Prevalence of Fosfomycin Resistance among Clinical Isolates of Klebsiella pneumoniae at a Tertiary Care Hospital in Bangladesh

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Abstract

Background: Fosfomycin, an old antimicrobial has aroused interest for the treatment of multidrug resistant infections. Unfortunately, the prevalence of fosfomycin resistance is increasing. Therefore, this study was done to investigate thepresence of fosfomycin resistance and identify the fosfomycin resistance genes by Polymerase Chain Reaction (PCR) among the clinical isolates of Klebsiella pneumoniae.

Materials and methods: This cross-sectional study was conducted in the Department of Microbiology of Dhaka Medical College, Dhaka, Bangladesh from January 2021 to December 2021. *K. pneumoniae* was isolated and identified by culture, Gram staining and biochemical tests. Minimum Inhibitory Concentration (MIC) of fosfomycin was determined by agar dilution method.PCR was used to identifyfosfomycin resistance genes *(fosA, fosA3, fosA4, fosA5, fosB,fosC, fosC2 and fosX).*

Results: Among 55 isolated *K. pneumoniae,* 10 (18.18%) were resistant to fosomycin detected by agar dilution method. MIC of fosfomycin showed a significant rise ranging from 256 to 4096 µg/ml.Out of 10 fosfomycin resistant *K. pneumoniae,* 8 (80%) were positive for fosA 5 followed by5 (50%) for fosA, 4 (40%) for fosA3, 3 (30%) *for fosA4 and 2 (20%) were positive for fosB.*

Conclusion: This study showed that fosfomycin resistance has been increasing gradually leading to raised multidrug resistance demanding the implementation of effective antibiotic policy.

Key words: Fosfomycin resistance genes; *Klebsiella pneumoniae*; Minimum inhibitory concentration; Polymerase Chain Reaction (PCR).

Introduction

Older antimicrobials are being considered to bereused due to worldwide increase in antibiotic resistance together with the shortage of new active drugs.[1] As a result, fosfomycin has recently aroused great interest for the treatment of multidrug resistant infections caused by *K. pneumoniae*.[2] It is currently used in several countries for treating uncomplicated urinary tract infections caused by *Enterobacterales* with activity against multidrug resistant *Klebsiella pneomoniaestrains.*[3] It has a unique bactericidal mechanism which interfere with cell wall synthesis in both gram positive and gram-negative bacteria by inhibiting peptidoglycan synthesis through binding irreversibly to UDP-N-acetylglucosamine enolpyruvyl transferase, murA.[4] Furthermore, fosfomycin reduces penicillin binding proteins.[5] The toxicity of fosfomycin is very little and high peak levels can be achieved in serum and urine.[6]

However, microbiological cure rates in patients treated with fosfomycin monotherapy have been low due to the development of fosfomycin resistance.[7] There are several mechanisms of fosfomycin resistance have been studied:

i) Reduced uptake of the drug by the pathogen owing to chromosomal (glpT or uhpT) gene mutations affecting the hexose-6-phosphate or L-a-glycerophosphate uptake systems

ii) Target site alteration in murAwhich decreases the affinity of fosfomycin

iii) Hydrolysis of the drug via diverse fosfomycin hydrolases encoded by fos genes, which are the main mechanism of resistance.[8] The fosfomycin hydrolases are classified into four main types, *fosA, fosB, fosC* and fosX and their subtypes *fosA, fosA2, fosA3, fosA4, fosA5* and *fosC2*.[9] High similarity was found among *fosA5, fosA6* and *fosA10* and they were proposed to originate from the *K. pneumoniae* chromosome.[10]

FosA (Glutathione S-transferase), the first to be described (in 1988), is a metalloenzyme transferred through plasmids in *Enterobacteriaceae*.[11] It catalyzes the reaction between glutathione and fosfomycin to an inactive adduct. All fos genes

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except fosA2 are located inplasmids. The second enzyme of the glyoxalase superfamily conferring resistance to fosfomycin is fosB which catalyzes the nucleophilic addition of either 1-Cys or bacillithiol to fosfomycin, resulting in a modified compound with no bactericidal properties.[12,13]

Fosfomycin,being an attractive option for treatment of multidrug resistant infections needs contineous monitoring of its resistance pattern inmicrobiology laboratory. Therefore, this study aimed to determine the prevalence of fosfomycin resistance among *Klebsiella pneumoniae* and to detect the fosfomycin resistance genes by polymerase chain reaction.

