Journal of Global Antimicrobial Resistance 23 (2020) 334–337

Contents lists available at ScienceDirect

Journal of Global Antimicrobial Resistance

journal homepage: www.elsevier.com/locate/jgar

Short Communication

Gold standard susceptibility testing of fosfomycin in *Staphylococcus aureus* and Enterobacterales using a new agar dilution panel®

Floriana Campanile[a,*], Mandy Wootton[b], Leanne Davies[b], Ausilia Aprile[a], Alessia Mirabile[a], Stefano Pomponio[c], Federica Demetrio[c], Dafne Bongiorno[a], Timothy R. Walsh[d], Stefania Stefani[a], Maria Lina Mezzatesta[a]

[a] Department of Biomedical and Biotechnological Sciences, section of Microbiology, University of Catania, Italy
[b] Specialist Antimicrobial Chemotherapy Unit, Public Health Wales, University Hospital of Wales, Cardiff, United Kingdom
[c] Scientific division, Liofilchem S.r.l. Roseto degli Abruzzi (TE), Italy
[d] Department of Zoology, University of Oxford, United Kingdom

ARTICLE INFO

Article history:
Received 21 July 2020
Accepted 27 August 2020
Available online 27 October 2020

Keywords:
Staphylococcus aureus
Enterobacterales
Fosfomycin
Agar dilution
Commercial AD panel

ABSTRACT

Objectives: Many clinical laboratories have difficulty in routinely performing *in vitro* fosfomycin susceptibility testing using the agar dilution (AD) method, considered to be the gold standard method. The objective of our work was to evaluate a rapid commercial fosfomycin agar dilution panel against clinical *Staphylococcus aureus* and Enterobacterales strains, in two different centres located in Italy and in the UK.

Methods: A total of 99 Enterobacterales (mostly *Escherichia coli* and *Klebsiella pneumoniae*) and 80 *S. aureus* clinical isolates was used to evaluate the commercial device, a 12-well panel containing fosfomycin incorporated into CA-MH agar supplemented with 25 mg/L of glucose-6-phosphate (Liofilchem S.r.l., Roseto degli Abruzzi, Italy). Testing was performed in two centres (Italy and UK) and kit results were compared against the gold standard in-house AD MIC method.

Results: According to the EUCAST breakpoints, fosfomycin inhibited 61% of the *S. aureus* strains, and 76% of the Enterobacterales isolates tested by the AD reference method. There was a Categorical Agreement (CA) of 100% and an Essential Agreement (EA) of 91.25% for *S. aureus*; while the Enterobacterales strains showed a CA of 94% and an EA of 97%. No evaluation errors were observed among *S. aureus*, while 5% Major Error and 1% Very Major Error were observed for the Enterobacterales.

Conclusions: Our results confirmed the feasibility of determining fosfomycin susceptibility using a commercial AD panel as a routine substitution for the AD test. The few differences observed were only in strains with MICs around the breakpoint used.

1. Introduction

Because of the increasing number of multidrug-resistant (MDR) bacteria, there has been a revival in the clinical use of older drugs [1]. Fosfomycin, first discovered in 1969, possesses broad-spectrum activity against both Gram-positive (*Staphylococcus aureus* including methicillin-resistant (MRSA) strains and most coagulase-negative staphylococci, less active against enterococci) and Gram-negative bacteria (including *Escherichia coli*, other Enterobacterales, and less active against *Pseudomonas aeruginosa*), by inhibiting bacterial cell wall synthesis [2]. Throughout Europe, the drug is widely used as a monotherapy for urinary tract infections, and is also used in combination to treat many severe infections including pneumonia, osteomyelitis, meningitis, surgical infections, arthritis, septicaemia, peritonitis, cervical lymphadenitis, diabetic foot infections, and typhoid fever [2,3].

Good efficacy for fosfomycin has been seen clinically against Enterobacterales with extended MDR, including extended-spectrum beta lactamase (ESBL) producers, being at least 90% susceptible [4].

