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In Vitro and in Vivo Evaluation of Antibiotic Combination against Multidrug Resistant *Enterobacter Species* Isolated From Patients of a Tertiary Care Hospital, Bangladesh

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Abstract The emergence of multidrug-resistant (MDR*) Enterobacter* as a worrying resistant pathogen is an important health concern, especially when there is scarcity of new antibiotics active against Gram-negative bacteria. However, currently no defined therapies available for MDR *Enterobacter* infections. In this study, in *vitro* and *in vivo* efficacy of different antimicrobial combinations were assessed. This cross-sectional study was carried out in the department of Microbiology of Dhaka medical college hospital, Bangladesh from July, 2018 to June, 2019. Multidrug resistance among isolated *Enterobacter species* were detected phenotypically by disk diffusion method. PCR and sequencing of fosfomycin resistance genes were done. In vitro activity of fosfomycin, amikacin, imipenem, piperacillin-tazobactam and their combinations were evaluated using agar dilution method and synergy was assessed by Fractional inhibitory concentration index. Mice models were made by using the MDR *Enterobacter* strain. We evaluate the efficacy of fosfomycin, amikacin, imipenem and their combination against multi-drug resistant *Enterobacter* infection in experimental mice models. Among 28 isolated *Enterobacter spp*. 53.33% were multidrug-resistant. Among the fosfomycin resistant *Enterobacter spp*. 70% , 50%, 40% were positive for *fosA*. *foA*$_5$ and *fosB*$_2$ respectively. The fractional inhibitory concentration index indicated that combining antibiotics resulted 2 to 8 fold reduction of MIC compared to single therapy. The ratio of synergy observed in imipenem-amikacin, fosfomycin-amikacin, fosfomycin-imipenem 16.67%, 87.33%, 50.0% respectively *in vitro*. No synergy observed in imipenem-piperacillin tazobactam combination. In mice model, compared to single antibiotic therapy, fosfomycin-amikacin, imipenem-amikacin, fosfomycin-imipenem showed increased sterile blood culture (100%, 60%, 80%). Fosfomycin plus amikacin or fosfomycin plus imipenem may be alternative treatment option against multidrug-resistant *Enterobacter* infection.

Keywords: *Enterobacter species, multidrug-resistance, combination therapy, in vitro and in vivo efficacy, fosfomycin resistance gene*

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1. Introduction

The genus *Enterobacter* is a member of the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species*), which contains the major resistant bacterial pathogens [1]. These pathogens are frequently associated with a multidrug resistance (MDR) phenotype, mainly due to their adaptation to the hospital environment [2,3]. *Enterobacter* infections can include bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis [4]. Specific risk factors for infection with nosocomial multidrug-resistant strains of *Enterobacter* species include the recent use of broad-spectrum cephalosporins or aminoglycosides and ICU care [5].

MDR strains of *Enterobacter spp.* is increasingly associated with resistance to the last-resort carbapenems [6]. *Enterobacter spp.* resistant to last resort antibiotics, tigecycline or colistin due to impaired uptake and enhanced pump out has been reported discourages the use of these agents as single therapies in the management of

infections caused by MDR pathogens [7]. The limited new options against these types of bacterial strains has meant that, over the last decade, antibiotics such as fosfomycin have gained considerable importance as rescue strategies or as combined therapy options for treating infections caused by these multidrug-resistant bacteria [8]. Although the contribution of fosfomycin-inactivating enzymes in emergence and spread of fosfomycin resistance currently seems low-to-moderate, their presence in transferable plasmids may potentially provide the best means for the spread of fosfomycin resistance in the future [9].

To best our knowledge, no study has been carried out in Bangladesh regarding use of different antibiotic combinations on multidrug resistant *Enterobacter spp*. In order to identify the best combination, we investigate the effectiveness of single drug and combination of fosfomycin, imipenem, amikacin, piperacillin-tazobactam against these multidrug resistant organisms and to compare the *in vitro* and *in vivo* activity. For *in vivo* experiment we developed a new MDR *Enterobacte*r experimental sepsis model in immunocompetent mice.

