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RESEARCH PAPER

Emergence of Fosfomycin Resistance among Isolates of Multidrug-resistant Enterobacter Species at a Tertiary Care Hospital in Dhaka, Bangladesh

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Abstract

Background: The emergence of multidrug-resistant *Enterobacter* species as a worrying resistant pathogen seriously threatened human health.

The rising rate of resistance to commonly used antibiotics limit the choice hence it is urgent to evaluate the antimicrobial activity of older drug like Fosfomycin.

Objective: The study aimed to seek the frequency of fosfomycin resistance in the clinical isolates of *Enterobacter* species and to detect the fosfomycin resistance gene along with antibiotic resistance pattern.

Methods: This cross-sectional study was conducted in the Department of Microbiology of a tertiary care hospital in Bangladesh from July 2018 to June 2019. *Enterobacter spp.* was isolated from a total of 350 samples by a standard microbiological method. Antibiotic susceptibility was performed by the disk diffusion technique. Antibiotic susceptibility and minimum inhibitory concentration (MIC) of fosfomycin were determined by the agar dilution method. Fosfomycin resistance gene *fosA*, *fosA3, fosA4, fosA5, fosB, fosB2, fosC*, *fosC2* and *fosX* among fosfomycin resistant *Enterobacter spp* detected by polymerase chain reaction (PCR) using specific primer. Sequencing of fosA and fosA… was performed by capillary method, and the nucleotide sequence of *fosA*… has been deposited to GenBank.

Results: Out of 28 *Enterobacter spp*. 7 (25%) fosfomycin resistant *Enterobacter spp.* were detected by agar dilution method. Out of 7 fosfomycin-resistant strains, 4 (57.14%) were isolated from urine samples. Fifteen (53.57%) isolates of *Enterobacter spp.* were multidrug-resistant detected by disc diffusion technique. All of the fosfomycin-resistant isolates were MDR. A significant rise in the MIC was found between 256µg/ml - ≥4096µg/ml to fosfomycin. PCR revealed that 100% of fosfomycin resistant isolates are positive for *fosA,* 71.43% and 28.57% were positive for *fosA*… and *fosB,* respectively. Sequencing of *fosA*… gene established the FosA family fosfomycin resistance glutathione transferase gene.

Conclusion: The results of this study showed a high proportion of fosfomycin resistance among multidrug-resistant *Enterobacter spp.* irrespective of fosfomycin usage in Bangladesh. FosA family fosfomycin resistance glutathione transferase gene is emerging in Bangladesh.

Keywords: Fosfomycin resistance gene, Multidrug resistance, Polymerase chain reaction, Sequencing, *Enterobacter spp.*

Introduction

Fosfomycin is a broad-spectrum bactericidal antibiotic considered as a potential treatment option against multi-drug resistant (MDR) bacteria especially extended-spectrum beta-lactamase and carbapenemase-producing Enterobacteriaceae.[1] Its re-emergence as an antibiotic of interest is due to the global increasing resistance of several bacteria to numerous antimicrobials.[2] There is a global interest in further investigating fosfomycin as monotherapy and in combination with other antimicrobial agents for the treatment of serious systemic infections due to multidrug-resistant (MDR) Gram-negative bacteria.[1,3-5] *Enterobacter spp* has gained immense recognition as an emerging pathogen in recent years and is increasingly associated with multidrug resistance, including the last resort carbapenem, and becomes a

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member of ESKAPE pathogens which are considered as the most concerning for healthcare institutions worldwide.[6,7] These organisms seem to have an innate resistance to older anti-microbial agents and have the propensity to rapidly develop resistance to newer anti-microbial agents.[8] *Enterobacter spp* is frequently associated with a multidrug-resistance phenotype, mainly due to their adaptation to the hospital environment and the pathogen's ability to easily acquire numerous genetic mobile elements containing resistance genes.[9,10]

The efficacy of fosfomycin against extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae is favorable although increasing fosfomycin resistance rates due to fosfomycin-modifying enzymes have been observed among ESBL-producing and carbapenemase-producing Enterobacteriaceae in the last decade, primarily in Asia but more and more globally.[11-14] There are several mechanisms of resistance to fosfomycin but the contribution of fosfomycin susceptibility rates varies in an evolving environment.[2]