Clinical use of fosfomycin requires *in vitro* testing of the drug, for correct categorization in the clinical reports. Disk diffusion, the main method for susceptibility testing in laboratories, and broth microdilution (BMD), the main reference method recommended by both EUCAST and CLSI, have been shown to give inaccurate results for fosfomycin [5]. Agar dilution (AD) is considered to be the

* Corresponding author.
E-mail address: f.campanile@unict.it (F. Campanile).

http://dx.doi.org/10.1016/j.jgar.2020.08.025

only approved method for testing fosfomycin susceptibility MIC values (EUCAST, 2020; CLSI, 2018) [6,7]; however, it is cumbersome, requires expertise not evident in most diagnostic laboratories, and is not routinely performed [8].

In this study, we evaluated the performance of a simpler, rapid, and less time-consuming panel to detect the *in vitro* activity of fosfomycin against a large sample of *S. aureus* and Enterobacterales clinical isolates, compared with the AD gold standard recommended.

2. Materials and methods

2.1. Isolate collection

In Italy, a total of 80 previously published *S. aureus* (70 MRSA and 10 methicillin-susceptible *S. aureus* (MSSA)) were selected from a collection of molecularly characterized (ST and SCC*mec*) clinical isolates recovered in 2012 from documented blood stream infections, lower respiratory tract infections, and skin and soft-tissue infections, as part of a national Italian survey [9,10]; the MRSA strains belonged to major worldwide spread clones (ST22/IVh; ST228/I; ST5/II; ST8/IV) and minor clones [9,10]; eight strains were not genotypically characterized. In the UK, a total of 99 Enterobacterales isolates (69 *E. coli*, 27 *Klebsiella pneumoniae*, and three other species) collected within the last 5 years from international sepsis samples was used. These isolates were sequenced, annotated, and possessed a range of ESBL genes and carbapenem-resistance genes. *S. aureus* ATCC® 29213 and *E. coli* ATCC® 25922 were used as reference strains.

2.2. MIC determination by in-house agar dilution

To determine the reference MIC values for fosfomycin, an in-house agar dilution method using cation-adjusted Mueller-Hinton agar (CA-MHB) (Difco, Detroit, MI) supplemented with 25 mg/L of glucose-6-phosphate (Sigma Aldrich Co, Italy & UK) was used [11], as recommended by Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2018) [7]. Fosfomycin (Sigma-Aldrich, St. Louis, MO, USA) was tested over a range of dilutions 0.25–256 mg/L. All experiments were repeated three times, using daily freshly prepared plates and inoculum. The fosfomycin breakpoints for the interpretative criteria for clinical isolates were used according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) [6]. Accordingly, *S. aureus* and Enterobacterales isolates with fosfomycin MICs of >32 mg/L were categorized as resistant.

2.3. Commercial AD fosfomycin panel

The commercial AD fosfomycin panel (Liofilchem S.r.l., Roseto degli Abruzzi, Italy) has 12 wells containing the antibiotic incorporated into an agar medium in different concentrations, i.e. 11 two-fold dilutions (0.25–256 mg/L) (www.liofilchem.com/ifu-sds), containing 25 mg/L of glucose-6-phosphate. The panel was used according to the manufacturer's guidelines. A 0.5 McFarland bacterial suspension was made for each isolate, then diluted 1:10 using sterile saline. Into each well, 2 μL of the diluted bacterial suspension was dispensed onto the agar surface (approximately 10^4 CFU/mL in each well). The growth-control well (no antimicrobial agent) was inoculated first followed by the antimicrobial-containing wells, starting with the lowest concentration (Fig. 1).

2.4. Definitions and analysis

Categorical Agreement (CA) and Essential Agreement (EA) *plus* Major Error (ME) and Very Major Errors (VME) were calculated. EA was defined as MIC differences ± 1 dilution; VME was defined when isolates were susceptible by the commercial AD panel but resistant by the reference AD method; and ME was defined when isolates were resistant by the commercial AD panel but susceptible by the reference AD method.

