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Evaluation of fosfomycin susceptibility in Pseudomonas aeruginosa isolates from patients with cystic fibrosis in a Palestinian hospital

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Abstract

Purpose

To evaluate the in vitro antimicrobial activity of fosfomycin against *Pseudomonas aeruginosa* isolates obtained from sputum samples of patients with cystic fibrosis treated at Caritas Baby Hospital in Palestine.

Methods

A total of 129 randomly selected *P. aeruginosa* isolates were classified as non-mucoid, mucoid, and carbapenem-resistant isolates. Fosfomycin susceptibility was assessed using disc diffusion (DD) and agar dilution minimum inhibitory concentration (MIC) methods following the relevant Clinical and Laboratory Standards Institute (CLSI) recommendations. As neither CLSI nor EUCAST provides susceptibility breakpoints for fosfomycin against *P. aeruginosa*, the existing CLSI breakpoints for Escherichia coli were applied to MIC results. Isolates with an MIC ≤ 64 mg/L were considered susceptible, whereas those with an MIC > 64 mg/L were considered non-susceptible. DD interpretive criteria were established by correlating zone diameters with MIC results, wherein zone diameter ≥ 16 mm are susceptible and those < 16 mm are non-susceptible.

Results

Overall, 39.53% of the isolates were susceptible to fosfomycin based on MIC testing. The highest susceptibility was observed among mucoid isolates, at 57.5%. A strong negative correlation was observed between DD zone diameters and MIC values.

Conclusion

Fosfomycin demonstrated in vitro activity against a proportion of *P. aeruginosa* isolates from patients with cystic fibrosis and may represent a potential therapeutic option. However, further studies, particularly in vivo investigations, are required to determine its clinical efficacy.

Introduction

Cystic fibrosis (CF) is a multisystem genetic disorder with lung involvement as the major cause of morbidity and mortality. Pulmonary infections account for most of the complications and deaths[1]. The lungs of patients with CF are often infected with organisms, such as *Staphylococcus aureus* and *Haemophilus influenzae*, which may damage the epithelial surfaces, leading to increased attachment of gram-negative bacilli such as *P. aeruginosa* and eventual replacement by it[2]. Chronic colonization of *P. aeruginosa* is associated with worse prognosis and death in children, adolescents, and adults[3].

Antibiotic therapeutic strategies aimed at preventing or delaying the progression of the initial *P. aeruginosa* infection to chronic infection is considered central to the management of patients with CF, which is one of the major reasons for increased patient survival. Therefore, the availability of effective antibiotics against colonizing *P. aeruginosa* is considered the major cornerstone for treating pulmonary exacerbation in patients with CF. Ceftazidime, gentamycin, tobramycin, ciprofloxacin, meropenem, and piperacillin-tazobactam are the most used antibiotics for treating *P. aeruginosa* infections in patients with CF.

Moreover, repeated administration of the same antimicrobial agent can increase the selection pressure on the bacteria, thus leading to the emergence of a new strain of resistant bacteria. Subsequently, the antibiotic becomes ineffective. Furthermore, repeated use of the same antibiotic results in patient intolerance and increased side effects.

Regarding the side effects, every antibiotic has adverse effects that can limit its use and make it debilitating to the patient, such as aminoglycosides causing ototoxicity and nephrotoxicity and penicillin causing rash and urticaria.[4]. Therefore, alternative antibiotics are needed.

Fosfomycin is a unique broad-spectrum bactericidal antibiotic[5], which interferes with cell wall synthesis in both Gram-positive and Gram-negative bacteria by inhibiting the initial step involving phosphoenolpyruvate synthetase. It is chemically unrelated to any other antimicrobial agent and is available in intravenous formulations, such as fosfomycin disodium and oral formulations, such as fosfomycin calcium or fosfomycin trometamol. Following intravenous administration of the disodium salt, 80–95% of the drug is excreted unchanged in the urine within 24 h (t½ = 1.5-2 hours); moreover, it is not bound to serum proteins, hence its volume of distribution is large[6]. The concentration of fosfomycin in the lung tissue can reach up to 50% of serum levels within 1–2 h of administration[7]; furthermore, it is actively taken up into *P. aeruginosa* cells through the α-glycerophosphate transport system and inhibits the initial step in cell wall synthesis[8]. Hence, it may be an alternative treatment option for *P. aeruginosa* infections in patients with CF with fewer side effects than commonly used antibiotics.