2. Methodology

2.1. Study Design

This cross-sectional study was conducted in the department of Microbiology of Dhaka Medical College Hospital, Bangladesh during July 2018 to June 2019. Ethical permission for this study was obtained from the institutional review board.

2.2. In Vitro Study

2.2.1. Isolation of Multidrug Resistant *Enterobacter spp.*

A total 350 samples (urine, wound swab, endotracheal aspirate and blood) were collected from adult patients admitted in Dhaka medical college hospital having clinically suspected infections were inoculated on MacConkey agar media and Blood 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 (catalase, oxidase, indole production, citrate utilization, urease activity, reaction in triple sugar iron medium and lysine decarboxylase test). Common *Enterobacter spp* (*E. cloacae* and *E. aerogenes*) were isolated [10]. Multidrug resistant *Enterobacter spp.* were identified by antimicrobial susceptibility testing by disk diffusion method using commercially available antibiotics disk (Oxoid Ltd, Basngstoke, United Kingdom*). Escherichia coli* ATCC 25922 was used for quality control [11]. 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 as per the Clinical Laboratory Standards Institute [11]. 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 [12].

The various beta-lactamases namely, the ESBL, AmpC, and MBL were screened using the combination discs. ESBL producers were detected by using combination discs of ceftriaxone, ceftazidime and clavulanic acid. AmpC producers were detected by cefoxitin-EDTA disk diffusion test while MBL producers were detected by Double-disk synergy test (DDST). All the isolates identified as multi-drug resistant based on the criteria of the European Centre for Disease Control (non-susceptible to ≥1 agent in ≥3 antimicrobial categories) [13].

Table 1. Primers used in this study

Genes	Sequence (5'-3')		Size (bp)	Reference
1. *fos A*	F	ATC TGT GGG TCT GCC TGT CGT	271	15
	R	ATG CCC GCA TAG GGC TTC T		
2. *fosA3*	F	CCT GGC ATT TTA TCA GCA GT	221	15
	R	CGG TTA TCT TTC CAT ACC TCA G		
3. *fosA4*	F	CTG GCG TTT TAT CAG CGG TT	230	16
	R	CTT CGC TGC GGT TGT CTT T		
4. *fosA5*	F	TAT TAG CGA AGC CGA TTT TGC T	177	15
	R	CCC CTT ATA CGG CTG CTC G		
5. *fosB*	F	CAG AGA TAT TTT AGG GGC TGA CA	312	17
	R	CTC AAT CTA TCT TCT AAA CTT CCT G		
6. *fosB2*	F	CCT GGC CGA GAA AGA GAT GAG	392	17
	R	AAC CGG TTT TGC AAA GTG CC		
7. *fosC*	F	CCT TGC TCA CTG GGG ATC TG	354	17
	R	TAC AAG ACC CGA CGC ACT TC		
8. *fosC2*	F	TGG AGG CTA CTT GGA TTT G	209	17
	R	AGG CTA CCG CTA TGG ATT T		
9. *fosX*	F	TGT CCC TCA CCT TCG ACT CT		17
	R	TTG CTG GTC TGT GGA TTTGC		

2.2.2. Molecular Characterization of Fosfomycin Resistance Gene

Polymerase chain reaction was done to detect 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 37°C, 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 to 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 -20°C until DNA extraction. Bacterial DNA was extracted by the boiling method [14]. The pair of primers were used to yield PCR products depicted in (Table 1) [15,16,17].

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

2.2.3. Procedure of DNA Sequencing

For sequencing of bacterial DNA, purification of amplified PCR product was done by using DNA purification kits (FAVOGEN, Biotech Corp.) Purified PCR products of 3 isolate of *Enterobacter cloacae* from urine sample were sent to 1st BASE laboratories, Malaysia for sequencing by capillary method (ABI 3500). BLAST analysis was performed to search for homologus sequences into Genebank database at www.ncbi.nlm.gov.