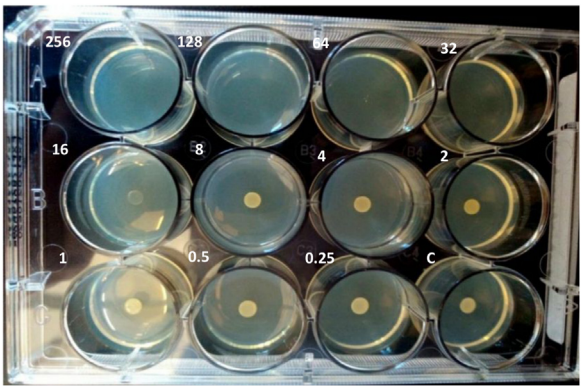


Fig. 1. Representation of the commercial AD fosfomycin panel. Fosfomycin MIC range: 0.25–256 mg/L; final *inoculum* 2 μL (10^4 CFU per spot); C, growth control.

3. Results

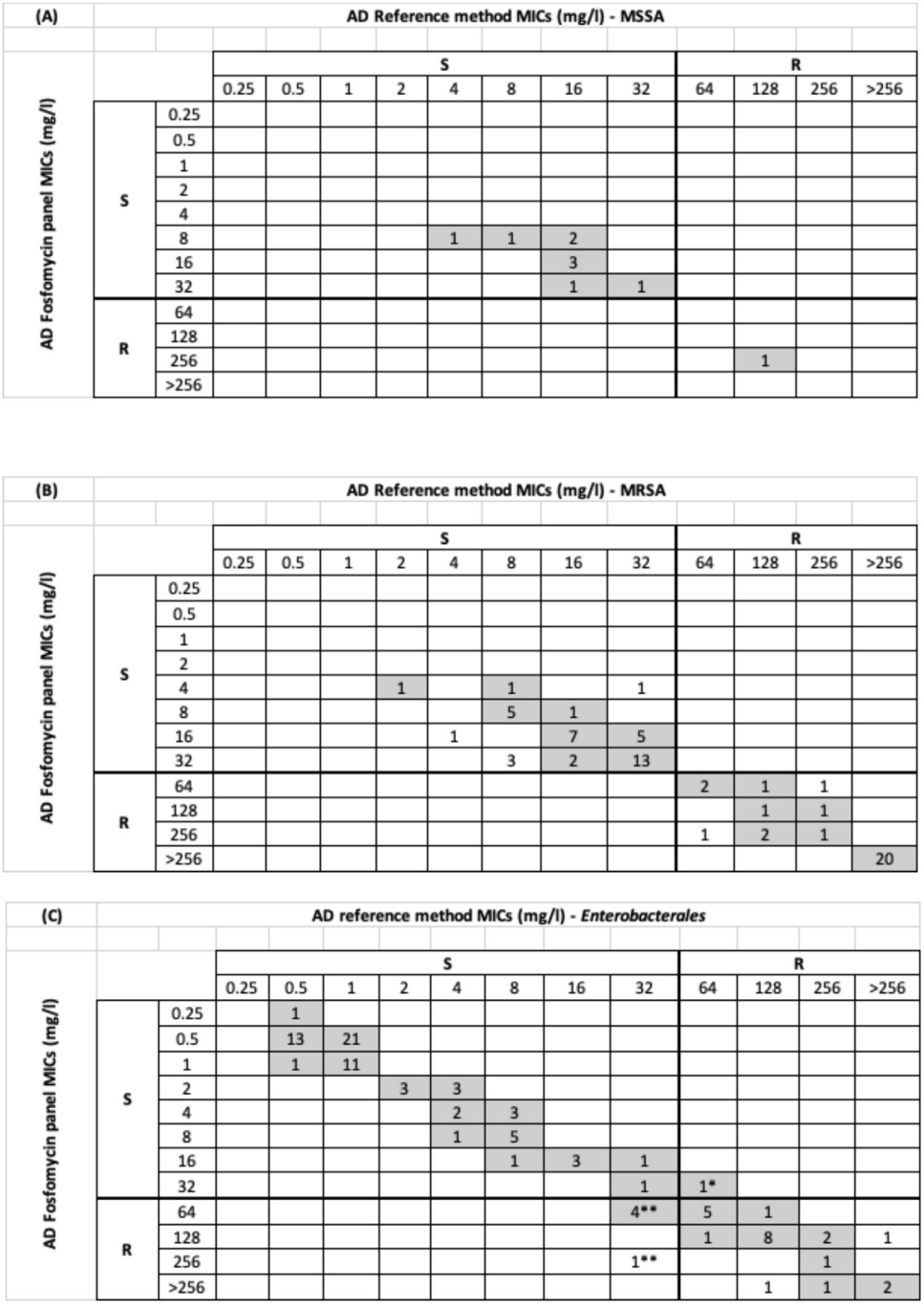
MIC distributions of the 10 MSSA, 70 MRSA, and 99 Enterobacterales, obtained by AD reference method compared with the commercial AD fosfomycin kit, are shown in Fig. 2a–c, respectively. The percentages of isolates classified as susceptible and resistant to fosfomycin, by each testing method, and the values of EA, ME, and VME are also included. According to the selected breakpoints (EUCAST 2020, CLSI 2018), fosfomycin inhibited 61% of all the *S. aureus* strains, with a MIC range from 2 to 32 mg/L; only one MSSA showed fosfomycin-resistance with an MIC of 128 mg/L), while 30 out of 70 MRSA strains were resistant (42.8%). No differences in MIC distribution were found among the diverse MRSA clones analysed.

