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Antimicrobial Susceptibility Studies

Discrepancies in fosfomycin susceptibility testing of KPC-producing *Klebsiella pneumoniae* with various commercial methods

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ABSTRACT

Fosfomycin susceptibility testing with Sensititre, Vitek2, Etest, Mic Strip Test and disk diffusion methodologies was compared versus reference agar dilution method (AD) with 78 clinical isolates of KPC-producing *Klebsiella pneumoniae*. All methodologies showed a Categorical Agreement and Essential Agreement of ≤69% and ≤72%, respectively, revealing a very low concordance with AD.

Carbapenem-resistant Enterobacteriaceae (CRE) have spread globally in the last decade, and represent one of the most critical challenges to antimicrobial chemotherapy (Nordmann et al., 2011; van Duin and Doi, 2017). Among CRE, *Klebsiella pneumoniae* is the species most affected by carbapenem resistance, and production of carbapenemases of different types (KPC, OXA-48-like, NDM, VIM and IMP) is the most prevalent resistance mechanism in this species (Nordmann et al., 2011; van Duin and Doi, 2017). In Italy, a country where CRE have achieved a high level of endemicity, *K. pneumoniae* strains producing KPC-type carbapenemases (KPC-KP) are by far the predominant CRE type (Giani et al., 2017).

Antibiotic treatment options for carbapenemase-producing *K. pneumoniae* are limited. Fosfomycin, an old antibiotic that inhibits the early stages of peptidoglycan synthesis (Michalopoulos et al., 2011), has long been used for treating uncomplicated urinary tract infections (Keating, 2013) and has recently attracted interest as an anti-CRE agent (Endimiani et al., 2010; Reffert and Smith, 2014). Indeed, recent studies revealed that many CRE strains retain susceptibility to fosfomycin (Kaase et al., 2014), and that fosfomycin has a synergistic effect with carbapenems (meropenem and ertapenem), colistin, aminoglycosides (netilmicin, amikacin) and tigecycline against KPC-KP (Evren et al., 2013; Samonis et al., 2012; Yu et al., 2017).

For these reasons, intravenous fosfomycin, in combination with other agents, is now considered a valuable anti-CRE option (Reffert and Smith, 2014), and the accuracy of susceptibility testing of fosfomycin with CRE strains has become a crucial issue in clinical microbiology laboratories.

According to EUCAST and CLSI, agar dilution (AD) is the reference method for fosfomycin susceptibility testing (CLSI, 2018; EUCAST, 2018). However, AD is not suitable for use in routine susceptibility testing.

The aim of this study was to determine the susceptibility to fosfomycin of a collection of clinical isolates of KPC-KP with agar dilution, and to compare the results with those obtained using automated, disk diffusion and gradient diffusion systems.

A total of 78 non-replicated KPC-KP clinical isolates (44 from rectal-swabs, 21 from urine cultures, 3 from sputum cultures, 7 from blood cultures, and 3 from wound swabs) were collected from 2015 to 2017 at the Clinical Microbiology Laboratory of San Luca Hospital (Lucca, Italy), and were investigated in this study. *Escherichia coli* ATCC 25922 was used as a quality control strain. Identification was carried out by MALDI-ToF (Vitek MS; bioMérieux, Marcy l'Étoile, France). Production of KPC was confirmed in all strains by the KPC K-Set immunochromatographic assay (Coris BioConcept, Gembloux, Belgium).