Materials and methods

This cross-sectional study was carried out in the Department of Microbiology of Dhaka Medical College, Bangladesh during January 2021 to December 2021. This research protocol was approved by the Research Review Committee of Dhaka Medical College.

Samples (Urine, wound swab, sputum, blood and endotracheal aspirates) were collected from the laboratory of Microbiology Department, Dhaka Medical College. All samples were inoculated in MacConkey agar and Blood agar media and incubated overnight aerobically at 37ºC. *Klebsiella pneumoniae* was identified on the basis of their colony morphology, Gram staining and biochemical tests (Catalase, oxidase, urease, indole test, gas production, motility, lactose fermentation and citrate utilization).

Fosfomycin stock solution was made by adding 150 ml of distilled water with 300 mg base of commercially available Fosfomycin (Beximco Pharma Limited) to make a concentration of 20 mg/ml. For each plate 50 ml Mueller-Hinton media containing 1.25 mg glucose-6-phosphate was impregnated with 80 µl, 160 µl, 320 µl, 640 µl, 1280 µl, 2560 µl, 5120 µl and 10240 µl of fosfomycin stock solution to achieve concentration of 32 µg/ml, 64 µg/ml, 128 µg/ml, 256 µg/ml, 512 µg/ml, 1024 µg/ml, 2048 µg/ml, 4096 µg/ml per plate respectively.

Inoculum preparation: The turbidity of bacterial suspension in normal saline was compared with 0.5 McFarland turbidity standard. As 0.5 McFarland turbidity standard contain 1.5×10^8 CFU/ml, to obtain 10^4CFU/ml on agar surface 1 µl of 10 times diluted inoculums were placed on Mueller-Hinton agar plate. The plate was then incubated aerobically at 37ºC overnight.

MIC of Fosfomycin was determined following EUCAST guideline, 2020.[14]

PCR was used to detect fosfomycin resistance genes *(fosA, fosA3, fosA4, fosA5, fosB,fosC, fosC2 and fosX).* GenomicDNA extraction was done by boiling method[15].

Table I Primers used for detection of colistin resistance genes in this study.[15,16,17]

Genes		Sequence (5'-3')	size (bp)
fosA	F	ATC TGT GGG TCT GCC TGT CGT	271
	R	ATG CCC GCA TAG GGC TTC T	
fosA3	F	CCT GGC ATT TTA TCA GCA GT	221
	R	CGG TTA TCT TTC CAT ACC TCA G	
fosA4	F	CTG GCG TTT TAT CAG CGG TT	230
	R	CTT CGC TGC GGT TGT CTT T	
fosA5	F	TAT TAG CGA AGC CGA TTT TGC T	177
	R	CCC CTT ATA CGG CTG CTC G	
fosB	F	CAGAGATATTTTAGGGGCTGACA	312
	R	CTCAATCTATCTTCTAAACTTCCTG	
fos C2	F	TGG AGG CTA CTT GGA TTT G	209
	R	AGG CTA CCG CTA TGG ATT T	
fosX	F	TGT CCC TCA CCT TCG ACT CT	
	R	TTG CTG GTC TGT GGA TTT GC	

The following cycling parameters were used:
After initial denaturation at 94ºC for one minute, the reaction was subjected to 32 cycles (Annealing at 57ºC for 45 seconds and elongation at 72ºC for one minute) followed by final extension at 72ºC for 10 minutes. The amplified DNA was analyzed by 1.5% agarose gel-electrophoresis at 100 volts for 35 minutes, stained with 1% ethidium bromide and visualized under ultraviolet light (Figure 1).