Among 99 Enterobacterales isolates, 75 (76%) were susceptible to fosfomycin, tested by the reference AD method, with MIC values between 0.5 and 32 mg/L. This included 54 *E. coli*, 19 *K. pneumoniae* and two other Enterobacterales.

When comparing the results of a commercial AD fosfomycin panel with those obtained from the in-house agar dilution, EA (within 1 $\log_2$) for MRSA was 90.0% and for MSSA was 100%. CA, for both MRSA and MSSA, was 100% and no discordant results, categorized as ME and VME, were observed. For Enterobacterales, the EA was 97% (96 out of 99) with CA of 94% and ME and VME rates of 5% and 1%, respectively. For one *E. coli* isolate, a VME was seen with a 1x$\log_2$ dilution difference, 64 mg/L using the reference in-house AD, and 32 mg/L with the commercial AD panel; MEs in five isolates: two strains of *E. coli* and two strains of *K. pneumoniae* showed a 1x$\log_2$ dilution difference (32 vs. 64 mg/L) while for one *E. coli* isolate, a ME was seen with a 2x$\log_2$ dilution difference (32 vs. 256 mg/L).

4. Discussion

The unremitting challenge against the rise of hospital infections sustained by MDR isolates is currently one of the most serious clinical problems, worsened by the lack of novel antimicrobials, with valid benefits in treating MDR bacteria [12]. At the same time, we are witnessing a revival of 'old' antibiotics developed decades ago [1]. Fosfomycin has drawn significant attention in recent years



(A)

AD Fosfomycin panel MICs (mg/l)		AD Reference method MICs (mg/l) - MSSA											
		S								R			
		0.25	0.5	1	2	4	8	16	32	64	128	256	>256
S	0.25												
	0.5												
	1												
	2												
	4												
	8					1	1	2					
	16							3					
	32							1	1				
R	64												
	128												
	256										1		
	>256												

(B)

AD Fosfomycin panel MICs (mg/l)		AD Reference method MICs (mg/l) - MRSA											
		S								R			
		0.25	0.5	1	2	4	8	16	32	64	128	256	>256
S	0.25												
	0.5												
	1												
	2												
	4				1		1		1				
	8						5	1					
	16					1		7	5				
	32						3	2	13				
R	64									2	1	1	
	128										1	1	
	256									1	2	1	
	>256												20

(C)