The same 0.5 McFarland inoculum, prepared from an overnight culture in blood agar plates, was used for fosfomycin testing with

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reference AD (EUCAST, 2018; http://www.eucast.org/clinical_breakpoints/) and the following methods: Sensititre (ITGNEGF panel, with fosfomycin wells added with glucose-6-phosphate (G6P), 25 μg/mL, Thermo Fisher Scientific, Waltham, MA, USA); Vitek2 (AST 201 card, bioMérieux); Etest (bioMérieux), disk diffusion (200 μg fosfomycin/50 μg G6P disks; Bio-Rad Laboratories, Hercules, CA); and MIC Test Strip (performed with 50 of the 78 KPC-KP isolates, randomly selected; Liofilchem, Roseto degli Abruzzi, Italy). All commercial methods were performed as recommended by the manufacturer. The MIC results were interpreted according to the EUCAST clinical breakpoints (susceptible ≤32 μg/mL, resistant >32 μg/mL) (EUCAST, 2018). For disk diffusion, reading of results was performed and interpreted as recommended by EUCAST (2018) for *E. coli*, since no interpretation criteria are present for *K. pneumoniae*.

Agreement of results obtained with different methods in comparison with reference AD was evaluated according to the International Organization for standardization ISO 20776–2, 2007 standard (ISO). Four parameters were used to compare different methods: essential agreement (EA), categorical agreement (CA), major errors (ME), and very major errors (VME). MIC values between doubling dilutions obtained by gradient diffusion methods were rounded up to the nearest doubling dilution. EA was fulfilled when the MIC value obtained with the commercial method was within ±1 doubling dilution compared with that by reference AD. CA was defined as the percentage of isolates classified in the same susceptibility category by the reference AD and the other method. Categorical discrepancies were classified as ME when an isolate was categorized as resistant by one of the methods but susceptible by AD, or VME when an isolate was categorized as susceptible by one of the methods but resistant by reference AD. Acceptable performance was evaluated according to the criteria established by the ISO as follows: ≥90% for EA or CA, ≤ 3% for VME or ME (ISO 20776–2, 2007).

Among the 78 KPC-KP isolates, 45 (57.7%) were susceptible to fosfomycin by AD. Table 1 shows MIC distribution, the percentage of isolates classified as susceptible and resistant to fosfomycin by each testing method, and the values of CA, ME, VME and EA. Overall, the EA and CA for all the commercial methods were in poor agreement with the reference method, with no method being acceptable according to the ISO criteria.

Sensititre and Etest exhibited high rates of VME (54.5% and 78.8%, respectively). Therefore, these two methods are more likely to underestimate MIC values compared to the AD reference method. On the other hand, Vitek2, disk diffusion and MIC Test Strip exhibited high rates of ME (75.6%, 84.4% and 76.7%, respectively). Therefore, these three methods are more likely to overestimate MIC values compared to the AD reference method (Fig. 1).

It should be noted that the reference method for fosfomycin susceptibility testing recommended by ISO 2776–1, 2006 standard is agar dilution since broth microdilution may not give reliable results (ISO 2776–1, 2006).

Altogether, our results indicated that the commercial systems evaluated in this study exhibit a poor correlation with reference AD for fosfomycin susceptibility testing with KPC-KP clinical isolates.