To date contribution of fosfomycin-inactivating enzymes in the emergence and spread of fosfomycin resistance currently seems low-to-moderate, but their presence in transferable plasmids may potentially provide the best means for the spread of fosfomycin resistance in the future. The multidrug resistance plasmid, pHN7A8, carries the bla (TEM-1b), bla (CTX-M-65), fosA3, and rmtB genes conferring resistance to penicillins, cephalosporins, fosfomycin, and aminoglycosides respectively.[15] Plasmid pKP96 carries nine genes (fosA among them), conferring resistance to several antibiotics, including penicillins, cephalosporins, fosfomycin, aminoglycosides, tetracycline, quinolones, and sulfamethoxazole.[16] The plasmids determinants of resistance to fosfomycin have been described as four main types: *fosA, fosB, fosC*, *fosX* genes and their subtypes: *fosA, fosA2, fosA3, fosA4, fosA5*, and *fosC2* genes. Unlike other fos-related genes, *fosA2* has been found in the chromosomes of *E. cloacae* isolates and flanked by Tn2961 sequences, indicating dissemination potential within *Enterobacter species*.[17-18] At least 10 kinds of *fos* genes have been identified of these, *fosA* (and *fosA* subtypes) and *fosC2* are primarily found in *Enterobacteriaceae*.[19]

Fosfomycin resistance is not routinely tested in most monitoring and surveillance studies and if so, the resistance mechanism is often not further analyzed and the molecular bases are rarely documented. Although the use of fosfomycin in Bangladesh is rare. To the best of our knowledge, no study has so far been carried out among *Enterobacter spp.* isolated from urine, wound swab and pus, blood, and endotracheal aspirates regarding detection of fosfomycin resistance and their molecular bases. Considering the public threat of dissemination of mobile fosfomycin resistance gene in multidrug-resistant clinical isolates, this study has been designed to obtain data on the resistance pattern of *Enterobacter spp* along with the detection of fosfomycin resistance gene by polymerase chain reaction (PCR) and sequencing.

Materials and methods

After obtaining approval from the institutional ethical committee, this cross sectional study was conducted in the Department of Microbiology of a tertiary care hospital of Bangladesh from July 2018 to June 2019. Informed written consent was taken from each patient or their legal guardian. Urine, wound swab and blood, endotracheal aspirates of adult patients having clinically suspected infections admitted in a tertiary care hospital of Bangladesh or attending in the Microbiology department for culture and sensitivity were included in this study irrespective of sex and antibiotic intake.

Identification of Enterobacter species: All samples were inoculated in MacConkey agar and blood agar media and incubated overnight aerobically at 37ºC. Trypticase soya broth was used for primary blood culture then subculture was done on blood agar and MacConkey agar media. Genus *Enterobacter* was identified by characteristics colonies (Lactose-fermenting, sometimes mucoid colonies), Gram staining pattern as Gram-negative bacilli, motility as motile, and standard biochemical reactions. Common *Enterobacter spp* (*E. cloacae and E. aerogenes*) were isolated.[20]

Antimicrobial susceptibility testing: Kirby–Bauer modified disc diffusion technique was used for antimicrobial susceptibility using Mueller Hinton agar plates and the zone of inhibition was interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines and criteria of the European Committee on Antimicrobial susceptibility testing were used for fosfomycin (Oxoid Ltd., UK).[21-22] Susceptibility testing for Amoxycillin/clavulanic acid

(20/10µg), Cefepime (30 µg), Ceftazidime (30µg), Cefuroxime sodium (30µg), Cefoxitin (30µg), Amikacin (30µg), Ceftriaxone (30µg), Ciprofloxacin (30µg), Piperacillin/Tazobactam (110/10µg), Imipenem (10µg), Tigecycline (15µgm), Aztreonam (30µgm) were performed. For fosfomycin susceptibility testing by the agar dilution method, Mueller-Hinton agar supplemented with 25 µg/mL of glucose-6-phosphate was used and interpreted according to EUCAST.[23] All the isolates identified as multi-drug resistant (MDR) based on the criteria of the European Centre for Disease Control (non-susceptible to ≥1 agent in ≥3 antimicrobial categories) were tested with Fosfomycin.[22] ESBL and carbapenemase producers were detected phenotypically by the double disc synergy (DDS) test and combined disc (CD) assay.

