Is a New Standard Needed for Diffusion Methods for In Vitro Susceptibility Testing of Fosfomycin against Pseudomonas aeruginosa?

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We analyzed fosfomycin susceptibility results in Pseudomonas aeruginosa clinical isolates obtained by MIC gradient strips and disk diffusion methods using two different inocula, 10⁸ and 10⁶ CFU/ml, and compared them to the agar dilution reference method. Essential and categorical agreements were 93.6% and 95%, respectively, for the 10⁶ CFU/ml alternative inoculum, and they were 67.6% and 78.2%, respectively, for the standard inoculum (10⁸ CFU/ml). The use of the 10⁶ CFU/ml inoculum improves the agreement values and inhibition zone readings.

The increase in antimicrobial resistance has forced researchers to look for new therapeutic strategies to treat infections. Fosfomycin has demonstrated efficacy when combined with other antimicrobials for the treatment of urinary and systemic infections against multidrug-resistant (MDR) Gram-negative bacteria, such as Pseudomonas aeruginosa (1). Fosfomycin-calcium and fosfomycin-tromethamine for oral use as well as fosfomycin-disodium for intravenous administration have been extensively used in some European countries and in Japan, while the intravenous formulation is now under clinical evaluation in the United States (2). However, studies documenting the in vitro activity of fosfomycin against P. aeruginosa remain scarce, and clinical breakpoints have not been established. EUCAST includes an epidemiological cutoff value (ECOFF) of 128 μg/ml (3). For in vitro susceptibility testing, agar dilution is considered the reference method, but the microdilution method has demonstrated adequate concordance in non-cystic fibrosis P. aeruginosa isolates. In contrast, MIC gradient strips and disk diffusion methods, widely used in clinical laboratories, did not demonstrate performance similar to that of the reference method (4).

The objective of our work was to analyze fosfomycin susceptibility testing results obtained when using MIC gradient strips and disk diffusion simultaneously performed with two different inoculum concentrations (10⁸ and 10⁶ CFU/ml) against a collection of 142 genetically unrelated P. aeruginosa clinical isolates (5). Molecular changes in the sequence of the glpT gene, responsible for coding the sole fosfomycin transporter (GlpT) in this species, were also assessed (6, 7).

P. aeruginosa ATCC 27853 was used as the control strain. Fosfomycin (Laboratorios Ern, Barcelona, Spain) MICs were determined by the reference agar dilution method (BBL Mueller Hinton II cation-adjusted agar; Becton-Dickinson, Sparks, MD), and their values were considered to be the reference (8). Since P. aeruginosa lacks the UhpT transport system, the addition of glucose-6-phosphate to the medium was unnecessary (6). MICs were also determined by Etest (bioMérieux, Marcy-l’Etoile, France) and disk diffusion (50 μg Oxoid; Thermo Fisher Scientific, Basingstoke, United Kingdom) methods with two different inocula: (i) a standard 1.5 × 10⁸ CFU/ml inoculum (0.5 McFarland standard) and (ii) a 1:100 dilution (1.5 × 10⁶ CFU/ml) of the standard suspension, which has been considered an alternative inoculum. This bacterial load was selected based on the results obtained in 5 isolates that were submitted to previous testing with an inoculum range of 10⁴ to 10⁸ CFU/ml. The growth was not confluent with an inoculum of <10⁶ CFU/ml, while mutant colonies appeared and affected the reading of the inhibition zone with a higher inoculum. Consequently, 10⁶ CFU/ml was chosen as the alternative inoculum. Susceptibility determination was performed in duplicate for each testing method. Fosfomycin ECOFF was applied for the susceptibility categorization of the isolates.

Statistical analysis was performed using the Stata statistical software (Data Analysis and Statistical Software version 11.0).

Comparison between the agar dilution and the Etest methods tested with 10⁸ and 10⁶ CFU/ml was performed by using the Bland-Altman method (9). The mean MIC values obtained with the two compared methods (x axis) were plotted against the difference between such MICs (y axis). Intraclass correlation coefficient (ICC) was also calculated. Values for the kappa coefficient were interpreted according to the Landis and Koch classification (10).

Categorical agreement (CA), essential agreement (EA), major errors (ME), and very major errors (VME) were calculated according to the published guidelines (11). Error levels were computed as percentages along with their corresponding exact 95% confidence intervals (CI).

Agar dilution and disk diffusion results were compared by regression analysis. Spearman correlation coefficient was also estimated.

The glpT gene was amplified by PCR and was sequenced according to previously published methods (6, 7). Nucleotide and amino acid sequences were analyzed with the sequence analysis software Clone Manager 9 (Scientific and Educational Software).