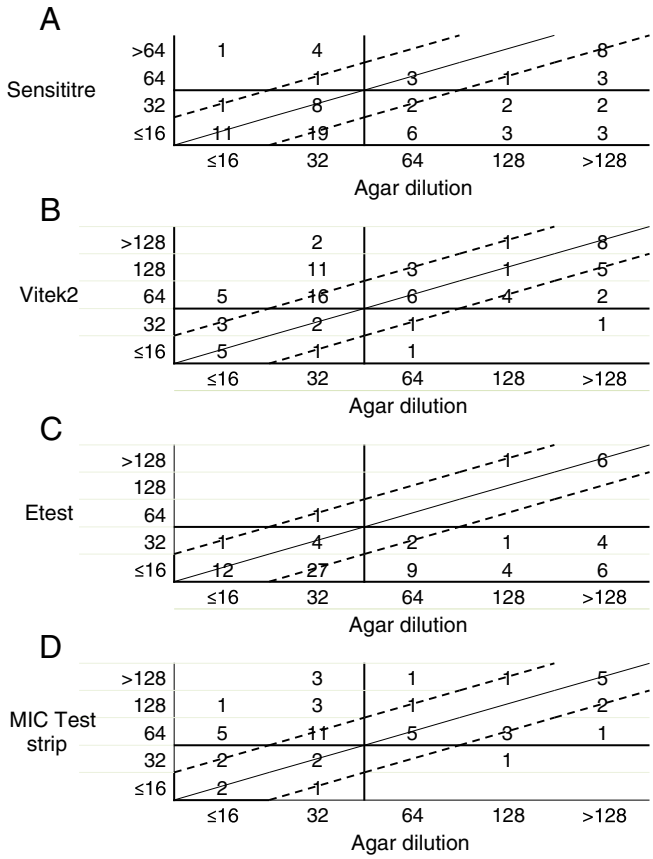

Fig. 1. Scattergram of fosfomycin MICs for KPC-KP tested measured by Agar dilution and Sensititre (A), Vitek2 (B), Etest (C), and MIC Test strip (D). MICs were indicated in μg/mL.

These results are in overall agreement with those of other studies, which have shown that the result of fosfomycin susceptibility testing is dependent on the method used and the microorganisms tested (de Cueto et al., 2006; Perdigão-Neto et al., 2014). Focusing the attention on carbapenemase producing *Enterobacteriaceae* other studies evaluating Etest and disk diffusion showed a poor correlation of these methods with agar diffusion (Kaase et al., 2014; Endimiani et al., 2010; Perdigão-Neto et al., 2014). However, since these studies tested a lower number of isolates, used different reference breakpoints and different methods, the results are not directly comparable.

With disk diffusion and MIC Test Strip, the colonies grown within the inhibition halo (observed with 31.5% and 25% of the strains, respectively) were not taken into account in the MIC reading, as recommended by EUCAST (2018) and by the manufacturer (Liofilchem), respectively. With Etest, the macrocolonies grown within the inhibition halo (observed with 29.7% of the strains) were not taken into account if the number was <5 as recommended by the

Table 1
Results of fosfomycin susceptibility testing with KPC-KP using reference Agar dilution, Sensititre, Vitek2, Etest, disk diffusion and MIC test strip. Seventy-eight KPC-KP strains were evaluated will all methods except for MIC Test Strip (N = 50).

	MIC (μg/mL)					Category (%)		CA (%)	ME	VME	EA
	≤16	32	64	128	>128	S	R				
Agar dilution	13	32	11	6	16	45 (57.7)	33 (42.3)				
Sensititre	42	15	8	13[a]	-	57 (73.1)	21 (26.9)	54 (69.2)	6 (13.3)	18 (54.5)	54 (69.2)
Vitek2	7	7	33	20	11	14 (17.9)	64 (82.1)	41 (52.6)	34 (75.6)	3 (9.1)	56 (71.8)
Etest	58	12	1	-	7	70 (89.7)	8 (10.3)	51 (65.4)	1 (2.2)	26 (78.8)	54 (69.2)
Disk diffusion[b]	-	-	-	-	-	8 (10.3)	70 (89.7)	39 (50)	38 (84.4)	1 (3)	-
MIC Test Strip	3	5	25	7	10	8 (16)	42 (84)	26 (52)	23 (76.7)	1 (5)	36 (72)

[a] Sensititre range ≤ 16- >64 μg/mL.
[b] Zone diameter interpretation breakpoint referred to *E. coli* (EUCAST, 2018).

manufacturer (bioMérieux). The presence of these colonies makes reading difficult, and could impair accuracy of results if reading is performed by unexperienced personnel.

A limitation of this study is that the isolates were collected from a single center and were only KPC-KP. It will be interesting to further investigate the performance of various fosfomycin-testing methods with larger collections of CRE representative of different species and different resistance mechanisms. Another limitation is that all strains were tested only once, and therefore reproducibility of results was not evaluated.

These findings therefore suggest that results of susceptibility testing of fosfomycin obtained with methods other than AD should be considered with caution. Since no results obtained with the investigated methods fell within the ISO parameters, laboratory routines should always carry out the AD to test fosfomycin susceptibility, especially for KP-KPC isolates from critical patients.

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