Determination of minimum inhibitory concentration of Fosfomycin: MIC of fosfomycin was determined by agar dilution method following EUCAST guideline 2018.[23]

Molecular Characterization of Fosfomycin Resistance Gene: PCR was done to detect the fosfomycin resistance gene. To prepare bacterial pellets, a loop full of bacterial colonies from MHA media was inoculated into a Falcon tube containing trypticase soy broth. After incubation overnight at 37ºC, the Falcon tubes were centrifuged at 4000 ×g for 10 minutes, after which the supernatant was discarded. A small amount of sterile trypticase soy broth was added into the Falcon tubes with pellets and mixed evenly. Then an equal amount of bacterial suspension was placed into 2 to 3 microcentrifuge tubes. The microcentrifuge tubes were then centrifuged at 4000 ×g for 10 minutes and the supernatant was discarded. The microcentrifuge tubes containing bacterial pellets were kept at -20ºC until DNA extraction. Bacterial DNA was extracted by the boiling method.[24] The pair of primers were used to yield PCR products depicted in the following table (Table I).[12,14,25]

PCR assays were performed in a DNA thermal cycler. After initial denaturation at 94ºC for one minute, the reaction was subjected to 32 cycles (annealing at 57ºC for 40 seconds, elongation at 72ºC for one minute) with a final extension at 72ºC for 10 minutes. The amplified DNA was loaded into a 2% agarose gel, electrophoresed at 230 volts for 30 minutes, stained with 1% ethidium bromide, and visualized under ultraviolet light (Figure i). under UV light (Figure 1)

Table I: Primers used in this study[12,14,25]

Target Genes	Sequence (5'-3')		Size (bp)
1.*fos A*	F	ATC TGT GGG TCT GCC TGT CGT	271
	R	ATG CCC GCA TAG GGC TTC T	
2.*fosA3*	F	CCT GGC ATT TTA TCA GCA GT	221
	R	CGG TTA TCT TTC CAT ACC TCA G	
3.*fosA4*	F	CTG GCG TTT TAT CAG CGG TT	230
	R	CTT CGC TGC GGT TGT CTT T	
4.*fosA5*	F	TAT TAG CGA AGC CGA TTT TGC T	177
	R	CCC CTT ATA CGG CTG CTC G	
5.*fosB*	F	CAG AGA TAT TTT AGG GGC TGA CA	312
	R	CTC AAT CTA TCT TCT AAA CTT CCT G	
6.*fosB2*	F	CCT GGC CGA GAA AGA GAT GAG	392
	R	AAC CGG TTT TGC AAA GTG CC	
7.*fosC*	F	CCT TGC TCA CTG GGG ATC TG	354
	R	TAC AAG ACC CGA CGC ACT TC	
8.*fosC2*	F	TGG AGG CTA CTT GGA TTT G	209
	R	AGG CTA CCG CTA TGG ATT T	
9.*fosX*	F	TGT CCC TCA CCT TCG ACT CT	
	R	TTG CTG GTC TGT GGA TTTGC	

