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Short Communication

Performance of disk diffusion and broth microdilution for fosfomycin susceptibility testing of multidrug-resistant clinical isolates of Enterobacterales and *Pseudomonas aeruginosa*

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ABSTRACT

Objectives: This study aimed to evaluate the susceptibility of clinical isolates of Enterobacterales and *Pseudomonas aeruginosa* to fosfomycin and to determine the concordance of disk diffusion (DD) and broth microdilution (BMD) with agar dilution (AD) for fosfomycin susceptibility testing.
Methods: The activity of fosfomycin against 225 clinical isolates of *Escherichia coli* ($n = 64$), *Klebsiella pneumoniae* ($n = 68$), *Enterobacter* spp. ($n = 28$) and *P. aeruginosa* ($n = 65$) was tested by AD, DD and BMD. For DD, results were recorded considering and not considering colonies growing within the inhibition halo as recommended by the CLSI and EUCAST, respectively. *Escherichia coli* breakpoints were used for all Enterobacterales. Results were reported as categorical agreement (CA), major error (ME; false-resistant), very major error (VME; false-susceptible) and minor error (any other discrepancies).
Results: Fosfomycin susceptibility of all tested species was >90% by AD. Following CLSI guidelines, DD was the only method reaching ≥90% CA with AD for *E. coli* and *K. pneumoniae*, albeit yielding 6% ME. Neither DD nor BMD achieved acceptable CA percentages for *Enterobacter* spp. Following EUCAST guidelines, none of the methods had CA ≥ 90%. For Enterobacterales, the best performance of DD is achieved when read as indicated by EUCAST but interpreted according the CLSI breakpoints (>97% CA; 0% VME; ≤2% ME). For *P. aeruginosa*, BMD yielded the best results (89% CA; 0% VME; 11% ME).
Conclusion: Neither DD or BMD provide accurate results owing to unacceptable ME and VME percentages even when performed as intended by the guidelines.

1. Introduction

The evolution and spread of various mechanisms of antimicrobial resistance among Enterobacterales and *Pseudomonas aeruginosa* has contributed to limited treatment options for infections caused by these micro-organisms [1]. *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter* spp. are among the most frequent Gram-negative bacteria causing multidrug-resistant (MDR) infections owing to the presence of β-lactamases such as extended spectrum β-lactamases (ESBLs) and carbapenemases, which can confer resistance to cephalosporins and carbapenems, respectively. Increasing β-lactam resistance rates worldwide has renewed interest in the use of fosfomycin, a phosphonic acid derivative that irreversibly inhibits MurA (UDP-*N*-acetylglucosamine-3-enolpyruvyl transferase), the enzyme responsible for the first step in peptidoglycan synthesis [2]. Fosfomycin is a broad-spectrum agent frequently active against MDR Enterobacterales and some isolates of MDR P. aeruginosa [3]. Fosfomycin is also regarded as an antibiotic with attractive pharmacokinetic properties, explaining its potential value in complicated and frequently deep-seated infections such as infections of the central nervous system, bone and joints, lungs and soft tissues as well as sepsis, complicated urinary tract infections and acute pyelonephritis [4,5].

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Despite numerous reports of its activity, broader clinical use of fosfomycin is hindered by the difficulty in performing susceptibility testing [6,7]. Only two methods for testing are approved by the Clinical and Laboratory Standard Institute (CLSI): the agar dilution (AD) reference method, supplemented with 25 mg/L of glucose-6-phosphate (G6P); and disk diffusion (DD) with 200 μg fosfomycin plus 50 μg G6P disks (200DD). Both methods have been approved for *E. coli* recovered from urinary tract infections but not for other Enterobacterales [8]. Likewise, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends using AD for Enterobacterales and *P. aeruginosa*, and DD with 200DD only for *E. coli* [9]. These two methods are time consuming for laboratory personnel as they require manual processing. Furthermore, in the DD test, growth of isolated colonies within the inhibition halo has generated confusion at reading and interpretation of the assay. Whilst the CLSI does not have any recommendation about this, EUCAST and the Comité de l'antibiogramme de la Société Française de Microbiologie (CA-SFM) [10] explicitly recommend to not take into account isolated colonies within the inhibition zone when reading the test. On the other hand, neither the CLSI nor EUCAST recommend the use of broth microdilution (BMD) [8,9], hence automated susceptibility testing, commonly used in most clinical microbiology laboratories, should not be used to assess fosfomycin susceptibility.