2.2.4. Minimum Inhibitory Concentration (MIC)

MIC of fosfomycin (Beximco Pharma Ltd), amikacin (ACI Pharma Ltd), imipenem, piperacillin-tazobactam (Reneta Pharma Ltd) was determined among resistant *Enterobacter spp*. MIC was performed by the agar dilution method. MICs were determined by using dilutions of individual antibiotics incorporated into Mueller Hinton agar (Oxoid Ltd, Basngstoke, United Kingdom). Seven doubling dilutions each antibiotic was prepared. To obtain 10^4cfu/spot on the agar surface, one microlitre of 10 times diluted 0.5 McFarland turbidity of test inoculums were placed on Mueller Hinton agar plates. After incubation at 35°C overnight, the lowest concentration of antibiotic impregnated Mueller-Hinton agar showing no visible growth on agar medium was considered the MIC of that drug of that strain. *Escherichia coli* ATCC 25922 was used as control strain [11]. The MIC was defined as the lowest concentration of antibiotic at which no growth was visible to the naked eye.

2.2.5. Antibiotic Combination Testing

Fifteen MDR *Enterobacter spp.* were isolated by susceptibility testing. Six MDR strains of *Enterobacter spp* (3 from urine sample, 1 from wound swab, 1 from endotracheal aspirate and 1 from blood) was randomly selected for combination studies. Combination of fosfomycin with amikacin, imipenem and imipenem with amikacin, piperacillin-tazobactam were examined by agar dilution method. Two fold serial dilutions of antibiotics were prepared from two fold higher dilutions of MICs up to eight fold lower dilutions of MIC. In evaluating the combination effect, synergy was present by the agar dilution method when there was a fourfold or greater reduction in the MICs of both antibiotics. A reduction of less than fourfold in the MICs of both antibiotics was considered additive. Indifference was found when neither drug exhibited a decrease in MIC, and an increase in the MIC was considered antagonism. Testing for synergy by the agar dilution technique is based on inhibitory rather than bactericidal endpoints [18]. Synergy was further assessed by calculation of the fractional inhibitory concentration index FICI as shown in the following equation.

$$\text{FICI} = \frac{MICofantibioticAincombination}{MICofantibioticalone} + \frac{MICofantibioticBincombination}{MICofantibioticalone}$$

The FICI were interpreted as follows: Synergy defined when FICI value ≤ 0.5; additivity, FICI of >0.5 to ≤1; indifference, FICI of >1 to ≤2; antagonism, FICI of >2. [19]

3. In Vivo Study

Mouse of swiss albino species was used for these experiments. Immunocompetent male and female mice weighing, 12-15 gram obtained from ICDDR'B, Breeding house, Dhaka, Bangladesh. Mice were housed in separated ventilated cages with food and water. Mouse was infected by intra-peritoneal injection of 12.5 Unit of approximately 10^4cfu/ml bacterial inoculums using a 100 IU insulin syringe in the lower right abdomen [20]. Bacterial inoculum was obtained through a 24 hours subculture of multidrug resistant *Enterobacter* in Mueller-Hinton agar media at 37°C. The animals were observed for 72 hours and the survival was recorded every 12 hours. Blood sample were taken as detailed below. All the samples were processed for microbiological studies.

3.1. Antibacterial Treatment

To evaluate the effectiveness or the different antibiotic regimens 40 mice were divided into, 8 groups (A, B, C, D, E, F, G and H) with 5 mouse each group. Group A, B, C, D, E, F, G was inoculated with bacterial inoculums. But Group H was not be inoculated with bacterial inoculums. Group H was regarded as negative control group. Group A,B,C,D,E, F and H received antimicrobial treatment. Group G was positive control group. Group A, B, C, D, E and F received antimicrobial treatment after 4 hours inoculation of bacterial inoculums in following treatment regimens over 72 hours. Group A- imipenem only-60mg/kg/day, Group B - amikacin only- 15 mg/kg/day, Group-C- fosfomycin only-400mg/kg/day, Group-D-

fosfomycin plus amikacin, amikacin- 15mg/kg/day and fosfomycin-400mg/kg/day, Group-E- Imipenem plus amikacin Imipenem- 60 mg/kg/day and amikacin- 15 mg/kg/day, Group-F- imipenem plus fosfomycin, fosfomycin-400mg/kg/day imipenem -60mg/kg/day. All the antibiotics were given intraperitoneally twice daily for 72 hours.