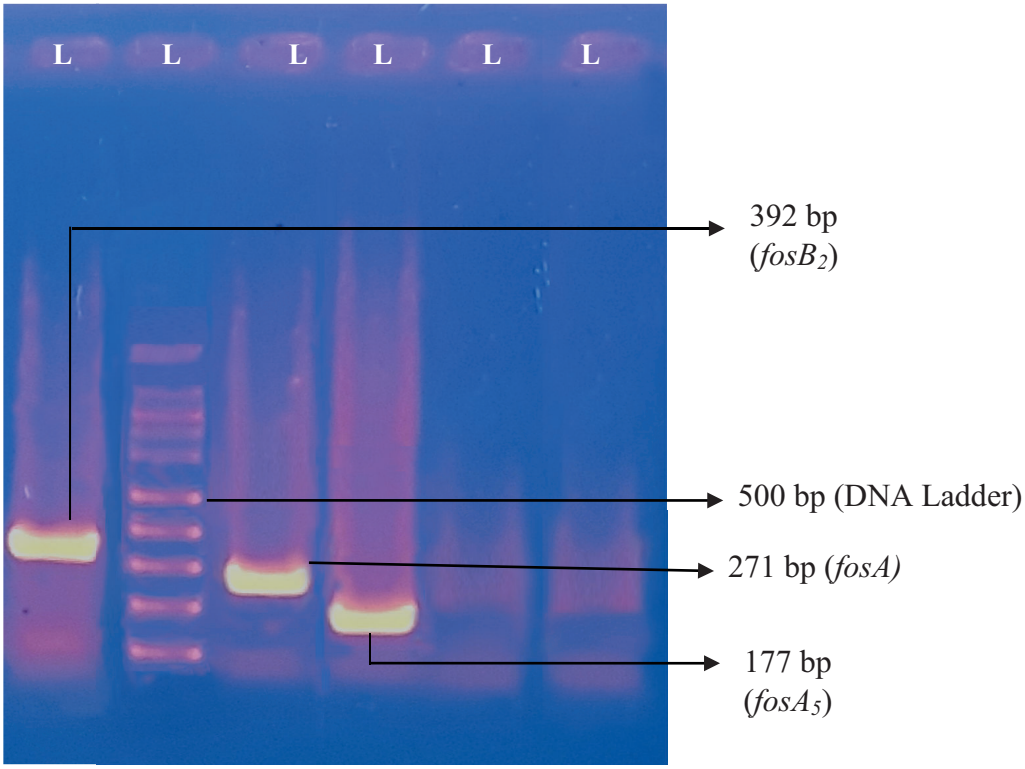

Figure 1: Photograph of gel electrophoresis of amplified DNA of 392bp for *fosB*$_2$ gene (lane 1), 100 bp DNA ladder (lane 2), amplified DNA of 271bp for *fosA* gene (lane 3), amplified DNA of *fosA*$_5$ gene (lane 4), negative control without DNA (TE buffer) (lane 5), negative control *Escherichia coli* ATCC 25922 (lane 6).

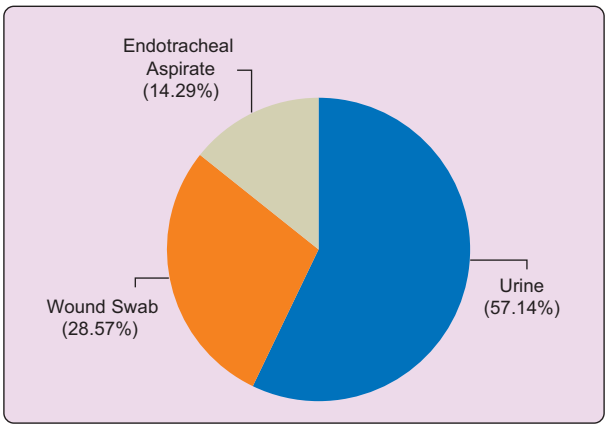

Figure 2: Distribution of Fosfomycin-resistant *Enterobacter* species

DNA sequence analysis: As this was the first detection of fosfomycin resistance gene in the tertiary care hospital, sequencing was performed. After PCR, purification of amplicons was done by using a DNA purification kit (FAVORGEN, Biotech Corp.) and subjected to automated DNA sequencing (ABI PRISM 3500). BLAST (Basic Local Alignment Search Tool) analysis was performed to search for homologous sequences in the GenBank database.

Statistical analysis: Data were analyzed by using Microsoft Office Excel (2013) software (Microsoft, Redmond, WA, USA).

Results

Among 350 samples, 28 E*nterobacter spp*. were identified. Of them, 22 (78.57%) were identified as *Enterobacter cloacae* and 6 (21.43%) were *Enterobacter aerogenes*. Out of 28 isolates, 7 (25%) were resistant to fosfomycin during the agar dilution method of which 6 (85.71%) were *Enterobacter cloacae* and 1 (14.29%) were *Enterobacter aerogenes*. *Enterobacter spp.* showed high resistance to most of the commonly used antibiotics whereas tigecycline was found the most sensitive drug followed by cefepime (Table II).

Table II: Resistant pattern of fosfomycin-resistant *Enterobacter spp*. (n=7)

Antimicrobial agent	*Enteraobacter cloacae* N= (6) n%	*Enterobacter aerogenes* N= (1) n%	Total N= (7) n%
Amoxiclav	6 (100)	1 (100)	7 (100)
Cefoxitin	6(100)	1(100)	7 (100)
Ceftriaxone	6(100)	1(100)	7 (100)
Ceftazidime	6(100)	1(100)	7 (100)
Amikacin	5(83.33)	1 (100)	6 (85.71)
Piperacillin-Tazobactam	5(83.33)	1(100)	6 (85.71)
Amikacin	5 (83.33)	1(0.00)	6 (85.71)
Ciprofloxacin	6(100)	0(0.00)	6 (85.71)
Imipenem	5(83.33)	0(100)	5 (71.43)
Colistin	3(50)	0 (0.00)	3 (42.86)
Cefepime	2(33.33)	0(0.00)	2 (28.57)
Tigecycline	1(16.67)	0 (0.00)	1 (14.28)