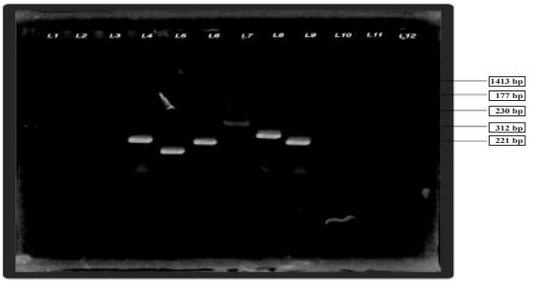

Figure 1 Photograph of gel electrophoresis of fosfomycin resistance genes showing amplified DNA of 271 bp of fosA gene (Lane 4) DNA of 177 bp for $fosA_5$ gene (Lane 5) DNA of 230 bp for $fosA_4$ gene (Lane 6) 100 bp DNA ladder (Lane 7) DNA of 312 bp for fosB gene (Lane 8) and DNA of 221 of fosA3 gene (Lane 9)

Statistical analysis was performed with SPSS software, versions 22.0 (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY: IBM Corp.). Continuous data that were normally distributed were summarized in terms of the mean, standard deviation, median, minimum, maximum and number of observations, Categorical or discrete data were summarized in terms of frequency counts and percentages.

All procedures of the present study were carried out in accordance with the principles for human investigations (i.e., Helsinki Declaration) and with the ethical guidelines of the Institutional research ethics. This research protocol was approved by the Research Review Committee and Ethical Review Committee of Dhaka Medical College (Reference number: MEU-DMC/ERC/2021/144). All participants were informed about the procedure and purpose of the study and confidentiality of information provided. All participants consented willingly to be a part of the study during data collection periods.

Results

A total of 340 samples were included in this studyout ofwhich 226 (66.47%) samples yielded growth in culture.Among the culture positive samples, 55 (24.34%) were identified as *K. pneumoniae* (Table II).

Table II Distribution of organisms isolated from different samples (n= 226)

Organisms	Number (%)
Escherichia coli	74 (32.74)
K. pneumoniae	55 (24.34)
K. ozaenae	1 (0.44)
K. oxytoca	1 (0.44)
Pseudomonas	32 (14.16)
Acinetobacter	11 (4.87)
Enterobacter	13 (5.75)
Proteus mirabilis	6 (2.65)
Proteus vulgaris	3 (1.33)
Citrobacter	4 (1.77)
Salmonella typhi	1 (0.44)
Gram positive bacteria	25 (11.06)
Total	226 (100.00)

Out of 55 isolated *K. pneumoniae,* 10 (18.18%) were resistant to fosomycin detected by agar dilution method. MIC of fosfomycin resistant isolates ranged from 256 to 4096 µg/ml (Table III).

Table III MIC of fosfomycin among fosfomycin resistant *K. pneumoniae* (n=10)

MIC of fosfomycin (µg/ml)		Number (%)
≥ 4096		1 (10.00)
	2048	2 (20.00)
	1024	4 (40.00)
	512	2 (20.00)
	256	1 (10.00)
	128	0 (0.00)
	64	0 (0.00)
	32	0 (0.00)
	Total	10 (100.00)

Among 10 fosfomycin resistant *K. pneumoniae,* 8 (80%) were positive for fosA5, 5 (50%) were positive for fosA, 4 (40%) were positive for fosA3, 3 (30%) were positive for fosA4 and 2 (20%) were positive for fosB. NofosC, fosC2 and fosX genes were found in any isolates (Table IV).