The first dose of every antibiotic was administered 4 hours after inoculation of the organism. In order to confirm that these drugs were not be toxic to the animal, another group of five uninfected mouse (Group H) was given each antibiotic for 72 hours (uninfected treat group/negative control). The infected animals were observed for 72 hours of treatment and the cumulative survival was recorded every 12 hours. Blood samples were taken as described below.

3.2. Microbiological Study

After 72 hours of antibiotic treatment, blood samples were collected from mice by cardiac puncture aseptically. At first, upper part of the chest was shaved by razor, and then washed with hexisol. After palpating the cardiac pulsation with the finger pulp, the area was washed with povidone iodine, and then 100 IU insulin syringe needle was introduced through the skin in the heart of mouse blindly. For blood culture 0.5ml of each mouse blood was inoculated in sterile conical flask containing 5 ml TSB and incubated for 24 hours at 37°C. Subculture was done in Blood agar and MacConkey agar media and incubated at 37°C. Then the incubated plates were observed for positive or negative growth. [21]

4. Result

4.1. In Vitro Test

Out of a total 350 samples 28 *Enterobacter species* were identified. Among twenty-eight isolated *Enterobacter spp*. fifteen multi drug resistant strains were detected by disk diffusion technique. MDR strains were isolated from urine. ETA, wound swab and one isolated from blood (Table 2).

Table 2. Distribution of multidrug resistant (MDR) *Enterobacter spp.* isolated from different samples

Sample	Total isolates N=28	MDR isolates n =15 (%)
Urine	11	5 (45.55)
Wound swab	9	5 (55.55)
Endotracheal Aspirate	7	4 (57.14)
Blood	1	1 (100.00)
Total	28	15 (53.57)

Among MDR *Enterobacter spp*. most of the isolates were resistant to cephalosporin, β-lactams, fluroquinolones, aminoglycosides (Figure 1).