N=Total number of bacteria; n=Number of resistant bacteria

Among 28 isolated *Enterobacter spp*. 32.14% were ESBL producers detected by DDS test. Among 7 fosfomycin resistant, *Enterobacter* isolates, 5 (71.43%) showed imipenem resistance of which 4 (80%) and 3 (60%) carbapenemase producers were detected by DDS test and CD assay respectively. MIC of 7 fosfomycin-resistant isolates ranges from 256µg/ml to 4096 µg/ml (Table III).

Among 7 fosfomycin-resistant isolates, most of the isolates were obtained from urine samples 4 (57.14%) (Figure ii). Seven (100%) Fosfomycin-resistant strains were found positive for *fosA*, followed by 5 (71.43%) were positive for *fosA…*, and 2 (28.57%) were positive for *fosB*, . The combinations of different genes in a single isolate were obseverd in the present study (Table IV). None of the isolates were positive for *fosAf , fosA„ , fosB, fosC, fosC, , fosX.*

Sequencing of *fosA…* gene had 97% identity with FosA family fosfomycin resistance glutathione transferase gene detected in *Klebsiella pneumoniae* (strain: sc-7) (GenBank accession: CP030269:1) isolated from urine.

Table III: Minimum inhibitory concentration of fosfomycin among Fosfomycin-resistant *Enterobacter species*

Resistant isolates	MIC of fosfomycin (µg/ml)					
	≥4096	2048	1024	512	256	128
Enterobacter cloacae(n=6)	1 (16.67)	1 (16.67)	1 (16.67)	3 (50.00)	-	-
Enterobacter aerogenes(n=1)	-	-	-	-	1 (100.00)	-
Total (n=7)	1(14.28)	1 (14.28)	1(14.28)	3 (42.86)	1 (14.28)	

The figure within parenthesis indicates the percentage. MIC: Minimum inhibitory concentration

Table IV: Detection *of fosA*, *fosA*$_3$, *fosA*$_4$, *fosA*$_5$, *fosB, fosB*$_2$, *fosC*, *fosC*$_2$ and *fosX* genes among fosfomycin resistant *E. cloacae* and *E. aerogenes* by PCR (n=7)

Samples	*fosA*	*fosAf*	*fosA„*	*fosA…*	*fosB*	*fosB,*	*fosC*	*fosC,*	*fosX*	Total
Urine	+	-	-	+	-	-	-	-	-	3 (42.86)
Urine	+	-	-	+	-	+	-	-	-	1 (14.28)
W/s and pus	+	-	-	-	-	-	-	-	-	1 (14.28)
W/s and pus	+	-	-	-	-	+	-	-	-	1 (14.28)
ETA	+	-	-	+	-	-				1 (14.28)
Total*	7(100.00)	0(0.00)	0(0.00)	5(71.43)	0(0.00)	2(28.57)	0(0.00)	0(0.00)	0(0.00)	7(100.00)*

*Denotes the column total, += Present, "= Absent. The total of the last row is more as some of the isolates had more than one gene. W/s=wound swab, ETA =Endotracheal aspirates

Nucleotide sequence accession number

The nucleotide sequence of *fosA…* gene of *E. cloacae* strains ID-23 obtained from urine sample has been deposited in the GenBank database under Accession no. MN755612.

Discussion

The shortage of new antimicrobial agents has led to the re-evaluation of old antibiotics such as fosfomycin as a potential regimen for treating multidrug-resistant bacteria especially extended-spectrum-beta-lactamase- and carbapenemase-producing Enterobacteriaceae but the detection of mobile fosfomycin-resistant genes in Enterobacteriaceae has increased in recent years.