Materials and methods

Bacterial samples

Following official approval and permission from Caritas Baby Hospital Medical Research Committee (MRC-043), this study evaluated 129 non-duplicate sputum samples from patients with CF. To ensure patient confidentiality, samples were handled by using the patients' hospital numbers, without accessing the identity of the patients who provided these samples. Bacterial isolates were kept frozen at -80°C at the Caritas Baby Hospital. Parents of the patients consented for the bacteriological cultures to be performed on their children respiratory samples as part of their cystic fibrosis medical management. These isolates were identified and classified by conventional methods in the hospital laboratory according to the protocols of the American Society of Microbiology. On January 1, 2020 we have

accessed the bacterial isolates and stratified them as 79 non-mucoid, 40 mucoid, and 10 carbapenem-resistant *P. aeruginosa* (CRPA) isolates.

P. aeruginosa mucoid phenotype is defined as the overproduction of the exopolysaccharide alginate. The mucoid or non-mucoid morphologies of *P. aeruginosa* were visually observed in strains cultured on both selective (MacConkey's agar) and non-selective (5% sheep blood agar) mediums.

Antibiotic susceptibility testing

Fosfomycin susceptibility testing was conducted using two different methods, disc diffusion (DD) (using 200 µg fosfomycin / 50 µg glucose-6-phosphate (G-6-p) and 50 µg fosfomycin discs) and agar dilution minimum inhibitory concentration (MIC) methods.

Susceptibility using agar dilution method was determined according to the CLSI guidelines using 0.5 McFarland inoculum and the Steers-Replicator apparatus. The performance of the agar dilution method was assessed by Ceftriaxone (1000 mg; Panpharma UK Ltd, Liverpool, England) against the reference strains *Escherichia coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213. The observed MIC values were compared with established CLSI quality control ranges to verify the performance of the testing procedure.

The isolates were collected from sample preservation tubes by sterile loops and inoculated directly on 5% sheep blood agar. The cultured bacteria were incubated at 35°C for 18–24 h Furthermore, the bacteria were re-cultured on MacConkey agar to avoid contaminated samples and incubated at 35°C for 18–24 hours. After eliminating any contaminated samples, the bacteria were re-cultured from the MacConkey plates onto 5% sheep blood agar and incubated at 35°C for 18–24 h. For sensitivity tests, *P. aeruginosa* antimicrobial susceptibility testing for carbapenems was conducted according to the CLSI M-100 guidelines. DD on Mueller Hinton Agar (MHA) was performed using Meropenem 10 µg discs. Colony suspensions equivalent to 0.5 McFarland standard were prepared. They were spread on MHA and incubated for 16–18 hours at 35°C in ambient air. *P. aeruginosa* strains were considered resistant to meropenem if the DD inhibition zone was ≤ 15 mm.

Fosfomycin powder (Sigma- Aldrich, Buchs, Switzerland) was diluted with distilled water (B. Braun, Melsungen, Germany) to make the desired concentrations 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, 128, and 256 µg/mL according to CLSI guidelines.

MHA was prepared with the required concentrations of fosfomycin according to the method described in reference [9]. After autoclaving, the G-6-P powder was added to MHA media (25 mg/L) when the temperature reached 42–50°C. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as positive controls. Normal saline was used as a negative control. After culturing the *P. aeruginosa* and quality control strains on appropriate plates and incubation for 18–24 hours, fosfomycin agar dilution was performed. MIC was defined as the lowest concentration of fosfomycin that prevented the visible growth

of microorganisms. MIC assay was conducted in triplicate for each strain at different MIC concentrations.