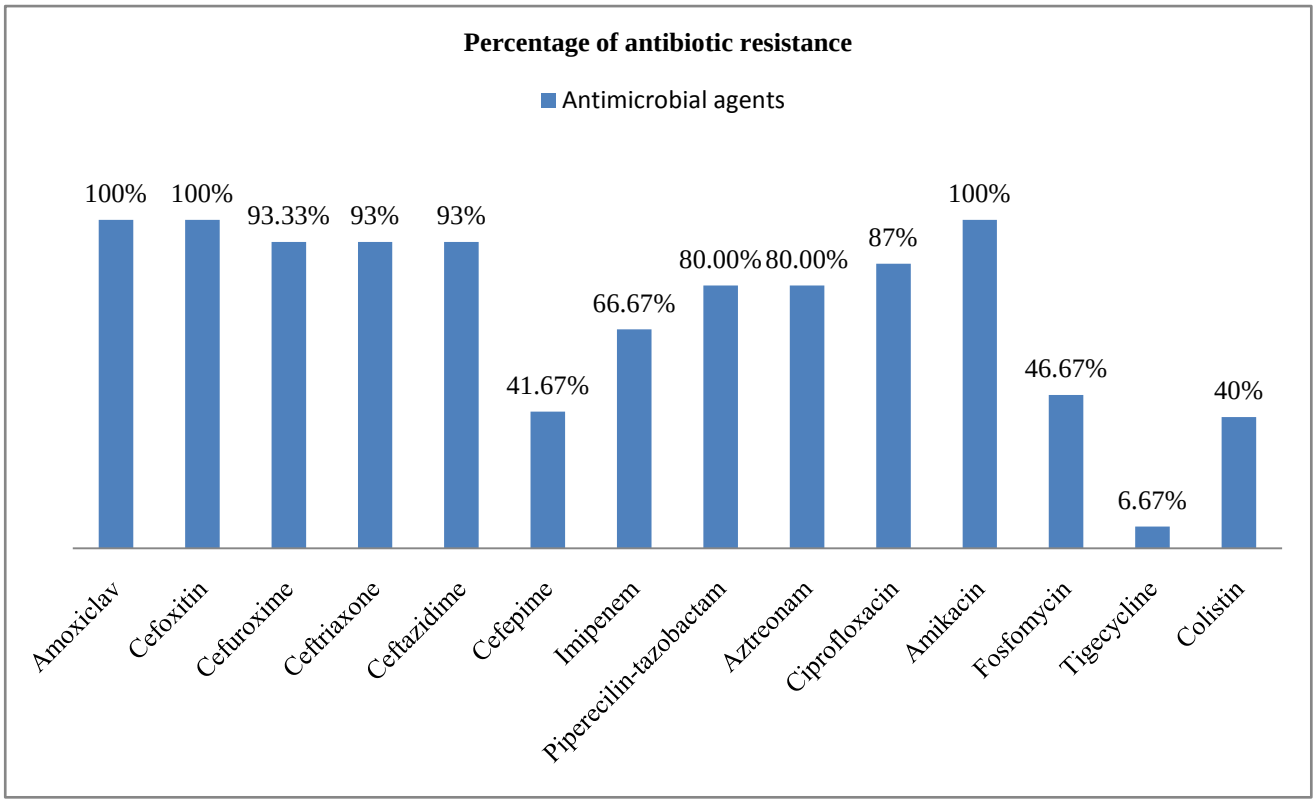

Figure 1. Antibiotic resistance pattern among MDR *Enterobacter* strains

Among the fosfomycin resistant *Enterobacter spp*. 70% were positive for *fosA*, 50% were positive for $fosA_5$ and 40% were positive for $fosB_2$ (Table 3). MIC of fosfomycin, amikacin, imipenem, piperacillin tazobactam among the MDR *Enterobacter* spp. were ranged from ≥2048-256 µg/ml, ≥2048-128 µg/ml, ≥128- 4µg/ml and ≥1024-64µg/ml respectively. Using different antibiotic combination against MDR *Enterobacter* isolates, resulted varying fold reduction in the MIC in agar dilution method which further assessed by FICI (Table 4).

Table 3. 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=10)

Genes	Urine n(%)	Wound swab n(%)	Endotracheal aspirate n(%)	Total n(%)
fosA	4 (40.00)	2 (20.00)	1(10.00)	7 (70.00)
fosA$_3$	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosA$_4$	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosA$_5$	3+1* (40.00)	1 (10.00)	0 (0.00)	5 (50.00)
fosB	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosB$_2$	2 (20.00)	2 (20.00)	0 (0.00)	4 (40.00)
fosC	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
fosC$_2$	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)

Table 4. Combinatory antibacterial activity of fofomycin, Imipenem, Amikacin and Piperacillin-Tazobactam against MDR *Enterobacter spp.*by agar dilution method (N=6)

MDR Strains	FOS+AK				AK+IM				FOS+IM				PIP-TZ+IM			
	FIC_a	FIC_b	FICI	Fold Reduction	FIC_a	FIC_b	FICI	Fold Reduction	FIC_a	FIC_b	FICI	Fold Reduction	FIC_a	FIC_b	FICI	Fold Reduction
1	0.25	0.25	0.50	4 fold	0.50	0.50	1	2 fold	1	1	2	N/R	1	1	2	N/R
2	0.125	0.125	0.25	8 fold	0.50	0.50	1	2 fold	0.25	0.05	0.50	4 fold	1	1	2	N/R
3	0.50	0.50	1	2 fold	1	1	2	N/R	0.50	0.50	1	2 fold	1	1	2	N/R
4	0.25	0.25	0.50	4 fold	1	1	2	N/R	0.50	0.50	1	2 fold	0.50	0.50	1	2 fold
5	0.25	0.25	0.50	4 fold	1	1	2	N/R	0.25	0.25	0.50	4 fold	1	1	2	N/R
6	0.125	0.125	0.25	8 fold	0.25	0.25	0.50	4 fold	0.25	0.25	0.50	4 fold	1	1	2	N/R

Note: FIC_a is fractional inhibitory concentration of one antibiotic in combination., FIC_b is fractional inhibitory concentration of other antibiotic in combination., FOS-Fosfomycin, AK-Amikacin, IM-Imipenem, PIP-TZ- Piperacillin-tazobactam, N/R- No reduction.