Because AD is so laborious, many clinical laboratories are using DD and/or BMD to evaluate the susceptibility to fosfomycin of other bacterial species, despite the facts that the DD zone diameter breakpoints have only been approved for *E. coli* and that BMD is not recommended for any species. Herein, in addition to determining the antimicrobial activity of fosfomycin against MDR clinical isolates of Enterobacterales and *P. aeruginosa*, we evaluated the performance of different susceptibility methods for *E. coli*, and the correlation of these assays with AD when used for other species.

2. Materials and methods

A total of 225 non-duplicate clinical isolates of *E. coli* ($n = 64$), *K. pneumoniae* ($n = 68$), *Enterobacter* spp. ($n = 28$) and *P. aeruginosa* ($n = 65$) were selected from our repository, which contains strains compiled through the Colombian Bacterial Resistance Surveillance Network. Isolates included in the study were collected between February 2016 and October 2017 from healthcare institutions located in 13 Colombian cities and were selected based on their antimicrobial susceptibility profile (isolates resistant to third-

Table 1
Clinical categorisation of fosfomycin susceptibility testing results by agar dilution (AD), disk diffusion (DD and DD*) and broth microdilution (BMD) according to CLSI and EUCAST breakpoints.

Species	No. of isolates	Method	CLSI			EUCAST	
			S	I	R	S	R
Escherichia coli	64	AD	97%	0%	3%	95%	5%
		DD	95%	0%	5%	81%	19%
		DD*	88%	3%	9%	67%	33%
		BMD	80%	8%	12%	69%	31%
Klebsiella pneumoniae	68	AD	99%	1%	0%	96%	4%
		DD	96%	3%	1%	62%	38%
		DD*	90%	4%	6%	59%	41%
		BMD	85%	9%	6%	76%	24%
Enterobacter spp.	28	AD	93%	0%	7%	89%	11%
		DD	93%	0%	7%	43%	57%
		DD*	75%	7%	18%	25%	75%
		BMD	75%	11%	14%	61%	39%
Pseudomonas aeruginosa	65	AD				98%	2%
		DD				77%	23%
		DD*				71%	29%
		BMD				88%	12%

CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; S, susceptible; I, intermediate; R, resistant; DD, disk diffusion without taking into account the isolated colonies (EUCAST); DD*, disk diffusion taking into account the isolated colonies (CLSI).
Shadowed rows represent the values obtained as recommend by each guideline.

generation cephalosporins or carbapenems). To avoid inclusion of genetically highly related isolates, those with any epidemiological, spatial or temporal links were excluded. Species identification of all strains was confirmed by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF/MS) (bioMérieux, Marcy-l'Étoile, France). AD was performed on Mueller–Hinton agar (MHA) using fosfomycin sodium disalt (Sigma Chemical Co., St Louis, MO, USA) supplemented with 25 mg/L G6P (Sigma Chemical Co.) as recommended by the CLSI [6].Two-fold dilutions across a range of 1–256 mg/L fosfomycin was used. The minimum inhibitory concentration (MIC) was considered as the lowest concentration of antimicrobial agent that completely inhibited visible growth, therefore single colonies or faint haze were disregarded [11]. DD was performed on MHA with 200DD (Becton Dickinson, Sparks, MD, USA) following the protocols described by the CLSI and EUCAST [8,9]. Two readings of the inhibition halo were recorded, taking and not taking into account the colonies growing within the inhibition zone, the later as recommended by EUCAST [8,9]. BMD was performed using customised Sensititre™ plates CMP1COLM (Trek Diagnostic Systems, East Grinstead, UK) following the manufacturer's recommendations. The MIC for BMD was recorded as the lowest concentration of antimicrobial agent that completely inhibited visible growth (which included any turbidity or small pellet) in the microdilution well [8]. *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as control strains in all assays.