In DD method, fosfomycin sensitivity test was conducted for all isolates using the Kirby Bauer DD method on MHA plates (Becton, Dickinson, Sparks, MD, USA) according to the CLSI guidelines using two different discs: fosfomycin 50 µg (Oxoid Ltd, Hamshire, UK) and fosfomycin 200 µg with G-6-P 50 µg (Becton, Dickinson, Sparks, MD, USA). Using each *P. aeruginosa* isolate, the tests were performed under same conditions once for fosfomycin 50 µg discs and twice for fosfomycin 200 µg with G-6-P 50 discs.

Interpretation of MIC results: Currently, due to to the lack of susceptibility breakpoint of fosfomycin for *P. aeruginosa* in CLSI and EUCAST guidelines, sensitivity to fosfomycin according to MIC results was determined based on the existing CLSI breakpoints for susceptibility, intermediacy, and resistance of *E.coli* at ≤ 64, 128, and ≥ 256 mg/L, respectively[10]. For the purposes of this research, we considered all isolates with an MIC ≤ 64 mg/L to be susceptible and > 64 mg/L to be non-susceptible.

DD results: The zone diameter interpretive results for the DD method were recorded for all the *P. aeruginosa* strains. By comparing the DD zones with the MIC results, we developed an interpretive criterion for the inhibition zone of fosfomycin in *P. aeruginosa.* Zone diameter ≥ 16 mm was interpreted as susceptible whereas, that < 16 mm was non-susceptible to fosfomycin.

The correlation between the MIC and DD methods was evaluated by computing Pearson correlation coefficients. Based on the CLSI guidelines, we also evaluated the errors between the two methods and classified them as minor, major, and very major [10].

Results

Agar dilution method validation results:

Method validation for agar dilution was performed on a Steer's Replicator apparatus in triplicate for each *P. aeruginosa* strain; and the results are illustrated in Table 1.

Table 1
Quality Control Results for ceftriaxone MIC testing using three ATCC reference strains.

MIC **Bacteria tested**	**MIC 1 (µg/ml)**	**MIC 2 (µg/ml)**	**MIC 3 (µg/ml)**	**Acceptable MIC QC range (µg/ml)**
Pseudomonas aeruginosa ATCC 27853	8	8	8	8–64
Escherichia coli. ATCC 25922	0.06	0.06	0.06	0.03–0.12
Staphylococcus aureus ATCC 29213	2	2	2	1–8

Note: ATCC, American Type Culture Collection; MIC, minimum inhibitory concentration; QC, Quality control

The MIC values obtained for the reference strains fell within the established quality control ranges for ceftriaxone, supporting the performance of the agar dilution procedure under the conditions tested.

Fosfomycin MIC and DD results:

The MIC assay was performed in triplicate for each isolate. For DD testing, the 50 µg fosfomycin disk was tested once and the 200 µg fosfomycin disc with 50 µg glucose-6-phosphate was tested in duplicate for each isolate.

Concentrations ≤ 64 mg/L are readily achievable in plasma and other sites, such as the subcutaneous, interstitial, and bone tissues at normal therapeutic doses[11].

According to the MIC results of *P. aeruginosa* strains, 39.5% of all isolates were susceptible to fosfomycin. The susceptibility rates for non-mucoid, mucoid, and CRPA isolates were 34.2%, 57.5%, and 10%, respectively (Table 2).

Table 2
Fosfomycin MIC distribution and susceptibility rates among *pseudomonas aeruginosa* isolates by phenotype and carbapenem resistance status.

Type of isolate	Number of isolates	Number of isolates with indicated MIC (µg/mL)								Susceptibility %
		4	8	16	32	64	128	256	More than 256	
Non-mucoid	79	2	1	-	1	23	26	15	11	**34.18%**
Mucoid	40	-	2	-	1	20	11	4	2	**57.5%**
CRPA	10	-	-	-	-	1	3	2	4	**10%**
Total	129	2	3	-	2	44	40	21	17	**39.53%**

Note: MIC, Minimum Inhibitor Concentration; CRPA, Carbapenem Resistant *Pseudomonas aeruginosa*.

Therefore, the mucoid *P. aeruginosa* strains were the most susceptible to fosfomycin, followed by the non-mucoid strains. The lowest susceptibility was observed for the CRPA strains.