While combiningfosfomycin and amikacin among six MDR *Enterobacter* isolates, 83.33% showed synergistic effect (FICI value ≤ 0.5), 16.67% showed additive effect (FICI value >0.5 to ≤1). In case of imipenem and amikacin combination, 16.67% showed synergistic effect, 33.33% showed additive effect and 50% showed indifferent effect (FICI value >1 to ≤2). While combining fosfomycin and imipenem, 50% showed synergistic effect, 33.33% showed additive effectand 16.67% showed indifferent effect. While combining imipenem plus piperacillin-tazobactam, none of the MDR isolates showed synergistic effect (Table 5).

4.2. In Vivo Tests

All the mice in the positive control group were bacteraemic and all the mice of negative control group went sterile. In the group treated with only fosfomycin, imipenem, amikacin 40%, 60% and 80% mouse were bacteraemic. However, in the group treated with fosfomycin plus amikacin, imipenem plus fosfomycin and in another group treated with amikacin plus imipenem 100%, 80% and 60% mouse were blood culture negative respectively (Table 6).

Table 5. Comparison of efficacy of different antibiotic combinations against MDR *Enterobacter spp.* (N=6)

Antimicrobial Combination	Synergistic FICI ≤ 0.5 total (n%)	Additive FICI 0.5−≤ 1 total (n%)	Indifference FICI > 1−≤ 2 total (n%)	Antagonism FICI > 2 total (n%)
Fosfomycin+Amikacin	5 (87.33%)	1 (16.67%)	0 (0.00%)	0 (0.00%)
Amikacin+Imipenem	1 (16.67%)	2 (33.33%)	3 (50.00%)	0 (0.00%)
Fosfomycin+Imipenem	3 (50.00%)	2 (33.33%)	1 (17.67%)	0 (0.00%)
Imipenem+Piperacillin-Tazobactam	0 (0.00%)	1 (16.67%)	5 (87.33%)	0 (0.00%)

Table 6. Result of Antibiotic therapy on the clearance of MDR *Enterobacter spp.* from the blood of mouse

Group	Blood culture positive (n%)
Positive control (3)	3 (100%)
Negative control (5)	0 (0.00%)
Amikacin (5)	4 (80.00%)
Fosfomycin (5)	2 (40.00%)
Imipenem (5)	3 (60.00%)
Fosfomycin+Amikacin(5)	0 (0.00%)
Amikacin+Imipenem(5)	3 (60.00%)
Fosfomycin+Imipenem(5)	1 (20.00%)

5. Discussion

Antimicrobial drug resistance is ranked as one of the 10 global health threats facing humanity [22]. In an era of increasing antibiotic resistance rates, multidrug-resistant (MDR) Gram-negative bacteria constitute a major problem in the treatment of affected patients all over the world [23].

The emergence of multidrug-resistant *Enterobacter spp.* isolates has had a negative impact on the clinical outcome of infected patients and increasing mortality rates [24]. *Enterobacter spp.* Causes significant morbidity and mortality, and infection management is complicated due to resistance to multiple antibiotics. The treatment of *Enterobacter* infection can be problematic because of the increasing level of resistance to multiple antibiotics especially to quinolones, aminoglycosides, and beta-lactams [25]. Because resistance to carbapenems and other broad-spectrum beta-lactams is increasing, and there is a lack of new antibiotics, it is urgent to explore the potential of combination therapy to enhance the antibacterial effects of available drugs [26].

Although several studies have been reported antimicrobial combination against MDR *Enterobacteriaceae, Acinetobacter baumanii,* and *Pseudomonas spp.*, but there has very few researchinto therapy against multidrug resistant *Enterobacter* infection. Therefore, the main goal of this study to compare the *in vitro* and *in vivo* efficacy of antibiotic combination in the treatment of multidrug resistant *Enterobacter* infection, using a new experimental model of immunocompetent mouse.

