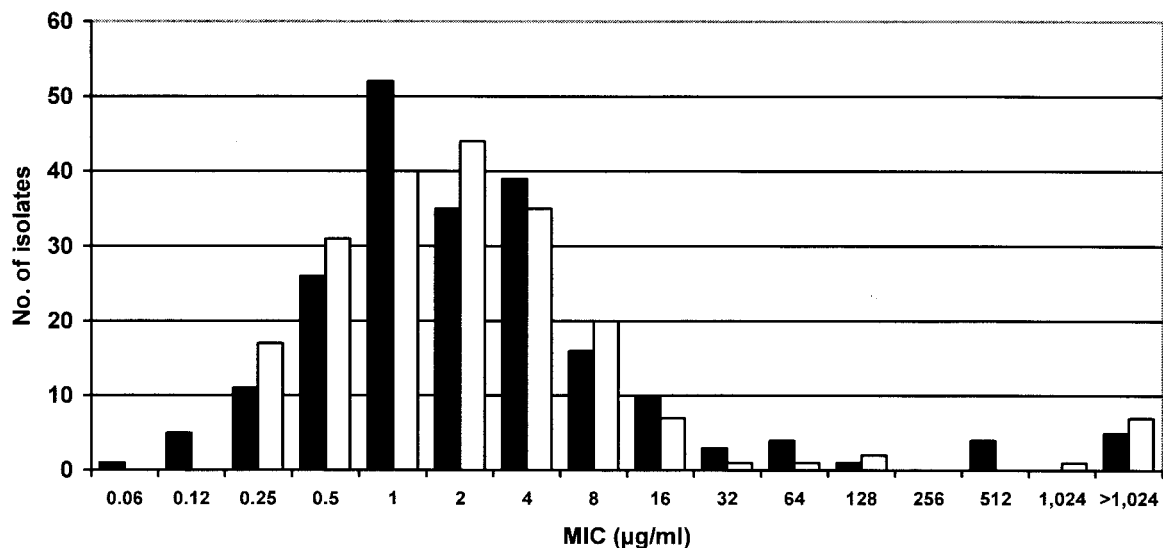


FIG. 1. Distribution of tobramycin MICs using agar dilution (■) and Etest (□) for the 206 CF *P. aeruginosa* isolates.

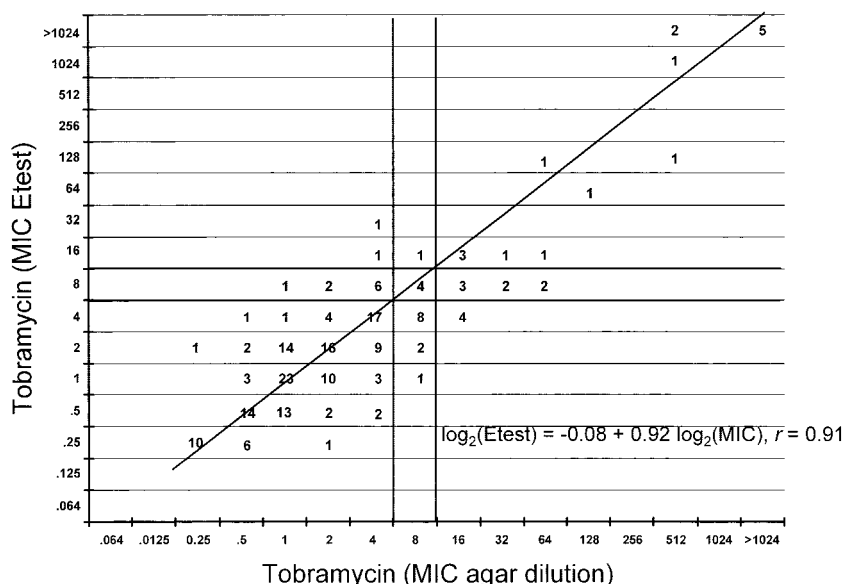


FIG. 2. Scattergram results for tobramycin comparing the reference agar dilution MICs with the high-range tobramycin Etest MICs against 206 *P. aeruginosa* isolates. The diagonal line represents, in each figure, complete agreement, and the numbers represent the occurrences observed at each point. Horizontal and vertical dotted lines represent CLSI breakpoint values for tobramycin.

respiratory samples using Etest strips has been successfully implemented (E. Cercenado, S. Cercenado, M. V. Rico, T. Vicente, and E. Bouza, Abstr. 42nd Intersci. Conf. Antimicrob. Agents Chemother., abstr. D-51, 2002).