Susceptibility results were interpreted according to available breakpoints set by the CLSI (susceptible at an MIC of ≤64 μg/mL for *E. coli* and extrapolated to the Enterobacterales) and EUCAST [susceptible at an MIC of ≤32 μg/mL for Enterobacterales and epidemiological cut-off (ECOFF) of ≤128 μg/mL for *P. aeruginosa*]. Results of BMD were interpreted according to AD breakpoints. DD results for Enterobacterales were interpreted according to the established zone diameter breakpoints for *E. coli* of the CLSI (≥16 mm, susceptible; 13–15 mm, intermediate; ≤12 mm, resistant) and EUCAST (≥24 mm, susceptible; <24 mm resistant); for *P. aeruginosa*, a zone diameter of 12 mm was used as the cut-off for susceptibility according to EUCAST [8,9]. Concordance of DD and BMD with the gold-standard method of AD was reported in terms of categorical agreement (CA), defined as matching results between the two methods based on the interpretative breakpoint proposed; false-resistant results were considered to be major errors (ME), false-susceptible results were considered to be very major errors (VME) and all other discrepancies were considered to be minor errors (mE). For a susceptibility test to be adequate, the CLSI recommends that it should yield <10% mE, 3% ME and 1.5% VME rates [12].

3. Results and discussion

In line with previously published data, susceptibility reports obtained by AD confirmed that fosfomycin is very active against MDR *E. coli*, *K. pneumoniae*, *Enterobacter* spp. and *P. aeruginosa* isolates regardless of the breakpoints applied (Table 1) [13–16]. However, due to the higher breakpoints established by the CLSI, susceptibility rates for all species increase when these are applied.

For instance, the MIC_{50} and MIC_{90} values were 2 μg/mL and 16 μg/mL for *E. coli*, 8 μg/mL and 32 μg/mL for *K. pneumoniae*, and 8 μg/mL and 64 μg/mL for *Enterobacter* spp. strains, respectively. Applying the CLSI established breakpoints to interpretation of the AD results, it was found that 97%, 99% and 93% of the *E. coli*, *K. pneumoniae* and *Enterobacter* spp. strains, respectively, were susceptible to fosfomycin. However, when EUCAST breakpoints are applied, these numbers decreased to 95%, 96% and 89%, respectively. Notably, susceptibility rates were similar between isolates displaying resistance to third-generation cephalosporins and carbapenems (data not shown). For *P. aeruginosa*, the MIC_{50} was 64 μg/mL, the MIC_{90} was 128 μg/mL, and 98% of the isolates had an MIC lower than or equal to the ECOFF (128 μg/mL).

Table 1 shows that susceptibility rates decreased when DD results were read and interpreted following each guideline compared with those obtained by AD. As indicated by the data

Table 2
Correlation of disk diffusion (DD and DD*) and broth microdilution (BMD) with agar dilution by the CLSI and EUCAST.

Species	No. of isolates	Method	CLSI (% of isolates)				EUCAST (% of isolates)		
			CA	VME	ME	mE	CA	VME	ME
Escherichia coli	64	DD	98	0	2	0	83	2	16
		DD*	91	0	6	3	69	1	30
		BMD	80	1	11	8	67	3	30
Klebsiella pneumoniae	68								
		DD	97	0	2	2	66	0	34
		DD*	90	0	6	4	63	0	37
		BMD	87	0	6	7	81	0	19
Enterobacter spp.	28	DD	100	0	0	0	54	0	46
		DD*	82	0	11	7	36	0	64
		BMD	79	0	11	11	64	4	32
Pseudomonas aeruginosa	65	DD					78	0	22
		DD*					72	0	28
		BMD					89	0	11