AD Fosfomycin panel MICs (mg/l)		AD reference method MICs (mg/l) - *Enterobacterales*											
		S								R			
		0.25	0.5	1	2	4	8	16	32	64	128	256	>256
S	0.25		1										
	0.5		13	21									
	1		1	11									
	2				3	3							
	4					2	3						
	8					1	5						
	16						1	3	1				
	32								1	1*			
R	64								4**	5	1		
	128									1	8	2	1
	256								1**			1	
	>256										1	1	2

Fig. 2. Scattergram of fosfomycin MICs for *Staphylococcus aureus* and Enterobacterales measured by agar dilution (AD) reference method and AD fosfomycin panel. (A) MSSA ($n = 10$).
(B) MRSA ($n = 70$); (C) Enterobacterales ($n = 99$).
The bold lines indicate the EUCAST breakpoint for susceptibility ($\leq$32 mg/L). Grey boxes indicate essential agreement (EA) between testing methods. * Very Major Error; ** Major Error.

because of its broad-spectrum activity against MDR Gram-positive bacteria, such as MRSA [13] and penicillin-resistant *Streptococcus pneumoniae*, and Gram-negative bacteria, such as ESBL-producing *E. coli* and, to some extent, KPC-producing *K. pneumoniae* [2,4,14,15].

Both CLSI and EUCAST have disk diffusion methods and criteria for susceptibility testing fosfomycin. However, the guidelines for reading the tests are different: CLSI count colonies inside the zone, whereas EUCAST do not, therefore, interpretation can be problematic for some laboratories. Some laboratories rely on automated systems for antimicrobial susceptibility testing of Fosfomycin, which predominantly use BMD; neither CLSI nor EUCAST recommend the use of BMD (EUCAST 2020; CLSI, 2018) [6,7]. Therefore, the majority of automated susceptibility testing systems should not be used to interpret fosfomycin, as they provide unreliable results compared with the reference AD gold standard method [6].

In some clinical scenarios, such as serious complicated infections, a fosfomycin MIC may be required. Although most

laboratories will struggle to perform the reference in-house AD method, the commercial AD fosfomycin kit described here could be offered as a useful and easy to use reference 'gold standard' method for obtaining a fosfomycin MIC.

In line with previously published study, our data confirm that fosfomycin is very active against MDR *S. aureus* clinical isolates, such as MRSA according to EUCAST or CLSI breakpoints [16,17]. The susceptibility rate of fosfomycin in *S. aureus* was estimated to be around 61%, and higher in Enterobacterales at around 76%.

The comparison between the commercial AD fosfomycin panel and the reference AD method demonstrated a robust consensus of the antibiotic susceptibility values, both for *S. aureus* and Enterobacterales isolates. Using the CLSI requirements for commercial antimicrobial susceptibility testing systems (EA $\geq$90%, CA $\geq$90%, VME $\leq$1.5%, ME $\leq$3.0%), the AD fosfomycin commercial method met the acceptance criteria with almost all values [18].

Only Enterobacterales ME values were above the CLSI criteria; however, the 5 isolates with discordant categorical results had MIC values at the breakpoint, thereby producing a major error rate of 5% (MICs of 32 and 64 mg/L).

In conclusion, this novel AD test is user-friendly, suitable and rapid to use, resulting in a feasible alternative to the reference AD method in the routine laboratory. Looking ahead, the reduction in turnaround time achieved using the AD fosfomycin panel could improve the clinical management of MDR isolates, providing a prompt suggestion for antibiotic therapy.

Funding

This work was partially supported by the National research grant "Piano per la ricerca 2016-2018—Linea di intervento 2". University of Catania (I).

Competing interests

None declared.

Ethical approval

Not required.

Acknowledgements

The AD fosfomycin panels used in the study were provided by Liofilchem S.r.l.

