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Short Communication

In vitro activity of fosfomycin against mucoid and non-mucoid *Pseudomonas aeruginosa* strains

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ABSTRACT

Objectives: Pseudomonas aeruginosa is the most frequent infectious agent in cystic fibrosis patients. *P. aeruginosa* resistance to first line antibiotics limits therapeutic options, but the therapeutic potential of older generation antibiotics, such as fosfomycin is under investigation. Fosfomycin does not belong to any other antibiotic class and acts by inhibiting the biosynthesis of the bacterial cell wall during the initial phases. A major problem for the use of fosfomycin against *P. aeruginosa* is the absence of a clinical breakpoint, the last one of 32 μg/mL was proposed in 2013 by the CA-SFM (Comité de l'Antibiogramme de la Société Française de Microbiologie).
Methods: Sixty-one strains of *P. aeruginosa* (thirty mucoid and thirty-one non mucoid) were collected from respiratory samples of cystic fibrosis patients. All isolates were identified by MALDI-TOF (Bruker, Bremen, Germany). Fosfomycin MICs against *P. aeruginosa* were measured using an automated system and confirmed by the gold standard method.
Results: There was no significant difference between mucoid and non-mucoid strains. MIC distribution and susceptibility rates were obtained by agar dilution method and from this data we measured MIC50 and MIC90 which were equal to 32 μg/mL and 64 μg/mL, respectively. From automated method results we measured a very major error (VME), major error (ME) and categorical agreement (CA) which were equal to 0%, 11% and 89%, respectively. Comparing automated and agar dilution methods, a Cohen's kappa equal to 73% (0.726) was measured.
Conclusions: Our data suggest that fosfomycin has good effect against mucoid and non-mucoid strains of *P. aeruginosa* and automated systems can be implemented in clinical microbiology laboratories to assess fosfomycin with rapid and reproducible results.

1. Introduction

Pseudomonas aeruginosa is a major opportunistic nosocomial pathogen and causes persistent infections, frequently associated with respiratory chronic diseases in cystic fibrosis patients. Chronic infections cause increasing morbidity and mortality in patients. Currently, the treatment of *P. aeruginosa* infections is based on the combinations of various antimicrobial agents, including aminoglycosides, carbapenems and colistin, since intrinsic and acquired resistance mechanisms of *P. aeruginosa* limited therapeutic choices [1,2]. Furthermore, biofilm producing *P. aeruginosa* showing resistance to beta-lactams demonstrated lower clearance by the host immune system [3]. Biofilm formation is typical of mucoid strains of *P. aeruginosa* [4].

Older generation antibiotics such as fosfomycin, a drug discovered 40 years ago, are nowadays intensively investigated as potential therapeutic agents against multi-drug resistant microorganisms [2]. Fosfomycin does not belong to any other antibiotic class, interferes with the first step of bacterial cell wall biosynthesis, and results in reduced growth and cell lysis, demonstrating good bactericidal activity against both Gram-positive and Gram-negative bacteria [5]. Biofilm does not hinder the effectiveness of fosfomycin [4].

Agar dilution is the method approved for determining fosfomycin Minimum Inhibitory Concentration (MIC), as

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published by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [6,7]. Nowadays, there is not a clinical breakpoint for fosfomycin against *P. aeruginosa* and no information is available about performance of automatic systems, when used for testing fosfomycin susceptibility. Until 2013, the Antibiotic Committee of the French Society of Microbiology (CA-SFM) had established a breakpoint value of 32 μg/mL for fosfomycin against *P. aeruginosa* [8]. Previous studies demonstrated that fosfomycin has an excellent distribution volume and it reaches high concentrations in plasma as well as in other sites such as subcutaneous, interstitial and bone tissue using intravenous formulation of fosfomycin. In a study performed by Matzi et al. [9] after a single intravenous dose administration of 4 g of fosfomycin lung concentrations of drug were measured by a microdialysis probe inserted in the infected lung tissue, and according to pharmacokinetic/pharmacodynamic calculations they estimated a breakpoint value for MICs of up to 32 μg/mL, hence *P. aeruginosa* isolates with MIC ≤ 32 μg/mL should be considered susceptible, while those with MIC > 32 μg/mL could be resistant to this therapeutic treatment. As suggest by Matzi et al., in this study we considered all isolates of *P. aeruginosa* with a MIC ≤ 32 μg/mL to be susceptible and those with a MIC > 32 μg/mL to be resistant to fosfomycin.

The aim of this study was to evaluate *in vitro* activity of fosfomycin against mucoid and non-mucoid isolates of *P. aeruginosa*. Moreover, we compared the MICs of fosfomycin measured by the agar dilution method with those obtained by the MicroScan WalkAway plus system (Beckman Coulter, Pasadena, CA).

2. Materials and methods

2.1. Sample collection

Sixty-one clinical isolates of *P. aeruginosa*, 30 mucoid and 31 non-mucoid, obtained from respiratory samples of cystic fibrosis patients were collected. The *P. aeruginosa* NCTC 12,924 strain (bioMérieux, Marcy-l'Étoile, France) was used as fosfomycin susceptible control. Total tested strains were 62.