CLSI, Clinical and Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; CA, categorical agreement, VME, very major error (false-susceptible); ME, major error (false-resistant); mE, minor error (any other discrepancies); DD*, disk diffusion taking into account the isolated colonies (CLSI); DD, disk diffusion without taking into account the isolated colonies (EUCAST).
Shading represents the values obtained as recommend by each guideline.

presented in Table 2 and in the scattergrams presented in Supplementary Figs. S1–S7, these methodologies yield a percentage of ME higher than that allowed by the CLSI (3%), overestimating resistance. Therefore, resistant isolates need to be confirmed by the gold-standard method (AD). However, our analysis revealed that, for Enterobacterales, the DD assay achieves the best performance (as measured by % of CA with AD, % VME and % ME) when read as recommended by EUCAST but interpreted using the CLSI breakpoints. This is especially relevant for *E. coli* and *Enterobacter* spp. given that 69% and 67% of the strains, respectively, displayed isolated colonies within the inhibition halo. In contrast, only 19% of *K. pneumoniae* and 18% of *P. aeruginosa* isolates showed this behaviour. Presumably, the differences in the presence of those 'breakthrough' colonies explain why BMD is the least concordant method to evaluate fosfomycin susceptibility for *E. coli* and *Enterobacter* spp., but is the most concordant assay with AD for *K. pneumoniae* and *P. aeruginosa* when the EUCAST breakpoints are used. Regardless, BMD cannot be recommended as an effective antimicrobial susceptibility test because for *E. coli* and *Enterobacter* spp. it increases the ME and VME to percentages that are unacceptable for an antimicrobial susceptibility test, and for *K. pneumoniae* it does not reach the minimum percentage of CA required (90%) [12]. The current results thus confirm the poor performance of DD and BMD for fosfomycin susceptibility testing of Enterobacterales, including KPC-producing *K. pneumoniae* isolates, reported previously by other groups [17,18]. Of note, we did not observe the 'skipped well' phenomenon reported by other authors when performing BMD [19].

In the case of *P. aeruginosa*, although BMD is the most concordant method with the gold standard (CA 89%), the results of the current study show that the percentage of ME exceeds the acceptable limit, hence resistant isolates should be confirmed by the gold-standard method (AD). This result is in line with a previous study by Díaz-Aguilar et al. in which following EUCAST recommendations the concordance between AD and BMD was higher than the concordance of AD and DD for assessment of fosfomycin susceptibility among 206 strains of *P. aeruginosa* in Spain [20]. Remarkably, the current analysis indicated that for *P. aeruginosa* BMD achieves the best performance in terms of CA (89%), VME (0%) and acceptable ME (11%).

4. Conclusions

In this study, we demonstrate the excellent activity of fosfomycin against this collection of Colombian MDR Enterobacterales and *P. aeruginosa* clinical strains. Studies of antimicrobial resistance rates are fundamental for therapeutic decisions, and the microbiology laboratory has an important role in the correct reporting of antimicrobial susceptibility profiles. Therefore, it is imperative to implement the best method to deliver reliable results in a timely manner. In this sense, the gold-standard method of AD is not a valid option for many clinical laboratories. However, according to the current analysis, the alternative methods as intended by the guidelines do not provide accurate results owing to unacceptable ME and VME percentages. Indeed, the best performance of DD is achieved when read as indicated by EUCAST but interpreted according the CLSI breakpoints. Although this study has limitations, including a small number of isolates and sampling bias for MDR isolates, the results suggest that a thorough revision of the approved methods and/or breakpoints is needed to ensure that the approved methods for fosfomycin susceptibility testing yield accurate and timely results.

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Competing interests

MVV and CJP have received consulting fees and/or research grants from Merck Sharp & Dohme, Pfizer, WEST and GPC Pharma; CH-G is currently an employee of MSD Colombia. All other authors declare no competing interests.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jgar.2020.01.003.

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