The correlations between the two methods, MIC and DD, were calculated and the results are presented in Table 3.

Table 3
Pearson correlation coefficients between fosfomycin MIC values and disc diffusion inhibition zone diameters.

Pearson Correlation Matrix				
		MIC	Zone 200	Zone 50
MIC	Pearson Correlation	1	-0.889-	-0.768-
	Sig. (2-tailed)		< 0.001	< 0.001
	N	129	129	129
Zone200	Pearson Correlation	-0.889-	1	0.932
	Sig. (2-tailed)	< 0.001		< 0.001
	N	129	129	129
Zone50	Pearson Correlation	-0.768-	0.932	1
	Sig. (2-tailed)	< 0.001	< 0.001	
	N	129	129	129

Zone 200, inhibition zone diameter for the 200 µg fosfomycin disc supplemented with 50 µg glucose-6-phosphate; Zone 50, inhibition zone diameter for the 50 µg fosfomycin disc; MIC, minimum inhibitory concentration. All reported correlates are statistically significant at $p < 0.001$ (two-tailed).

These results demonstrated strong negative correlations between MIC values and zone diameters for both 200 µg and 50 µg discs. The absolute values of the Pearson correlation coefficients were > 0.5.

The MIC values showed a stronger correlation with the inhibition zone diameters of the 200 µg fosfomycin disc than with those of the 50 µg disc (r = -0.889 and r = -0.768, respectively; Table 3).

The negative correlation between MIC values and inhibition zone diameters for the 200 µg fosfomycin disc is illustrated in Fig. 1.

Regarding the CLSI guidelines, interpretation errors between the two methods were noted in only one sample, where the fosfomycin MIC was 128 mg/L and the DD provided a 17 mm zone diameter. Herein, we used an intermediate interpretation point. Hence, this single error was considered as a minor error where the isolate was interpreted as intermediately resistant to fosfomycin.

Discussion

In patients with CF, when the lungs are colonized with *P. aeruginosa*, which is considered chronic colonization, the patients receive the same antibiotics several times. These repeated courses lead to the emergence of bacterial resistance in future. Repeated administration of the antibiotics exacerbates the side effect profile and toxicity[7].

Fosfomycin has several pharmacological characteristics that make it a good candidate for studying its activity against *P. Aeruginosa* isolated from patients with CF. It has a unique chemical structure and a different mechanism of action from other previously used antibiotics [12]. It has a mild side-effect profile than previously used antibiotics, and reaches the lung tissues at a good concentration [7]. Moreover, literature demonstrates that fosfomycin has good in vitro activity against several organisms, including gram-positive and gram-negative bacteria, such as *P. aeruginosa*[7, 12, 13]. Furthermore, it has a synergistic effect when used in a combination with other antibiotics. In the in vitro study, 53.5% of the MDR *P. aeruginosa* isolates were susceptible to fosfomycin when used in combination with aminoglycosides, beta-lactams, or ciprofloxacin. Clinically, six studies used fosfomycin in combination with other antibiotics to treat MDR *P. aeruginosa* (predominantly in pulmonary exacerbation of CF), and 91% of the patients demonstrated clinical improvement[14].

The only method approved for testing fosfomycin sensitivity is agar dilution, as published by the CLSI and EUCAST[12].

Owing to the lack of breakpoints for fosfomycin on *P. aeruginosa* in CLSI or EUCAST guidelines, several studies have used the breakpoint of fosfomycin on *E. coli* as a reference[15].

Isolates with a breakpoint of ≤ 64 mg/L were considered susceptible in our study. *P. aeruginosa* demonstrated a good susceptibility towards Fosfomycin; approximately 40% of all isolates were susceptible, especially in mucoid type where its susceptibility was approximately 50%.

A comparison of our findings with other studies demonstrated that fosfomycin reveals good susceptibility towards *P. aeruginosa* isolates[12, 14] [16].

In future, fosfomycin may be an option for treating *P. aeruginosa* infection in patients with CF. However, more studies may be required as there is a difference in the mechanism of resistance between *P. aeruginosa* and *E. coli*, which may result in differences in MIC. Therefore, a better understanding of the relationship between fosfomycin pharmacokinetics and pharmacodynamics on *P. aeruginosa* is needed[15].