2.2. Species identification

All isolates were identified by Matrix Assisted Laser Desorption Ionization-Time of Flight mass spectrometry (MALDI-TOF, Bruker, Bremen, Germany). Modified extraction procedure was used for the mucoid strains, as previously described [10].

2.3. Agar dilution method

0.5 McFarland suspension was used for both agar dilution and automated methods. The agar dilution method was set up with 250 μl of the 0.5 McFarland bacterial suspension. The inoculum replicator had 1 mm pins which delivered 0.1–0.2 μL of bacterial suspension (approximately 1–3 × 10^5 CFU).

Standard reference MICs for fosfomycin (Nordic Pharma S.r.l, Milan, Italy) were determined in triplicate for each isolate by agar dilution on Mueller-Hinton agar (Liofilchem, Roseto degli Abruzzi, Italy), supplemented with 25 mg/L of glucose-6-phosphate (Sigma–Aldrich Co, Milan, Italy) according to CLSI guidelines [11]. Fosfomycin 1–512 μg/mL concentration range was tested. Plates were incubated at 35–37 °C for 16–18 h. Quantitative and purity inoculum control for each strain, was performed plating the bacterial suspension on agar.

Table 1
Description of the phenotype of the strains and their minimum inhibitory concentration.

N°	Phenotype[a]	MIC Agar dilution[a] method (μg/mL)	MIC MICROSCAN[a] (μg/mL)
1[b]	non mucoid	4	≤32
2	mucoid	32	≤32
3	mucoid	128	64
4	mucoid	4	≤32
5	mucoid	8	≤32
6	mucoid	32	≤32
7	mucoid	64	>64
8	mucoid	8	≤32
9	mucoid	32	≤32
10	mucoid	16	≤32
11	mucoid	8	≤32
12	mucoid	4	≤32
13	mucoid	64	64
14	mucoid	32	64
15	mucoid	32	≤32
16	mucoid	4	≤32
17	mucoid	64	>64
18	mucoid	256	>64
19	mucoid	64	>64
20	mucoid	4	≤32
21	mucoid	32	64
22	mucoid	32	≤32
23	mucoid	32	64
24	mucoid	128	>64
25	mucoid	32	≤32
26	mucoid	32	≤32
27	mucoid	32	≤32
28	mucoid	8	≤32
29	mucoid	32	≤32
30	mucoid	8	≤32
31	mucoid	8	≤32
32	non mucoid	32	≤32
33	**non mucoid**	**4**	**64**
34	non mucoid	32	≤32
35	non mucoid	8	≤32
36	non mucoid	32	≤32
37	**non mucoid**	**8**	**64**
38	non mucoid	32	≤32
39	non mucoid	32	≤32
40	non mucoid	16	≤32
41	non mucoid	16	≤32
42	non mucoid	32	≤32
43	non mucoid	4	≤32
44	non mucoid	8	≤32
45	**non mucoid**	**32**	**>64**
46	non mucoid	32	≤32
47	**non mucoid**	**32**	**>64**
48	non mucoid	4	≤32
49	non mucoid	8	≤32
50	non mucoid	64	>64
51	non mucoid	64	64
52	non mucoid	16	≤32
53	non mucoid	8	≤32
54	non mucoid	64	64
55	non mucoid	128	>64
56	non mucoid	128	>64
57	non mucoid	256	>64
58	non mucoid	8	≤32
59	non mucoid	32	≤32
60	non mucoid	64	64
61	non mucoid	16	≤32
62	non mucoid	32	≤32

[a] The strains in which a major error (ME) was observed are indicated in bold.
[b] Strain n. 1: control strain NCTC.

2.4. Automated method for measuring fosfomycin MIC

The automated method was set up with 100 μl of the 0.5 McFarland bacterial suspension, the same used for the agar dilution method. The NF50E MicroScan panel for the WalkAway plus System (Beckman Coulter, Pasadena, CA) was used. This

Table 2
Minimum inhibitory concentration (MIC) distribution and susceptibility rates of fosfomycin by the agar dilution method.

Type of isolates	Number of strains	Number of isolates with indicated MIC (μg/mL)										MIC50	MIC90
		1	2	4	8	16	32	64	128	256	>512		
MUCOID	30	0	0	4	6	1	12	4	2	1	0	**32**	**64**
NON-MUCOID	32	0	0	4	6	4	11	4	2	1	0	**32**	**64**
ALL ISOLATES	62	0	0	8	12	5	23	8	4	2	0	**32**	**64**

MIC50, minimum inhibitory concentration required to inhibit the growth of 50% of organisms; MIC90, minimum inhibitory concentration required to inhibit the growth of 90% of organisms.

system measures 32 μg/mL–64 μg/mL dilutions for fosfomycin. This method consists of a microdilution of the bacterial suspension in water, which is used to rehydrated lyophilized broth containing glucose-6-phosphate and the fosfomycin. The system reads the turbidity at the initial time and after 18 h of incubation at 35 ± 2 °C, following the producer guidelines.

