Role of mutant frequency on fosfomycin MIC discrepancies by agar dilution and microdilution method

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Background: Fosfomycin is an old antibiotic currently re-evaluated for the treatment of infections caused by multidrug resistant Enterobacteriaceae. Important discrepancies in the MIC have been described depending on the methodology used. The agar dilution (reference method for CLSI) uses 2-8 times less inoculum than microdilution method (10⁴ cfu/spot vs 2-8x10⁴ cfu/well). The aim of the study is to understand the role of the inoculum effect in fosfomycin activity in vitro.

Material/methods: 220 clinical isolates of E. coli and K. pneumonia were studied. MIC was determined by agar dilution and broth microdilution using the CLSI recommendations. In both methods initial inocula were measured. The mutant frequencies were assessed in a subset of 21 E. coli (MIC =1 mg/l) and 21 K. pneumoniae (MIC = 16 mg/l) isolates. Mutants were recovered in MHA plates supplemented with glucose-6-phosphate (25 mg/l) and fosfomycin (4, 16, 64 and 256x MIC for E. coli and 4 and 16x MIC for K. pneumoniae).

To evaluate the critical inoculum that selects resistant subpopulations, both resistant and total subpopulations were monitored for 4 clinical isolates of E. coli (MIC =1 mg/l) and E. coli ATCC 25922 (MIC =1 mg/l). For this purpose, a microdilution method with 0, 4 and 8 mg/l of fosfomycin during 24 h incubated at 37ºC, in a multimode microplate reader (Infinite 200Pro; OD₅₉₅nm) was used. Eight different bacterial inocula were used, ranging from 5x10⁵ cfu/well to 3.91x10³ cfu/ml (1:2 dilutions).

Results: Using agar dilution method as reference, 149 isolates were susceptible, 9 intermediate and 62 resistant to fosfomycin according to CLSI. For E. coli, 86.4% of categorical agreement (CA), 9.1% very major errors (VME), 3.3% major errors (ME) and 9.9% minor errors (mE) were found. For K. pneumoniae, CA was 51.1%, VME 15.7%, ME 28.4% and mE 25.2%. Essential agreement (+/- 1-log) was observed in 55.45%. By microdilution, however, 35.9% of isolates showed discrepancies of >= 2 dilutions compared to the agar dilution method. The initial inoculum count (mean) was 5.63 times higher in the microdilution method.
Fosfomycin mutant frequencies for *E. coli* were: $6.05 \times 10^{-5}$ (4xMIC), $5.21 \times 10^{-6}$ (16xMIC), $4.37 \times 10^{-6}$ (64xMIC), and $5.59 \times 10^{-7}$ (256xMIC). For *K. pneumoniae*: $1.49 \times 10^{-4}$ (4xMIC), and $1.58 \times 10^{-5}$ (16xMIC).

Subpopulations with increased MIC arose mainly after 8-hour incubations with inocula higher than $3.13 \times 10^4$ cfu/well (figure 1).

**Conclusions:** Important discrepancies were observed between agar dilution and microdilution method in the fosfomycin MIC. Discrepancies seem to be partially due to the presence of mutants in the initial inoculum used for each method. Small differences in the initial inoculum are enough to observe subpopulations with increased MIC to fosfomycin.