In the case of CF patient isolates, Etest methodology merits further consideration. It assures a timely evaluation of the problem of resistance within a group of patients for whom the need of an adequate therapeutic behavior is essential, since they are usually colonized with multiresistant strains for which few treatment options exist. The antimicrobial concentrations displayed in the case of the tobramycin high-range Etest strips give a more precise reflection of the true levels reached in pulmonary secretions when this compound is delivered by aerosolization (11). Evidently, this spanned antimicrobial scale permits the evaluation of strain susceptibilities with a more

effective approach to clinical situations of CF patients using inhaled antimicrobials.

When the results obtained using the conventional CLSI susceptibility criteria (7) and those tentatively proposed by The MENSURA Group (20) are compared, a better interpretive category correlation between the reference agar dilution method and the tobramycin high-range Etest was obtained with the newly proposed inhaled tobramycin breakpoints. If the goal is to avoid interpretive discrepancies, this can be reached by using either agar dilution or Etest by adopting the proposed breakpoints for inhaled tobramycin. Thus, the complete agreement of results between the two methodologies strengthens the possible validation of the confident performance of high-range Etest strips and the rationale of the new proposed criteria. However, some authors (4) have suggested that, although the resistance for parenteral administration does not apply to inhaled tobramycin, the definition of a new breakpoint is still unnecessary based upon favorable clinical response and the fact that few *P. aeruginosa* isolates display tobramycin MICs of $\geq 16 \mu\text{g/ml}$. Nevertheless, in our collection, 13% of isolates had this resistance level, and it was also the MIC of this compound at which 90% of the isolates tested are inhibited for the tested collection. Unlike a previous study comparing tobramycin disk diffusion and broth microdilution susceptibility testing results (5), our correlation was poorer, since 16 of 27 tobramycin-resistant isolates were categorized as susceptible with the disk diffusion technique. It is of note that the majority of discrepant isolates (11 of 16) were nonmucoid isolates, which have been reported to be more susceptible to tobramycin (5).

In the light of this situation, the question to be posed seems to be whether conventional definitions of susceptibility and resistance require reexamination for the particular case of

TABLE 3. Use of high-range tobramycin Etest strips for susceptibility testing of *P. aeruginosa* strains with CLSI-conventional breakpoints and MENSURA-proposed criteria for inhaled tobramycin

Performance values	Interpretive criteria ^a			
	CLSI		MENSURA	
	n/total	%	n/total	%
Categorical agreement	172/206*	83.5	204/206*	99.0
Categorical errors				
Minor	28/206*	13.6	NA	NA
Major	2/163†	1.2	1/196†	0.5
Very major	4/27‡	14.8	1/10‡	10.0

^a *, considering the total number of isolates; †, considering the number of susceptible isolates; ‡, considering the number of resistant isolates; NA, not applicable.

inhaled tobramycin. Using agar dilution and Etest methodologies, a *P. aeruginosa* subpopulation within the first peak of MIC distribution could be observed (Fig. 1) that was clearly different from organisms only inhibited by higher tobramycin concentrations. These high MICs could be associated with the presence of aminoglycoside modifying enzymes and/or that of the still-infrequent 16S rRNA methylase encoded by the *rmtA* gene (26). Conversely, low to moderate resistance has been associated with impermeability or so-called adaptive resistance (2), which has mostly been observed in CF isolates. An inducible multidrug efflux system, MexXY-OprM, appears to be responsible for this adaptive mechanism of resistance (13). Such induction may explain the increase in transient low to moderate tobramycin resistance upon exposure of *P. aeruginosa* to antimicrobial concentrations near those of the MICs of aminoglycosides, a situation that may occur even under inhaled therapy (33). Long intervals between inhaled tobramycin courses (on-off drug cycles) might limit the impact of adaptive resistance by allowing its reversion (1, 4, 3, 28, 29)

In summary and according to the present results, we propose that, whenever tobramycin is considered for therapy, *P. aeruginosa* strains from CF patients categorized as intermediate or resistant to tobramycin according to the CLSI criteria should be retested with high-range Etest strips and recategorized, when necessary, by using the MENSURA interpretive criteria. CLSI criteria should still be applied when intravenous tobramycin is used in CF patients, particularly during the course of exacerbations.

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