2.5. Data analysis and comparison between methods

MIC50 and MIC90, defined as the antibiotic concentration required to inhibit the growth of 50% and 90% of the pathogen, respectively, were determined by agar dilution method. Very Major Error (VME) rates were calculated using the total number of resistant isolates as denominator, whereas Major Error (ME) rates were calculated using the total number of susceptible isolates as denominator. Minor errors were not calculated as no intermediate (I) category has been established. Results obtained from agar dilution and automated methods were compared using Cohen's kappa, which gives a measure of the percentage of agreement between MICs obtained by the different methods beyond that expected by chance. Results were interpreted according to the classification described by Landis and Koch [12].

3. Results

The MICs obtained with agar dilution and automated methods of the 62 strains (61 clinical strains and 1 control) are shown in Table 1.

MIC50 and MIC90 values, resulted from the agar dilution method, were 32 μg/mL and 64 μg/mL respectively, as shown in Table 2. There was not significant difference between mucoid and non-mucoid strains. The lowest MIC value found was 4 μg/mL, obtained, as expected, for the *P. aeruginosa* NCTC 12,924 and for 8 clinical isolates, 4 mucoid and 4 non mucoid.

The highest MIC was 256 μg/mL, obtained for 2 clinical isolates, 1 mucoid and 1 non mucoid (Tables 1 and 2).

For the purposes of this study we considered all isolates with MIC ≤ 32 μg/mL as susceptible and those with MIC > 32 μg/mL as resistant to fosfomycin. Using this cut off, the percentage of susceptibility measured by the agar dilution method was 77% and 78% for mucoid and non-mucoid strains, respectively.

Comparing MICs results obtained by the agar dilution and automatic methods, VME and ME were 0% and 11%, respectively, with a Cohen's kappa equal to 73% (0.726), while the categorical agreement was 89%.

4. Discussion and conclusion

Fosfomycin has a good activity to treat infections caused by multidrug-resistant microorganisms [13,14]. The susceptibility of *P. aeruginosa* to fosfomycin can vary substantially and it has been hypothesized that the antimicrobial activity may depend on the bacterial physiological state and growth conditions [15]. This bacterium shows the ability to adapt to environment changes and increases its survival chance by forming a biofilm, which protect it from host defences. This is particularly relevant for chronic pulmonary infections in patients with cystic fibrosis and ventilator-associated pneumonia [4,16]. Biofilm does not hinder the effectiveness of antibiotics like fosfomycin [4].

The only method approved to test the susceptibility to fosfomycin is the agar dilution as published by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Nevertheless, automated systems are indispensable tools for clinical laboratories. With automated system is possible to obtained rapid and reliable results about antimicrobial susceptibility of isolate. MicroScan WalkAway automated system was used for assessing *in vitro* susceptibility of fosfomycin against *P. aeruginosa* with respect to the gold standard. MicroScan WalkAway plus showed a good performance with the mucoid morphotype, which is very difficult to process with other methods.

In this study we considered all isolates of *P. aeruginosa* with a MIC ≤ 32 μg/mL to be susceptible and those with a MIC > 32 μg/mL to be resistant to fosfomycin. This breakpoint value was chosen following studies of literature that evaluated the tissue concentration of fosfomycin at normal therapeutic doses after the administration of the intravenous formulation of the drug. Fosfomycin has an excellent distribution volume and it reaches high concentrations in plasma as well as in other sites such as subcutaneous, interstitial and bone tissue. In a study performed by Matzi et al. [9] after a single intravenous dose administration of 4 g of fosfomycin lung concentrations of drug were measured by a microdialysis probe inserted in the infected lung tissue, and according to pharmacokinetic/pharmacodynamic calculations they estimated a breakpoint value for MICs of up to 32 μg/mL.

Evaluation of the concordance between the chosen automatic method is described by values of kappa coefficient (Cohen's kappa), which gives a measure of the percentage of agreement between MICs obtained by the different methods beyond that expected by chance (interpreted according to Landis and Koch, 1977). The Cohen's kappa value of 73% (0.726) can be considered as substantial strength of agreement [12]. However, MicroScan WalkAway plus system showed ME of 11%, and VME of 0% and categorical agreement of 89% (0.887). The ME and the categorical agreement values were below the acceptable performance criteria for antimicrobial susceptibility test devices, as requested by the most recent ISO 20776-2:2007 guideline, but it must be considered that these results were obtained with an unofficial clinical breackpoint so performance evaluation of MicroScan WalkAway plus system had some limits. MicroScan WalkAway plus system, unlike other automated systems, provides the result of 100% of the antibiograms performed, especially as regards the mucoid strains. In fact, another automated system was also initially used for testing fosfomycin susceptibility, but for mucoid isolates test interruption was observed, and the method was not further evaluated in this study. Finally, our data suggest that fosfomycin has good *in vitro* activity against mucoid and non-mucoid strains of *P. aeruginosa* and with opportune experience and limitations, automated systems may be implemented in clinical microbiology laboratories to assess fosfomycin. These

preliminary data require clinical confirmation of *in vivo* efficacy of MICs measured *in vitro*.

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Ethical approval

Not required.

Conflict of interest

None declared.

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