In the current study, among the 28 isolated *Enterobacter spp*. 7 (25%) were resistant to fosfomycin. A study in India reported that 40% of *Enterobacter spp*. were resistant to fosfomycin and all the resistant strains were isolated from urine samples which is similar to the present study.[26] In the present study, 57.14% of *Enterobacter spp*. resistant to fosfomycin were isolated from urine. Among isolated *Enterobacter spp* 32.14% were ESBL producers detected by the DDS test. In a study in India, the prevalence of ESBL among *Enterobacter spp.* was 33.33%.[27] Clinical use of fosfomycin in Bangladesh is rare and there is no data regarding fosfomycin resistance in clinical isolates. The reason behind fosfomycin resistance in the current study might be due to the horizontal transfer of resistance genes between different species. Plasmids containing ESBL and fos genes may facilitate the dissemination of antibiotic resistance. Recent studies indicate that the recombination of plasmid-encoding carbapenemase and fosfomycinase occurs via mobile elements, thus presenting new treatment challenges.[28]

In the present study, among the fosfomycin resistant *Enterobacter spp*. 7 (100%) were positive for *fosA,* 5 (71.43%) were positive for *fosA5* and 2 (28.57%) were positive for *fosB2.* The fosfomycin resistance protein FosA, are metalloenzymes that catalyze the nucleophilic addition of the tri-peptide glutathione to the C1 position of the antibiotic, cleaving the epoxide ring and rendering it ineffective as an antibacterial drug. Among glutathione transferase (FosA type) enzymes found to be plasmid-borne are *FosAƒ , FosA„ , FosA…* and *FosC, .*[29] A study in China by Zhang *et al*. (2017) reported that 80% *of Enterobacter cloacae* isolates were positive for *fosA* and 10% *E. cloacae* were positive for *fosA5*. The plasmid-mediated fosfomycin enzyme *fosA…* gene was found in *E. coli* first in 2014. In China, among 73 *Klebsiella pneumoniae* isolates all produce *fosA… gene*.[30] However, among 7 fosfomycin-resistant strains, 5 (71.43*%) E. cloacae* isolates were positive for *fosA…* gene in the present study, which might indicate that fosA… genes are transferred from *K.* pneumoniae to *Enterobacter spp.* No data is found to compare the resistance rate of *fosB2* genes among fosfomycin-resistant *Enterobacter spp.* Acquisition of fosfomycin resistance by antibiotics modifying enzyme that shows a higher incidence in multidrug-resistant strains. The multidrug resistance plasmid, pKP46 carries nine genes (fosA among them) conferred resistance to several antibiotics including penicillins, cephalosporins, fosfomycin, aminoglycosides, quinolones.[15] In this study, among 28 isolated *Enterobacter spp*. 15 (53.57%) isolates were multidrug-resistant. A study by Adhikari *et al.* (2018) in Nepal reported that 52.90% *of Enterobacter spp.* were MDR.[31]. This multi-resistance plasmid might be the reason behind increasing fosfomycin resistance among MDR *Enterobacter spp.* Moreover, resistance developed mainly in strains of *P. aeruginosa, Klebsiella spp., Proteus spp.*, and *Enterobacter spp*.[2]

In the present study, MIC of fosfomycin among the fosfomycin-resistant *Enterobacter spp*. ranged 512 µg/ml - 4096 µg/ml. The reason behind the high MIC of fosfomycin might be due to moderate susceptibility of *Enterobacter spp* to fosfomycin (MIC ranged from 16-64 µg/ml) whereas other *Enterobacteriacae* including *E. coli, K. pneumoniae* are highly susceptible (MIC <16 µg/ml).[32] The present study reported that *Enterobacter* species were resistant to most of the commonly used antibiotics in Bangladesh with the emergence of resistance to fosfomycin which is considered as a reserve drug for the treatment of multidrug-resistant Enterobacteriaceae. Data from recent studies suggest that the therapeutic use of other antibiotic classes promotes the emergence and dissemination of fosfomycin-resistant Entero-bacteriaceae since additional resistance genes are often present.[33] Although, it is not surprising that the resistance rate is higher in the most commonly used antibiotics among tested *Enterobacter* clinical isolates, what is perhaps more unexpected is the detection of fosfomycin resistance among

Enterobacter isolates which is not routinely used to date in Bangladesh. The high antibiotic resistance in the present study might be due to the indiscriminate use of antibiotics that provide selective pressure.

Conclusion

Fosfomycin resistance genes are emerging in Bangladesh which was detected in *Enterobacter cloacae*. The acquisition of plasmid-mediated fosfomycin-modifying enzymes accounted for a majority of the fosfomycin resistance in the clinical isolates of *Enterobacter spp* in the current study may be of major concern for physicians because this organism is increasingly associated with multidrug resistance and one of the common threats for hospital patients of critical care unit. Continuous monitoring will be necessary to prevent further dissemination of fosfomycin resistance genes as well as the optimization of therapeutic strategies is necessary.

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