References

[1] Theuretzbacher U, Paul M. Revival of old antibiotics: structuring the re-development process to optimize usage. Clin Microbiol Infect 2015;21:878–80.
[2] Michalopoulos AS, Livaditis IG, Gougoutas V. The revival of fosfomycin. Int J Infect Dis 2011;15:e732–9.
[3] Sastry S, Doi Y. Fosfomycin: resurgence of an old companion. J Infect Chemother 2016;22:273–80.
[4] Falagas ME, Kastoris AC, Kapaskelis AM, Karageorgopoulos DE. Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum beta-lactamase producing, Enterobacteriaceae infections: a systematic review. Lancet Infect Dis 2010;10:43–50.
[5] Mojica MF, De La Cadena E, Hernandez-Gomeza C, Correa A, Appela TM, Pallaresa J, et al. Performance of disk diffusion and broth microdilution for fosfomycin susceptibility testing of multidrug-resistant clinical isolates of *Enterobacterales* and *Pseudomonas aeruginosa*. J Glob Antimicrob Resist 2020;21:391–5.
[6] The European Committee on Antimicrobial Susceptibility Testing. Routine and extended internal quality control for MIC determination and disk diffusion as recommended by EUCAST. Version 10.0, valid from 01/01/2020. http://www.eucast.org.
[7] Clinical and Laboratory Standards Institute (CLSI). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. 11th ed. Wayne, PA: CLSI standard M07; 2018.
[8] Mezzatesta ML, La Rosa G, Maugeri G, Zingali T, Caio C, Novelli A, et al. *In vitro* activity of fosfomycin trometamol and other oral antibiotics against multidrug-resistant uropathogens. Int J Antimicrob Agents 2017;49:763–6.
[9] Campanile F, Bongiorno D, Perez M, Mongelli G, Sessa L, Benvenuto S, et al. AMCLI – S. aureus Survey Participants et al. Epidemiology of Staphylococcus aureus in Italy: First nationwide survey, 2012. J Glob Antimicrob Resist 2015;3:247–54.
[10] Bongiorno D, Mongelli G, Stefani S, Campanile F. Genotypic analysis of Italian MRSA strains exhibiting low-level ceftaroline and ceftobiprole resistance. Diagn Microbiol Infect Dis 2019;95:114852.
[11] Andrews JM. Determination of Minimum Inhibitory Concentrations. J Antimicrob Chemother 2001;48:5–16.
[12] Gona F, Bongiorno D, Aprile A, Corazza E, Pasqua B, Scuderi MG, et al. Emergence of two novel sequence types (3366 and 3367) NDM-1- and OXA-48-co-producing *K. pneumoniae* in Italy. Eur J Clin Microbiol Infect Dis 2019;38:1687–91.
[13] Gudiol C, Cuervo G, Shaw E, Pujol M, Carratala J. Pharmacotherapeutic options for treating Staphylococcus aureus bacteremia. Expert Opin Pharmacother 2017;18:1947–63.
[14] Vardakas KZ, Legakis NJ, Triarides N, Falagas ME. Susceptibility of contemporary isolates to fosfomycin: a systematic review of the literature. Int J Antimicrob Agents 2016;47:269–85.
[15] Falagas ME, Roussos N, Gkegkes ID, Rafailidis PI, Karageorgopoulos DE. Fosfomycin for the treatment of infections caused by Gram-positive cocci with advanced antimicrobial drug resistance: a review of microbiological, animal and clinical studies. Expert Opin Investig Drugs 2009;18:921–44.
[16] Flamm RK, Rhomberg PR, Watters AA, Sweeney K, Ellis-Grosse EJ, Shortridge D. Activity of fosfomycin when tested against US contemporary bacterial isolates. Diagn Microbiol Infect Dis 2019;93:143–6.
[17] Perdigão-Neto LV, Oliveira MS, Rizek CF, Carrilho CMDM, Costa SF, Levin AS. Susceptibility of multiresistant gram-negative bacteria to fosfomycin. Antimicrob Agents Chemother 2014;58:1763–7.
[18] Clinical and Laboratory Standards Institute. Verification of commercial microbial identification and antimicrobial susceptibility testing systems. 1st ed. CLSI document M52-Ed Wayne PA: CLSI; 2017.