In the present study, 15 (53.57%) multidrug resistant *Enterobacter spp.* were isolated from different samples (urine, wound swab, ETA, blood). A study [27] in Nepal reported that 52.90% *Enterobacter spp.* were multidrug-resistant which is similar to our present study. Among 15 MDR *Enterobacter* isolates, which were resistant to at least three (or more) groups of antibiotics that are aminoglycosides, fluroquinolones and third generation cephalosporin. Present study observed, 100% MDR strains were resistant to amikacin. In a study [28] in India reported, among MDR *Enterobacter*, 94% isolates were resistant to amikacin.

In this study, *in vitro* while combining fosfomycin with amikacin against MDR *Enterobacter spp*. 87.33% showed synergistic effect, 16.33 % showed additive effect, no indifference or antagonism was observed. A retrospective study [29] showed that 71.4% synergism while combining fosfomycin with amikacin against *Klebsiella* infection which is close to the present finding. Studies of multidrug-resistant *Acinetobacter baumannii, Pseudomonas aeruginosa, K. pneumoniae* and heterogeneous methicillin resistant *Staphylococcus aureus* (MRSA) reveal that FM + AMK shows mostly synergistic and additive effects *in vitro* [30,31]. Similar findings were reported for combination therapies of fosfomycin and aminoglycosides (amikacin or gentamicin) against MDR bacterial isolates including *Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Escherichia coli* [32,33,34]. This may be because fosfomycin increase uptake of amikacin into the cell, resulting in an intracellular concentration sufficient to inhibit protein synthesis. Also fosfomycin can destroy bacterial cell wall and biofilm which create conditions for the accumulation of other drugs [35]. To best our knowledge there is no *in vitro* study regarding fosfomycin and amikacin combination against MDR *Enterobacter infection.*

In the present study, while fosfomycin combine with imipenem against MDR *Enterobacter* isolate, 50% showed synergistic effect, 33.33% showed additive effect, 17.67% showed indifferent effect. No antagonism was observed. A study [36] reported 42% synergism against *Cp-Klebsiella pneumoniae* infection. In another study [37] reported 74% synergy while fosfomycin combined with imipenem against MDR *Klebsiella pneumoniae*. The synergistic effect between fosfomycin and β-lactam antibiotics is proposed to arise from the inhibition of cell wall synthesis at separate steps; fosfomycin inhibits the first enzymatic step, whereas β-lactam antibiotics inhibit the final stage in the cell wall synthesis process [37]. A study reported that fosfomycin has been successfully applied in combined therapy with other agents including aminoglycosides, carbapenems & TZP, tigecycline against MDR *Enterobacteriaceae* [38].

In this study, while imipenem combine with amikacin, 15% showed synergistic effect, 33.33% additive effect and 50% showed indifferent effect even though these strains had resistance to both imipenem and amikacin. In a study [39] reported 15% synergistic effect against MDR *Pseudomonas* infection which is close to present finding. Although there was no additive effect, rest all showed indifferent effect. Previous studies have indicated the mechanism by which carbapenems disrupts the cell walls and helps amikacin to act on the bacteria [40]. However, in many of these studies antibiotic combinations have demonstrated synergistic or bactericidal effects against bacteria that have been resistant to the individual drug. The results from published in vitro studies are conflicting, which may be due to differences in methods, antibiotic concentrations, bacterial inocula, and strain-dependent factors [26]. Although, *in vitro* data to choose of combination against MDR *Enterobacter* isolates were insufficient.