However, the agar dilution method is labor-intensive and time-consuming. Generally, susceptibility testing using the DD method is faster and easier to perform than agar dilution. In this study, we also studied fosfomycin susceptibility using the DD method and observed a strong negative correlation between MIC values and DD zone diameters. The findings suggest that a zone diameter of ≥ 16 mm may indicate fosfomycin susceptibility; however, one isolate with a zone diameter of 17 mm had an MIC of 128 mg/L, which was classified as intermediate and therefore non-susceptible for the purposes of this study. The inhibition zone diameters obtained using fosfomycin 200 µg fosfomycin discs supplemented with G-6-P showed a stronger correlation with MIC values than those obtained using 50 µg fosfomycin discs [15],[17].

On evaluation of the errors between the two methods, we observed only one minor error in the 129 samples tested. The error percentages were as follows: 0% very major error, 0% major error, and 0.77% minor error, which are acceptable according to the CLSI standards (< 10% for minor errors and < 3% for major and very major errors)[10].

Fosfomycin demonstrated good susceptibility towards *P. aeruginosa* isolates in Palestinian patients with CF. Fosfomycin should be considered among the antibiotics used to treat *P. aeruginosa* infection in patients with CF, especially as it has good safety and side effect profiles.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and relevant ethical guidelines and regulations. The study was reviewed and approved by the Caritas Medical Research Committee at Caritas Baby Hospital, Palestine, on January 1, 2019 (MRC Project No. MRC-43). The study was retrospective, and patient confidentiality was maintained by using hospital identification numbers without accessing patients' identities. CBH Medical Research Committee approved the publication of this study. Patient's confidentiality was maintained with blinded isolates. Patients guardians sign consent form for all laboratory testing including culturing the sputum samples.

Consent for publication:

Not applicable.

Competing Interests:

The authors declare no competing interests.

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Author Contribution

B.S.conducted the laboratory work, collected and analysed the data. H.H and M.H. supervised study and critically revised the manuscript. all authors read and approved the final manuscript.

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Data Availability

data was generated and it is available at any time

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Figures

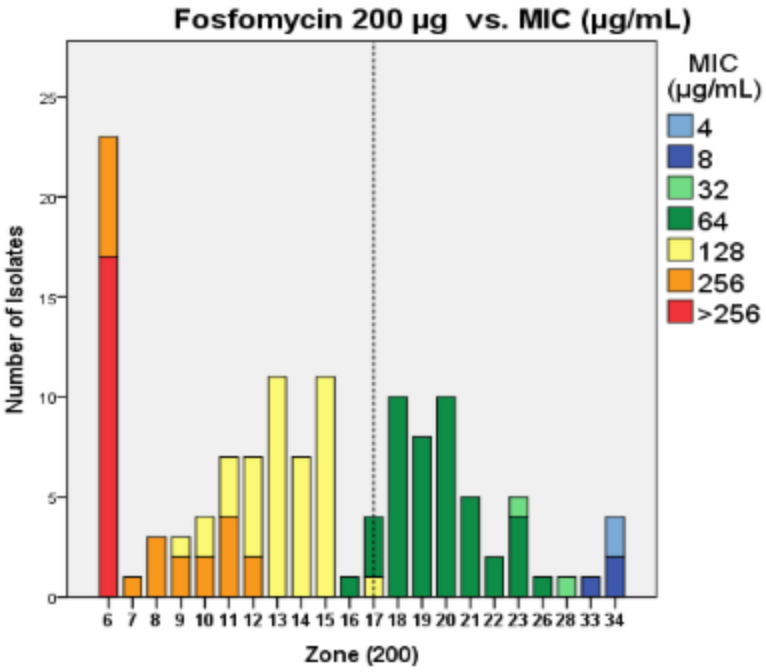

Figure 1

Correlation between fosfomycin minimum inhibitory concentration (MIC) and disc diffusion (DD) inhibition zone diameters for *Pseudomonas aeruginosa* isolates.