Table IV Distribution of fosfomycin resistance genes among fosfomycin resistant *K. pneumoniae* in different samples (n= 10)

Genes	Wound swab n=3 n (%)	Urine n=5 n (%)	Sputum n=1 n (%)	ETA n=1 n (%)	Total n=10 n(%)
fosA	1 (33.33)	3 (60.00)	0 (0.00)	1 (100.00)	5 (50.00)
fosA3	1 (33.33)	2 (40.00)	0 (0.00)	1 (100.00)	4 (40.00)
fosA4	0 (0.00)	2 (40.00)	0 (0.00)	1 (100.00)	3 (30.00)
fosA5	2 (66.67)	4 (80.00)	1 (100.00)	1 (100.00)	8 (80.00)
fosB	0 (0.00)	1 (20.00)	0 (0.00)	1 (100.00)	2 (20.00)
fosC	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosC2	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosX	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)

N= Total number of fosfomycin resistant *K. pneumoniae* in different samples.
n= number of different samples positive for different fosfomycin resistance genes.
*ETA= Endotracheal Aspirates.

Discussion

Drug resistant *K. pneumoniae* has become a global threat causing pneumonia, septicemia, infections of wound, urinary tract and central nervous system with high mortality rates and warrants the search for alternative therapeutic option like fosfomycin.[18] However, the emergence of fosfomycin resistance has been increased.

In this study, out of 226 (66.47%) culture positive samples most of the organisms were *Escherichia coli* (32.74%) followed by *Klebsiella pneumoniae* (24.34%). A study in India reported *Escherichia coli*

(36.11%) as the most common organism followed by *K. pneumonia* (26.79%).[19] This similarity may be attributed to the fact that these two studies were conducted in same geographic areas.

In the present study, among the isolated *Klebsiella pneumoniae,* 10 (18.19%) fosfomycin resistant *K. pneumoniae* were identified. A study in India reported 22% fosfomycin resistance in *K. pneumoniae.*[20] There are a limited number of studies on fosfomycin resistance and the prevalence of resistance can vary and change over the time within and between the countries. In developing countries, owing to poor diagnostic facilities, circulation and co-circulation of resistance genes may remain undetected, underreported and poorly characterized.[21]

In the present study, MIC of fosfomycin among the fosfomycin resistant *K. pneumoniae* ranged from 256 to ≥ 4096 µg/ml which is in accordance with the study in Dhaka Medical College Hospital who reported MIC value of fosfomycin ranging from 512 to ≥ 4096 µg/ml.[22] Elliot et al. reported that *K. pneumoniae* isolates carrying fosA genes were associated with higher MIC distribution.[23]

In this study, among 10 fosfomycin resistant *K. pneumoniae,* fosA5 was the most prevalent resistance gene (80%) followed by fosA(50%) fosA3 (40%), fosA4 (30%) and fosB (20%). Huang et al. reported 100% prevalence of fosA5 in fosfomycin resistant *Klebsiella pneumoniae*.[24] According to a previous study, fosA5 showed high similarity to the chromosomal $fosA^{Kp}$ of the *K. pneumoniae*, which can be found in most of *K. pneumoniae.*[25] The prevalence of fosA3 was reported as 36.3% among KPC-Kp in China.[8] Another study showed 60% fosfomycin resistant *K. pneumoniae* harbouring fosA.[26]

Limitation

All other genes responsible for fosfomycin resistance could not be detected due to time and resource constraints.

Conclusion

This study shows an increasing rate offosfomycin resistance among the clinical isolates of *K. pneumoniae*. Plasmid mediated dissemination of fosfomycin resistance genes from one species to anotheris a major concern for public health. Implementation of antibiotic stewardship programme along with surveillance of fosfomycin resistance is necessary to control the spread of resistance genes.

Recommendation

Further study can be done to evaluate the efficacy of fosfomycin based antibiotic combination against fosfomycin resistant *K. pneumoniae*.

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Contribution of authors

FA-Design, acquisition of data, analyzed the data, interpreted the results, draft manuscript & final approval.

SMS-Analysis of data, critical revision & final approval.

Disclosure

Both the authors declared no conflict of interest.

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