The Present study observed periodic observation of mouse after antibiotic therapy on survival of mouse. All the mice of every group were survived except the positive control group. Two out of five mice died after 72 hour of infection before collecting the sample in the positive control group indicating that, without any antimicrobial therapy, multidrug resistant *Enterobacter* infection is severe and lethal. However, no immunosuppressive agent like cyclophosphomide was used in the present study to mouse become neutropenic. But in a study [41] cyclophosphomide was used as immunosuppressive agent to rats become neutropenic and significant number of rats were died in different groups of rats in that study. We performed animal experiment to determine whether fosfomycin and amikacin, fosfomycin and imipenem, and amikacin plus imipenem combination may present any advantage in the treatment. The best *in vivo* result appeared in the group treated with fosfomycin and amikacin combination. *In vivo* combination of fosfomycin plus amikacin showed 100% bacterial clearance of the mouse from blood sample. Bacterial clearance of the mouse from blood sample was 80% with the combination of imipenem and fosfomycin. Present study also observed

60% clearance of organism from blood while combining imipenem with amikacin, 20% with amikacin alone, 40% with imipenem and 60% fosfomycin alone. Fosfomycin in combination with aminoglycoside has improved therapeutic effect against biofilm producing *pseudomonas* infection [30]. Fosfomycin is reported to mitigate *in vivo* toxicity of aminoglycosides [42]. To best our knowledge, there are no *in vivo* animal study that could compare the activity of such combination against MDR *Enterobacter* infection.

The studies made *in vivo* agree with the results obtained *in vitro*. From these *in vivo* and *in vitro* experiment, it may be clear that fosfomycin and amikacin is the best effective combination against MDR *Enterobacter spp*. The second best *in vivo* and *in vitro* effective combination is fosfomycin and imipenem.

In the present study, among the fosfomycin resistant *Enterobacter spp*. 70% were positive for *fosA*, 50% were positive for $fosA_5$ and 40% were positive for $fosB_2$. A study [43] in China reported that 80% *Enterobacter cloacae* isolates were positive for *fosA* and 10% *E.cloacae* were positive for $fosA_5$. No data is found to compare resistance rate of $fosB_2$ 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 gene (fosA among them) conferred resistance to several antibiotics including penicillins, cephalosporins, fosfomycin, aminoglycosides, quinolones [44]. This multi resistance plasmid might be the reason behind increasing fosfomycin resistance among MDR *Enterobacter spp.* To best our knowledge this is the first identification of *fosA*, $fosA_5$ *and* $fosB_2$ gene in *Enterobacter cloacae* in Dhaka medical college hospital, which was further validated by sequencing. In the present study, DNA sequence of amplified PCR product of $fosA_5$ gene detected in *E.cloacae* which was 97% identical with *Klebsiella pneumoniae* which is available in gene bank (Accession no-CP0302269) suggested that the $fosA_5$ gene found in *E. cloacae* in the present study might have been transferred from *K.pneumoniae* through plasmid.DNA sequence of amplified PCR product of *fosA* gene detected in *E.cloacae* which was 84% identical with *Enterobacter ludwigii* strain I42 chromosome, complete genome which is available in gene bank (Accession no-CP040606) and DNA sequence of amplified PCR product of $fosB_2$ gene detected in *E.cloacae* which had 99% identity with *Klebsiella pneumoniae* strain KP36, complete genome which is available in gene bank (Accession no-CP017385).

In this study, DNA sequence of amplified PCR product and translated nucleotide base sequence of *fosA*, $fosA_5$, $fosB_2$ showed point mutations including base substitution and frame shift mutation at multiple positions. A study [45] reported that large and small deletions are the main source of gene-inactivating mutations followed by insertions/duplications.

Multi drug resistance is emerging among *Enterobacter spp.* leaving limited therapeutic options for the management of serious infections. The present study reported both *in vitro* and *in vivo* efficacy of combination of two antimicrobials against MDR *Enterobacter spp*. We believe that repurposing of older antimicrobial like fosfomycin and fosfomycin combination therapy may be good options for the treatment of infection caused by them.

6. Conclusion

In the present study we determined *in vitro* and *in vivo* efficacy of antibiotic combination compared to single antibiotic therapy against multidrug resistant *Enterobacter spp*. Fosfomycin+ amikacin and fosfomycin + imipenem showed great synergistic activity and could provide promising therapeutic avenue to combat MDR *Enterobacter* infection. Another fosfomycin based combination and combinations of more than two drugs can also be evaluated.

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Conflict of Interest

There are no conflicts of interest in this study.

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