Considering the Etest results (Table 1), EA was 93.6% for MICs
performed with $10^6$ CFU/ml and 67.6% for the standard inoculum. CA was 95% and 78.2% for the $10^6$ CFU/ml inoculum and the $10^8$ CFU/ml inoculum, respectively. Regarding error rates, ME values were higher for the standard inoculum (22.2%) than for the alternative one (1.5%). In contrast, VME presented a rate of 31.2% for $10^6$ CFU/ml and 18.7% for $10^8$ CFU/ml.

However, a search for mutations in the $glpT$ gene in all 5 isolates that contributed to the VME with the alternative inoculum revealed no codifying mutations in this gene.

Kappa values were 0.73 (good agreement) and 0.35 (fair agreement) for the alternative and the standard inoculum, respectively. ICCs of 0.88 and 0.78 were obtained for the alternative inoculum and for the standard inoculum, respectively. Using Bland-Altman analysis, an enhanced level of agreement was also observed with the alternative inoculum (Fig. 1), as the majority of values were distributed between a range (EA). Linear fit presented a slightly negative slope but was also between a range. However, with the standard inoculum, plots were distributed in a wide range of values, and the linear fit presented a marked negative slope because at high fosfomycin concentrations, Etest using $10^8$ CFU/ml showed higher MICs exceeding the lower limit.

Concordance between disk inhibition zone diameters and agar dilution MICs was established by regression slope graphics (Fig. 2) and the Spearman coefficient, which was −0.81 for $10^6$ CFU/ml and −0.71 for $10^8$ CFU/ml.

Many clinical laboratories routinely perform in vitro fosfomycin susceptibility testing against $P. aeruginosa$ using diffusion methods; however, these methods have not been validated nor have they presented concordance with the reference method. The standard inoculum density of 0.5 McFarland standard has been accepted as a dogma for all microorganisms when performing these in vitro testing procedures (12, 13).

Fosfomycin mutant frequency in $Pseudomonas$ is $10^{-6}$ for the wild-type phenotype (6). The final inoculum used in microdilution and agar dilution methods is $10^4$ CFU (per well and prong, respectively) (14). However, when performing diffusion methods (initial inoculum, $10^6$ CFU/ml), $10^6$ CFU are plated onto the Mueller-Hinton agar (ca. 25 l are normally absorbed by a standard swab) (15), thus allowing the presence of an inherently resistant subpopulation. These mutants render colonies inside the inhibition zone nonreadable and lead to nonreproducible results. The use of $10^6$ CFU/ml as an alternative inoculum improves agreement values and the inhibition zone readings of the major bacterial population (Fig. 3). The influence of inoculum size was observed in previous studies (16).

The only discrepant value when using Etest with the alternative inoculum was the VME rate of 31.2%. Isolates (5/16) were resistant by agar dilution method (1 to 2 dilutions higher than the ECOFF value) but were susceptible with Etest (MIC, 128 µg/ml). However, they presented no codifying mutations in the $glpT$ sequence. This reinforces that these isolates belonged to the wild-type population rather than to the resistant one and should not be taken into consideration for VME. This discrepancy may be hypothetically attributed to the presence of undetermined borderline resistance mechanisms or even to the total number of resistant isolates ($n = 16$), which is lower than that required according to guidelines ($n = 30$) (11). Nevertheless, we cannot rule out the possibility of other potential resistance mechanisms that might
affect fosfomycin susceptibility. A homolog of the fosA gene that codifies a plasmid-encoded fosfomycin-inactivating enzyme in Enterobacteriaceae has been identified in the chromosome of P. aeruginosa, but its relevance in fosfomycin resistance still has to be demonstrated (17).

Finally, the validation of this alternative inoculum when testing fosfomycin and P. aeruginosa may allow for the more confident use of diffusion methods in clinical laboratories, giving more precise and reliable information about the actual susceptibility status of this species against this compound and the possibility of its use in combination with other antibiotics. Our study reinforces that different antibiotics might need different testing conditions, including inoculum size. This has been the case with the addition of 2% NaCl when testing oxacillin against staphylococci by agar or broth dilution, 50 μg/ml Ca²⁺ when testing daptomycin, or polysorbate 80 (P-80) when testing new lipoglycopeptides. Inoculum should be included as a new variable condition in susceptibility testing, particularly in antibiotics (such as fosfomycin) in which the frequency of resistance mutants is close to the inoculum used.

FIG 2 Scattergram results comparing the reference fosfomycin agar dilution MICs with disk diffusion inhibition zones (mm) resulting from two different inoculum concentrations: (A) 10⁶ CFU/ml and (B) 10⁸ CFU/ml.

FIG 3 Etest and disk diffusion techniques performed with an inoculum of 10⁸ CFU/ml (A) and 10⁶ CFU/ml (B) with a fosfomycin MIC of 64 μg/ml (reference agar dilution). Etest: (A) >1,024 μg/ml and (B) 32 to 48 μg/ml.
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